Characterization and redesign of galactonolactone dehydrogenase, a flavoprotein producing vitamin C

Nicole G. H. Leferink

Promotor:	Prof. dr. S.C. de Vries Hoogleraar in de Biochemie Wageningen Universiteit
Co-promotor:	Dr. W.J.H. van Berkel Universitair hoofddocent Laboratorium voor Biochemie Wageningen Universiteit
Promotiecommissie:	Prof. dr. H.J. Bosch (Plant Research International, Wageningen) Prof. dr. D. Haltrich (Universität für Bodenkultur Wien, Austria) Prof. dr. J. van der Oost (Wageningen Universiteit) Prof. dr. ir. A.G.J. Voragen (Wageningen Universiteit)

Dit onderzoek is uitgevoerd binnen de onderzoeksschool VLAG

Characterization and redesign of galactonolactone dehydrogenase, a flavoprotein producing vitamin C

Nicole Gerharda Henrica Leferink

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. M.J. Kropff,
in het openbaar te verdedigen
op woensdag 8 april 2009
des namiddags te vier uur in de Aula

Characterization and redesign of galactonolactone dehydrogenase, a flavoprotein producing vitamin C

Leferink, N.G.H.

PhD thesis Wageningen University, The Netherlands (2009)
With references and summary in Dutch

ISBN: 978-90-8585-352-7

Contents

chapter 1

General introduction

Nicole G. H. Leferink[1], Willem J. H. van Berkel[1]

[1] Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

Flavoenzymes

Flavoenzymes are ubiquitous proteins in nature, taking part in a large variety of biochemical reactions. Processes involving flavoenzymes include biosynthesis, energy production, light emission, protein folding, neuronal development, detoxification, apoptosis, chromatin remodeling and DNA repair (Joosten and van Berkel, 2007). Flavoenzymes are attractive biocatalysts because of their intrinsic (enantio-)selectivity and catalytic efficiency, which are often difficult to achieve by conventional chemical approaches. The discovery of new flavoenzymes together with novel insights into their catalytic mechanisms will increase the biocatalytic potential for application of these enzymes in the pharmaceutical, fine-chemical and food industries (Fraaije and van Berkel, 2006; Joosten and van Berkel, 2007).

Flavoenzymes contain a flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) as redox active prosthetic group, giving the enzymes their characteristic yellow appearance (flavus means yellow in Latin). FMN and FAD are synthesized from riboflavin (vitamin B2) by plants and microorganisms. The isoalloxazine ring system is the reactive part of the flavin molecule. It can undergo one- or two-electron reductions, making the flavin a chemically versatile cofactor (Fig. 1.1).

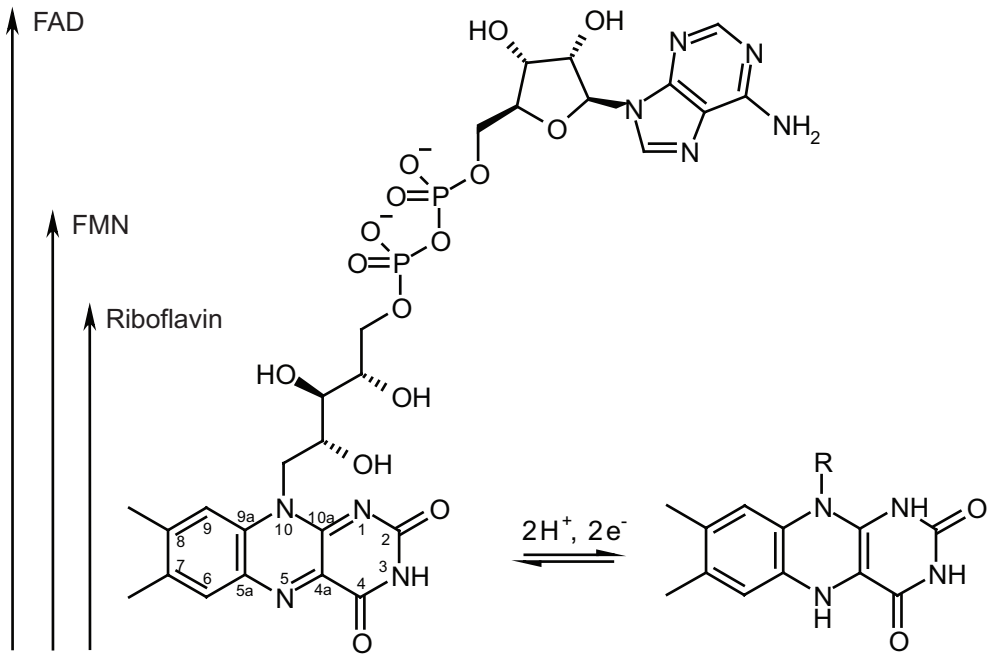
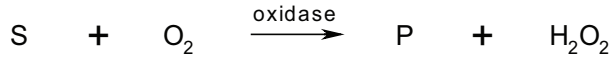


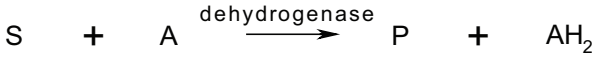
Figure 1.1. Structure of riboflavin, FMN and FAD in the oxidized and reduced state.

Flavoenzymes are capable of catalyzing a wide variety of redox reactions, including oxidations, reductions and monooxygenations. This thesis focuses on flavoenzymes that catalyze the oxidation of organic substrates, which involves the breaking of a C-H bond with

the concomitant transfer of two electrons and two protons to an electron acceptor. Such reactions vary from simple oxidation reactions, like alcohol or amine oxidations, to more complex reactions like the oxidative cyclization of plant alkaloids (Kutchan and Dittrich, 1995; Sirikantaramas et al., 2004). Oxidation reactions catalyzed by flavoproteins involve two substrates: an electron donor (the substrate) and an electron acceptor. Flavoprotein oxidases use molecular oxygen as electron acceptor, producing hydrogen peroxide (scheme 1.1), while dehydrogenases use alternative electron acceptors such as $NAD(P)^+$, quinones, or electron transfer proteins (scheme 1.2):



Scheme 1.1. Reaction catalyzed by oxidases



Scheme 1.2. Reaction catalyzed by dehydrogenases

Free reduced flavin reacts poorly with molecular oxygen (2.5×10^2 M^{-1} s^{-1}) (Massey, 1994). The oxygen reactivity of the reduced flavin can be highly modulated in a protein environment. Whereas flavoprotein oxidases generally react fast with molecular oxygen (10^4 - 10^6 M^{-1} s^{-1}), dehydrogenases react extremely slow or not at all with molecular oxygen. The molecular determinants for these differences in oxygen reactivity are largely unknown (Mattevi, 2006).

The reaction cycle of flavoenzymes consists of two half-reactions. In de reductive half-reaction, reducing equivalents are transferred from the substrate to the flavin resulting in reduced flavin and oxidized product. In the oxidative half-reaction, the flavin reacts with the electron acceptor yielding re-oxidized flavin. The cycling between oxidized and reduced flavin results in characteristic spectral properties, enabling the separate study of both half-reactions.

Most flavoproteins contain a tightly but non-covalently bound flavin cofactor, whereas a subset (about 10% in humans) of flavoproteins have their cofactor covalently bound to the protein (Mewies et al., 1998). Several distinct types of covalent flavinylation have been recognized, involving a histidine, cysteine or a tyrosine residue linked to the 8α or 6 position of the isoalloxazine ring. Recently, several flavoenzymes were identified that contain a bi-covalently linked flavin attached to both a cysteine and histidine residue (Huang et al., 2005; Winkler et al., 2007; Heuts et al., 2008). The rationale for covalent flavinylation is not

completely understood but the covalent binding may be beneficial for tuning the redox properties by increasing the redox potential, saturation of the active site with cofactor, protein stability and preventing flavin modification.

Carbohydrate oxidoreductases

Carbohydrate oxidoreductases are valuable enzymes with great biocatalytic potential. They use either molecular oxygen as electron acceptor (oxidases) or alternative electron acceptors (dehydrogenases). Carbohydrate oxidases can be applied, for example in diagnostic applications, in the food and drinks industry as oxygen scavenger, and for the synthesis of carbohydrate derivatives. Oxidation of carbohydrates results in changed physical properties such as solubility, rheology, gelatinization strength, swelling and chelation. Oxidized carbohydrates are applied, for example, as thickeners or emulsifiers in the food industry, as water binders in the paper industry, as metal chelators or as anti-oxidizing agents in organ preservation. Oxidized carbohydrates are also used as building blocks in chemical synthesis, as precursors for further chemical modification or for conjugation to other biomolecules. The occurrence and biocatalytic potential of carbohydrate oxidases are summarized in a recent review (van Hellemond et al., 2006).

Carbohydrate oxidoreductases often contain a flavin cofactor as redox-active group and belong to structurally different flavoprotein families. A surprising large number of carbohydrate oxidases contain a covalently linked FAD cofactor (Table 1.1). Carbohydrate oxidases have mainly been isolated from fungi and are predominantly active on mono- and/or disaccharides. The reactions catalyzed by several characterized carbohydrate oxidases are shown in Figure 1.2.

Glucose oxidase is the best known carbohydrate oxidase, it catalyzes the oxidation of D-glucose into D-glucono-1,5-lactone (Fig. 1.2A) and has been isolated from various fungi (Witteveen et al., 1992; Witt et al., 1998; Simpson et al., 2006). Other well known fungal carbohydrate oxidoreductases include pyranose 2-oxidase, which catalyzes the oxidation of D-glucose and other pyranoses into the corresponding ketoaldoses (Fig. 1.2B) (Giffhorn, 2000) and cellobiose dehydrogenase, a flavocytochrome that oxidizes cellooligosaccharides to their corresponding lactones (Henriksson et al., 2000). All the abovementioned enzymes belong to the Glucose-Methanol-Choline (GMC) family. Members of the GMC family adopt a two-domain folding topology, a FAD-binding domain with an ADP-binding $\beta\alpha\beta$-fold and a substrate binding domain (Cavener, 1992). Fungal carbohydrate oxidoreductases have been suggested to be involved in the degradation of cellulose and lignin, either directly or via the supply of hydrogen peroxide for peroxidases (van Hellemond et al., 2006).

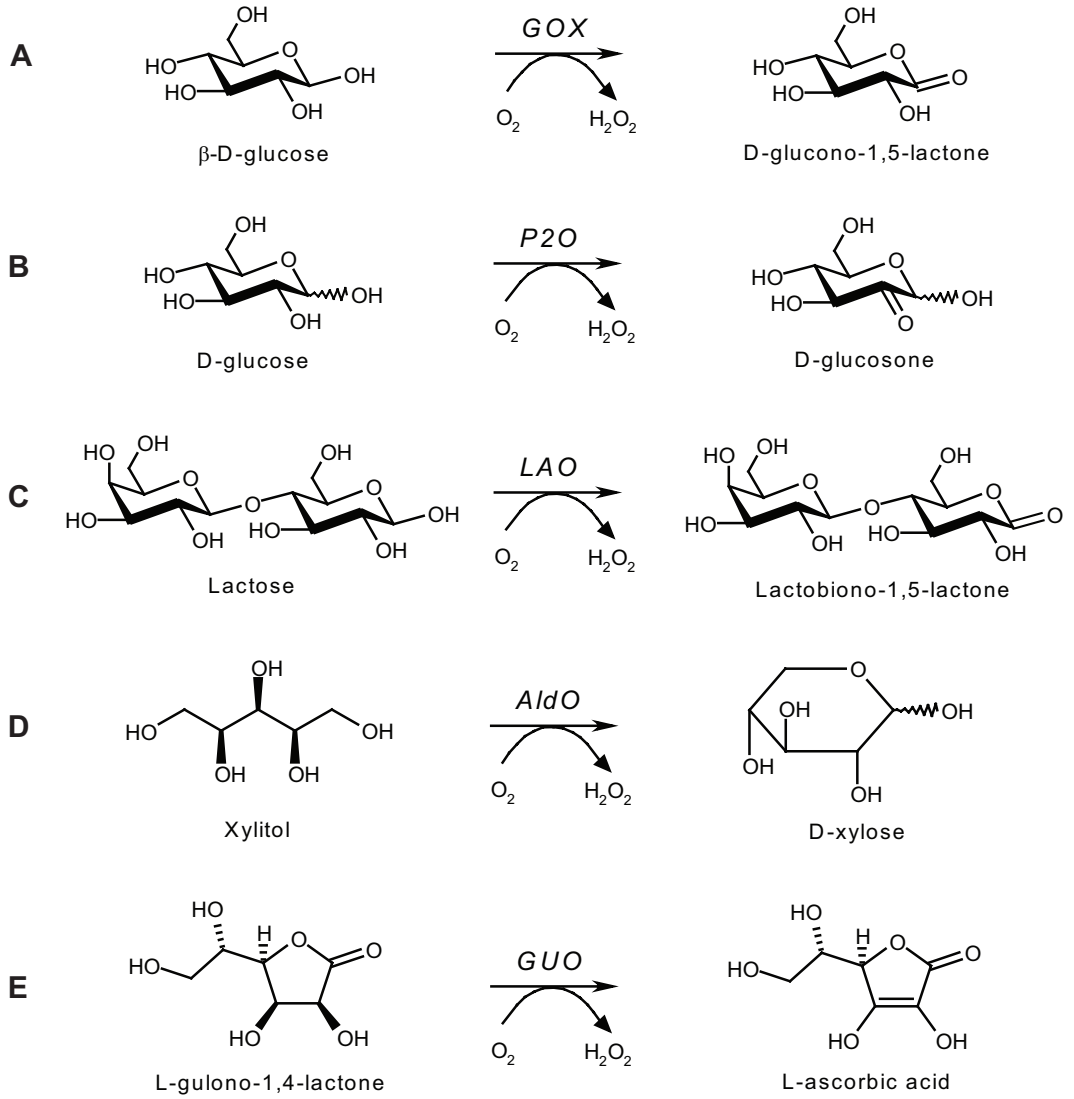


Figure 1.2. Examples of reactions catalyzed by carbohydrate oxidases. A) Glucose oxidase (GOX), B) Pyranose 2-oxidase (P2O), C) Lactose oxidase (LAO), D) Alditol oxidase (AldO), E) Gulonolactone oxidase (GUO).

Several polyol oxidases have been isolated from *Streptomyces* bacteria, including a xylitol, sorbitol and alditol oxidase (Hiraga et al., 1998; Yamashita et al., 2000; Heuts et al., 2007[a]). Alditol oxidase catalyzes the oxidation of primary alcohols into the corresponding aldehydes, the oxidation of xylitol by alditol oxidase yields D-xylose (Fig. 1.2D) (Heuts et al., 2007[a]). All three known polyol oxidases contain a covalently bound 8α-*N*1-histidyl FAD cofactor and belong to the vanillyl-alcohol oxidase (VAO) family. Recently a fungal glucooligosaccharide oxidase of the VAO family was identified, which contains a bi-covalently bound 6-*S*-cysteinyl, 8α-histidyl FAD (Huang et al., 2005). A bi-covalent binding mode has also been recognized in other carbohydrate oxidases with a VAO-fold, including hexose oxidase from a red algae (Rand et al., 2006), chitooligosaccharide oxidase from *Fusarium graminearum* (Heuts et al., 2008), and aclacinomycin oxidoreductase from *Streptomyces* (Alexeev et al.,

2007), and is predicted for lactose oxidase from *Microdochium nivale* (Leferink et al., 2008[a]). This unusual bi-covalent flavin linkage has been implicated in tuning the redox potential and increased structural integrity (Heuts et al., 2008; Huang et al., 2008). Members of the VAO family adopt a two-domain folding topology with a N-terminal FAD-binding domain that contains a PP-loop which interacts with the pyrophosphate moiety of the flavin, and a C-terminal cap-domain that determines the substrate specificity (Fraaije et al., 1998).

Flavoprotein carbohydrate oxidases with another fold include the fungal fructosyl amino acid oxidases (FAO) or amadoriases. These enzymes act on amadori compounds, which are formed upon reaction of reducing sugars with amino groups of proteins or peptides. FAOs catalyze the oxidative cleavage of these protein carbohydrate linkages (Wu et al., 2000). FAOs share the same fold as other flavin-dependent amine oxidases, including D-amino acid oxidase, monomeric sarcosine oxidase, and monoamine oxidase (Collard et al., 2008). FAO contains a covalent 8α-*S*-cysteinyl FAD, a linkage not found in the VAO or GMC flavoprotein families.

Plants appear to be a rich source of carbohydrate oxidoreductases, but the function and identity of these enzymes is largely unknown. They are presumed to play a role in the biosynthesis and organization of the plant cell wall, the biosynthesis of vitamins and hormones and in active defense through the production of hydrogen peroxide. A few VAO-type carbohydrate oxidases have been isolated from plants, including a glucose oxidase from tobacco nectar (Carter and Thornburg, 2004) and a sunflower carbohydrate oxidase involved in plant defense (Custers et al., 2004). The genome of the model plant *Arabidopsis thaliana* encodes many putative flavoproteins with a predicted VAO-fold and unexplored catalytic activities. Several of these putative oxidoreductases have a high sequence identity with sunflower carbohydrate oxidase and the tobacco nectar glucose oxidase (Carter and Thornburg, 2004; Custers et al., 2004). Furthermore, a number of putative oxidoreductases are related to bacterial alditol oxidases and to the aldonolactone oxidoreductases. The latter enzymes are involved in the biosynthesis of vitamin C (Fig. 1.2E).

Tabel 1.1. List of characterized flavoprotein carbohydrate oxidoreductases

Enzyme	Fold	Cofactor	Source	Substrate(s)	Electron acceptor
Cellobiose dehydrogenase[1]	GMC	FAD	Fungi	Disaccharides	Cytochrome *c*
Glucose oxidase[2]	GMC	FAD	Fungi	Glucose	Oxygen
Pyranose 2-oxidase[3]	GMC	8α-*N*3-His FAD	Fungi	Monosaccharides	Oxygen
Aclacinomycin oxidoreductase[4]	VAO	6-*S*-Cys, 8α-*N*1-His FAD	Bacteria	Glycated polyketides	Oxygen
Alditol oxidase[5]	VAO	8α-*N*1-His FAD	Bacteria	Polyols	Oxygen
Aldonolactone oxidoreductase[6]	VAO	FMN	Trypanosomes	Aldonolactones	Cytochrome *c*
Arabinonolactone oxidase[7]	VAO	8α-*N*1-His FAD	Yeast	Arabinonolactone	Oxygen
Carbohydrate oxidase[8]	VAO	6-*S*-Cys, 8α-*N*1-His FAD[a]	Plants	Oligosaccharides	Oxygen
Chitooligosaccharide oxidase[9]	VAO	6-*S*-Cys, 8α-*N*1-His FAD	Fungi	Chitooligosaccharides	Oxygen
Glycopeptide hexose oxidase[10]	VAO	6-*S*-Cys, 8α-*N*1-His FMN	Bacteria	Glycopeptides	Oxygen
Galactonolactone dehydrogenase[11]	VAO	FAD	Plants	Galactonolactone	Cytochrome *c*
Glucooligosaccharide oxidase[12]	VAO	6-*S*-Cys, 8α-*N*1-His FAD	Fungi	Oligosaccharides	Oxygen
Gulonolactone oxidase[13]	VAO	8α-*N*1-His FAD	Animals	Gulonolactone	Oxygen
Hexose oxidase[14]	VAO	6-*S*-Cys, 8α-*N*1-His FAD	Algae	Monosaccharides	Oxygen
Lactose oxidase[15]	VAO	6-*S*-Cys, 8α-*N*1-His FAD[a]	Fungi	Oligosaccharides	Oxygen
Nectarin 5[16]	VAO	6-*S*-Cys, 8α-*N*1-His FAD[a]	Plants	Glucose	Oxygen
Sorbitol oxidase[17]	VAO	8α-*N*1-His FAD[a]	Bacteria	Polyols	Oxygen
Xylitol oxidase[18]	VAO	8α-*N*1-His FAD[a]	Bacteria	Polyols	Oxygen
Fructosyl amine oxidase[19]	DAAO/MSOX	8α-*S*-Cys FAD	Fungi	Fructosyl amines	Oxygen

[a] predicted from the amino acid sequence. References used: 1, (Henriksson et al., 2000); 2, (Gibson et al., 1964); 3, (Giffhorn, 2000; Halada et al., 2003); 4, (Alexeev et al., 2007); 5, (Heuts et al., 2007[a]); 6, (Wilkinson et al., 2005; Logan et al., 2007); 7, (Huh et al., 1994; Huh et al., 1998); 8, (Custers et al., 2004); 9, (Heuts et al., 2007[b]; Heuts et al., 2008); 10, (Li et al., 2007); 11, (Mapson and Breslow, 1958; Ôba et al., 1995; Yabuta et al., 2000); 12, (Lin et al., 1991; Huang et al., 2005); 13, (Nishikimi et al., 1976; Kiuchi et al., 1982); 14, (Groen et al., 1997; Rand et al., 2006); 15, (Xu et al., 2001); 16, (Carter and Thornburg, 2004); 17, (Hiraga et al., 1998); 18, (Yamashita et al., 2000); 19, (Collard et al., 2008)

Vitamin C biosynthesis

Vitamin C or L-ascorbic acid (ascorbate, Fig. 1.3A) is an important sugar derivative that acts as antioxidant, redox buffer and as enzyme cofactor in a number of metal-dependent oxygenases involved in e.g. collagen and carnitine biosynthesis (Englard and Seifter, 1986). Most organisms can synthesize ascorbate to their own requirements. Humans and other primates, however, have lost the ability to synthesize ascorbate during evolution, hence ascorbate is a vitamin for men and also for a number of other mammalian species including guinea pigs and bats, a few bird species, and some fish (Chatterjee, 1973). Humans completely depend on their diet, to meet the daily ascorbate requirements. A diet deficient in ascorbate can lead to scurvy, a disorder characterized by abnormal collagen synthesis. Ascorbate is named after its anti-scorbutic properties in humans.

Ascorbate is particularly abundant in plants, the main dietary source of vitamin C for humans. In plants ascorbate plays in addition to its antioxidant capacity, a pivotal role in the control of photosynthesis, cell expansion and growth, and trans-membrane electron transport (Smirnoff and Wheeler, 2000). During photosynthesis excess absorbed light can generate reactive oxygen species, which can damage proteins, unsaturated fatty acids and DNA. Plant cells in green tissues can contain up to 5 mM ascorbate, representing 10% of the total soluble carbohydrate pool (Smirnoff and Wheeler, 2000).

While ascorbate is widespread in the animal and plant kingdom, microorganisms possess ascorbate analogs. D-Erythorbic acid (isovitamin C, Fig. 1.3B) is a C5 epimer of ascorbate and is only found in the filamentous fungus *Penicillium* (Salusjärvi et al., 2004). Yeasts contain another ascorbate analog, D-erythroascorbic acid (Fig. 1.3C), which is a five-carbon analog of ascorbate.

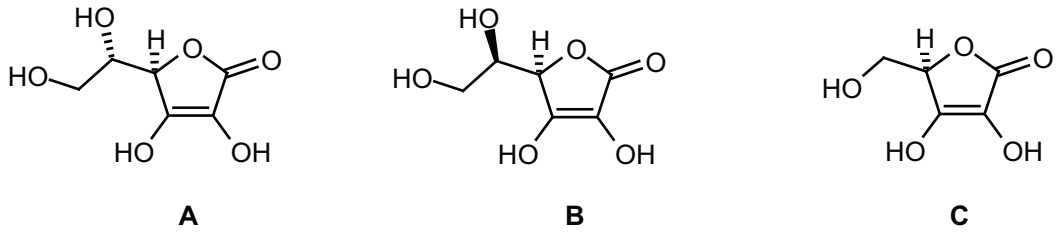


Figure 1.3. Chemical structures of L-ascorbic acid (A), D-erythorbic acid (B) and D-erythroascorbic acid (C).

Vitamin C is widely applied as preservative and antioxidant, besides its traditional use in the food and drinks industry, its application in animal feed and the cosmetics industry is rapidly growing. About 50% of the synthetic vitamin C produced is used in vitamin supplements and in pharmaceutical preparations (Hancock and Viola, 2002). To meet the increasing world demand, vitamin C is currently produced from glucose via two main routes.

The Reichstein process, already developed in the 1930s, involves a single pre-fermentation step followed by six purely chemical steps. The two-step fermentation process, developed in China in the 1960s, uses an additional fermentation step to replace part of the chemical steps of the Reichstein process. Both processes yield about 50% vitamin C from the glucose feed (Hancock and Viola, 2002). The world production of synthesized vitamin C is estimated at 11 $\times$ 10^7 kg/year, most of which is produced in China. The need to reduce capital costs, protect the environment and increase the process efficiency have urged to develop alternative manufacturing processes. Innovations in recombinant DNA technology, the availability of genome sequences and recent advances in protein engineering techniques may be exploited for the biotechnological production of vitamin C. Metabolic engineering can be applied, for example, to increase the vitamin C content in plants (Ishikawa et al., 2006) or modify microorganisms for the biotechnological production of vitamin C (Hancock and Viola, 2001). To achieve this, it is of utmost importance to have a detailed understanding of the biosynthesis of vitamin C and the enzymes involved.

Different pathways have evolved for ascorbate biosynthesis in animals, plants and fungi. Ascorbate and its analogs are synthesized from various sugars and their production involves the action of several carbohydrate oxidoreductases. The biosynthesis of ascorbate has been extensively studied by feeding experiments with radiolabeled substrates (Smirnoff and Wheeler, 2000; Ishikawa et al., 2006; Linster and van Schaftingen, 2007). The ascorbate biosynthesis pathway in animals has been elucidated first and is well established. D-Glucose is the ultimate precursor and the first committed step is the conversion of D-glucuronate into L-gulonate by the action of glucuronate reductase (Smirnoff, 2001; Linster and van Schaftingen, 2007). The final step is carried out by the microsomal flavoprotein L-gulono-1,4-lactone oxidase (GUO), which catalyzes the two-electron oxidation of L-gulono-1,4-lactone into L-ascorbate (Burns et al., 1956).

The biosynthesis of vitamin C in plants seems to follow a multitude of pathways and has only recently been elucidated (Fig. 1.4) (Ishikawa et al., 2006). The majority of ascorbate biosynthesis in plants follows the so-called Smirnoff-Wheeler pathway, which starts from GDP-D-mannose and runs via L-galactose (Wheeler et al., 1998). The sugar L-galactose is oxidized by a cytosolic NAD^+-dependent L-galactose dehydrogenase to the final precursor L-galactono-1,4-lactone (Gatzek et al., 2002). The terminal step in this pathway is catalyzed by the mitochondrial flavoenzyme L-galactono-1,4-lactone dehydrogenase (GALDH), which catalyzes the two-electron oxidation of L-galactono-1,4-lactone into L-ascorbate (Mapson et al., 1954). More recently other routes towards L-ascorbate have been identified in plants. One route involves D-galacturonic acid, a major constituent of plant cell walls (Agius et al., 2003) and another one involves L-gulono-1,4-lactone as final precursor resembling part of the

animal pathway (Lorence et al., 2004). Not all enzymes involved in these routes have been identified up to now.

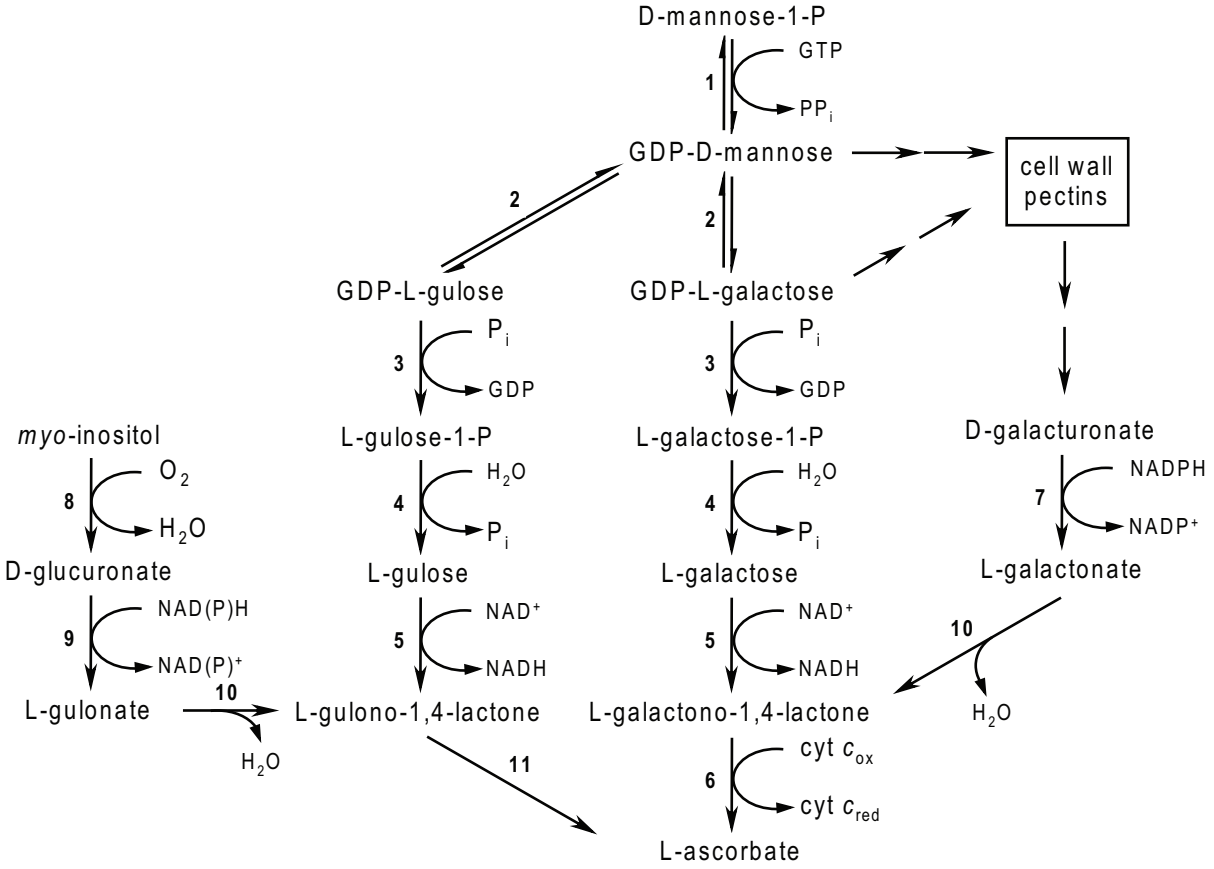


Figure 1.4. Biosynthesis of vitamin C in plants. Enzymes involved: 1, GDP-D-mannose pyrophosphorlyase; 2, GDP-D-mannose-3'-5'-epimerase; 3, GDP-L-galactose phosphorylase (GDP-L-galactose orthophosphate guanyltransferase); 4, L-galactose 1-phosphate phosphatase; 5, L-galactose dehydrogenase; 6, L-galactono-1,4-lactone dehydrogenase; 7, D-galacturonate reductase; 8, *myo*-inositol oxygenase; 9, D-glucuronate reductase; 10, aldonolactonase; 11, L-gulono-1,4-lactone oxidase or dehydrogenase. GDP, guanosine diphosphate; P_i, phosphate; cyt *c*, cytochrome *c*.

The biosynthesis of ascorbate has also been studied in several protists. Ascorbate production in trypanosome parasites, the causative agents of African sleeping sickness (*Trypanosoma brucei*) and Chagas' disease (*T. cruzi*), is likely to proceed via L-galactose and L-galactono-1,4-lactone (Wilkinson et al., 2005), resembling the Smirnoff-Wheeler pathway in plants. The final step occurs in a unique peroxisome related single-membrane organelle, called the glycosome (Wilkinson et al., 2005; Logan et al., 2007). Photosynthetic algae synthesize ascorbate via D-galacturonic acid and L-galactonate (Ishikawa et al., 2008), analogous to the alternative pathway in plants.

The biosynthesis of the microbial analogs erythorbate and erythroascorbate is less complicated and involves only two oxidative steps. In *Penicillium* sp. D-glucose is converted

to D-gluconolactone by glucose oxidase, which is subsequently converted to D-erythorbic acid by D-gluconolactone oxidase (GLO) (Takahashi et al., 1976; Harada et al., 1979; Salusjärvi et al., 2004). In yeasts, D-erythorascorbic acid is synthesized from the pentose sugar D-arabinose, which is oxidized to D-arabinono-1,4-lactone by the action of D-arabinose dehydrogenase (Amako et al., 2006). The lactone is then oxidized to D-erythroascorbic acid by D-arabinono-1,4-lactone oxidase (ALO) (Huh et al., 1998).

Aldonolactone oxidoreductases

The terminal step in the biosynthesis of ascorbate and its analogs is catalyzed by a group of closely related flavin-dependent aldonolactone oxidoreductases. These enzymes share a conserved FAD domain and do all belong to the vanillyl-alcohol oxidase (VAO) family (Fraaije et al., 1998; Leferink et al., 2008[a]).

L-Gulono-γ-lactone oxidase (GUO; L-gulono-1,4-lactone: oxygen oxidoreductase; EC 1.1.3.8) catalyzes the final step of vitamin C biosynthesis in animals; the oxidation of L-gulono-1,4-lactone with the concomitant reduction of molecular oxygen into hydrogen peroxide. GUO activity was first demonstrated in rat liver microsomes (Burns et al., 1956), and has been isolated from rat and goat liver microsomes (Nishikimi et al., 1976), and chicken kidney microsomes (Kiuchi et al., 1982). GUO is an integral membrane protein localized at the ER/microsomal membrane, with the active site facing the lumen of the ER (Puskas et al., 1998). GUO contains a covalently 8α-*N*1-histidyl FAD linked to a histidine located in the N-terminal FAD-binding domain (Kenney et al., 1976). The enzyme is most active with L-gulono-1,4-lactone, but also with other aldonolactones showing the same configuration of the C2 hydroxyl group as in L-gulono-1,4-lactone (Kiuchi et al., 1982). GUO is inhibited by various thiol reactive reagents, indicating that sulfhydryl groups are involved in catalysis (Nakagawa and Asano, 1970). The GUO gene is defective in humans and other primates, which makes them susceptible to scurvy (Nishikimi et al., 1994).

L-Galactono-γ-lactone dehydrogenase (GALDH; L-galactono-1,4-lactone: ferricytochrome *c*-oxidoreductase; EC 1.3.2.3) catalyzes the oxidation of L-galactono-1,4-lactone into L-ascorbate with the concomitant reduction of cytochrome *c*. GALDH is presumably localized in the mitochondrial intermembrane space where it is involved in feeding electrons into the electron transport chain (Heazlewood et al., 2003). GALDH is an essential enzyme for the plant, besides from producing the antioxidant ascorbate, GALDH has also been associated with the assembly of respiratory complex I and the proper functioning of plant mitochondria (Alhagdow et al., 2007; Pineau et al., 2008). GALDH has been isolated for the first time from cauliflower florets (Mapson and Breslow, 1958) and has since then been isolated from the mitochondria of a number of other plant species (Mutsuda et al., 1995; Ôba et al., 1995;

Østergaard et al., 1997; Imai et al., 1998; Yabuta et al., 2000). In contrast to GUO, GALDH contains a non-covalently linked FAD and reacts poorly with molecular oxygen. GALDH shows a high specificity for L-galactono-1,4-lactone as substrate. All known GALDH enzymes are, like GUO, inhibited by sulfhydryl reactive agents.

D-Arabinono-γ-lactone oxidoreductase (ALO; D-arabinono-1,4-lactone: oxygen oxidoreductase; EC 1.1.3.37) from yeast is responsible for the oxidation of D-arabinono-1,4-lactone into D-erythroascorbic acid using oxygen as electron acceptor. ALO is a membrane bound mitochondrial oxidase. The enzyme has been isolated from the mitochondria from *Saccharomyces cerevisiae* (Nishikimi et al., 1978; Bleeg and Christensen, 1982; Huh et al., 1998) and *Candida albicans* (Huh et al., 1994). ALO contains, like GUO, a 8α-*N*1-histidyl FAD linked to a histidine in the N-terminal FAD-binding domain (Kenney et al., 1979). ALO is active with D-arabinono-1,4-lactone, L-galactono-1,4-lactone, and L-gulono-1,4-lactone, showing a similar substrate specificity as GUO (Nishikimi et al., 1978; Huh et al., 1994). ALO is, like other aldonolactone oxidoreductases, inactivated by thiol modifying agents (Huh et al., 1994).

Penicillium fungi are capable of converting D-gluconolactone into D-erythorbic acid by the action of D-gluconolactone oxidoreductase (GLO). GLO is an extracellular enzyme and is the only known aldonolactone oxidoreductases that is active as a dimer (Salusjärvi et al., 2004). GLO contains a covalently bound FAD (Harada et al., 1979) and is presumably active with both D-glucono-1,4-lactone and D-glucono-1,5-lactone, the latter substrate is the direct product of glucose oxidation (Salusjärvi et al., 2004). GLO is not inactivated by thiol reactive compounds (Takahashi et al., 1976).

GALDH homologs were recently identified in the trypanosome parasites *T. brucei* and *T. cruzi* (Wilkinson et al., 2005; Logan et al., 2007). Both enzymes, TbALO and TcGAL, are active with L-galactono-1,4-lactone and D-arabinono-1,4-lactone and use cytochrome *c* as electron acceptor. Both TbALO and TcGAL are reported to contain a non-covalently bound FMN cofactor (Logan et al., 2007). TcGAL is an interesting drug target since *T. cruzi* can not take up ascorbate from its environment (Logan et al., 2007).

Recently a L-gulono-1,4-lactone dehydrogenase (GUDH) was identified in *Microbacterium tuberculosis* which oxidizes L-gulono-1,4-lactone to L-ascorbate, using both cytochrome *c* and phenazine methosulfate as electron acceptors (Wolucka and Communi, 2006). GUDH homologs were detected in the genomes of other bacteria, but they have most likely a different physiological substrate, since ascorbate is exclusively made by eukaryotes (Smith et al., 2007). This is supported by the relatively high K_m value of GUDH for L-gulono-1,4-lactone of 5.5 mM and a rather low k_{cat} of < 0.1 s^{-1} (Wolucka and Communi, 2006). No flavin could be detected in the recombinant enzyme, despite the presence of the conserved N-terminal FAD binding domain.

Aim and outline

The work described in this thesis is the result of the research performed in the project "Genome-based discovery and characterization of novel carbohydrate oxidoreductases" of the Carbohydrate Research Centre Wageningen. The aim of this project was to identify, characterize and redesign carbohydrate oxidoreductases from the model plant *Arabidopsis thaliana*, that are involved in plant cell wall maintenance, plant defense or ascorbate biosynthesis.

This thesis mainly deals with the molecular characterization and redesign of GALDH, the ultimate vitamin C producer in plants. The first part of this thesis (chapters 3-5) describes the molecular cloning and characterization of GALDH from *A. thaliana*. The second part of this thesis (chapters 6-7) reports on the different aspects of the oxygen reactivity of GALDH. Finally, a GALDH homolog from trypanosome parasites was studied (chapter 8). The knowledge obtained provides a sound basis for the future design of stable biocatalysts suitable for the biotechnological production of vitamin C or other valuable carbohydrates with new functional properties.

In **Chapter 2** an overview is given of the VAO flavoprotein family. Within this family different types of (covalent) flavin binding modes are recognized. The catalytic activities of some recently discovered members, including several carbohydrate oxidoreductases, are reviewed.

Chapter 3 describes the heterologous production, purification and biochemical characterization of GALDH from *A. thaliana*. The recombinant enzyme contains a non-covalently bound FAD as redox active cofactor and is highly active with its natural substrates L-galactono-1,4-lactone and cytochrome *c*. The role of a leucine residue in the FAD-binding domain, which is specific for plant GALDH enzymes, was investigated by site-directed mutagenesis.

So far, no three-dimensional structure data are available for the aldonolactone oxidoreductase subfamily. **Chapter 4** reports on the active site of GALDH. Two putative active site residues were identified by sequence comparison with alditol oxidase and cholesterol oxidase, and their function was addressed by site-directed mutagenesis.

Chapter 5 describes the interaction of GALDH with its natural electron acceptor cytochrome *c*. A nuclear magnetic resonance (NMR) chemical shift perturbation analysis of ^{15}N labeled yeast iso-1-cytochrome *c* revealed that GALDH forms a transient complex with cytochrome *c* facilitating fast electron transfer. The interaction with cytochrome *c* remains intact after removing 9 charged residues at the GALDH protein surface. The crystallization properties of GALDH are not significantly improved by the surface mutations.

In **Chapter 6** the sensitivity of GALDH towards oxidative stress is investigated. GALDH is irreversibly inactivated by hydrogen peroxide due to the specific oxidation of an active site cysteine. The critical involvement of this cysteine in substrate recognition together with the fact that plants produce high amounts of ascorbate, provide a rationale for the poor oxygen reactivity of GALDH.

Chapter 7 reports on the discovery of a gatekeeper residue in VAO-type flavoprotein dehydrogenases that prevents them from acting as oxidases. Replacing the corresponding residue in GALDH (Ala113→Gly) yields a catalytically competent oxidase with properties similar to other flavoprotein oxidases. The A113G mutations creates space near the flavin C4a locus allowing oxygen to reach and react with the reduced flavin.

In **Chapter 8** the aldonolactone oxidoreductase of *Trypanosoma cruzi*, the causative agent of Chagas' disease, is characterized. In contrast to an earlier report, we found that this enzyme uses non-covalently bound FAD as redox active cofactor rather than FMN. Furthermore, in line with the predicted space near the C4a-N5 locus of the flavin, the enzyme can use molecular oxygen as electron acceptor in addition to cytochrome *c*.

In **Chapter 9** the findings described in this thesis are summarized and discussed, and some final conclusions are drawn.

chapter 2

The growing VAO flavoprotein family

Nicole G. H. Leferink[1], Dominic P. H. M. Heuts[2], Marco W. Fraaije[2], Willem J. H. van Berkel[1]

Archives of Biochemistry and Biophysics (2008) 474(2):292-301

[1] Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
[2] Laboratory of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Abstract

The VAO flavoprotein family is a rapidly growing family of oxidoreductases that favor the covalent binding of the FAD cofactor. In this review we report on the catalytic properties of some newly discovered VAO family members and their mode of flavin binding. Covalent binding of the flavin is a self-catalytic post-translational modification primarily taking place in oxidases. Covalent flavinylation increases the redox potential of the cofactor and thus its oxidation power. Recent findings have revealed that some members of the VAO family anchor the flavin via a dual covalent linkage (6-*S*-cysteinyl-8α-*N*1-histidyl FAD). Some VAO-type aldonolactone oxidoreductases favor the non-covalent binding of the flavin cofactor. These enzymes act as dehydrogenases, using cytochrome *c* as electron acceptor.

Keywords: Alditol oxidase; chitooligosaccharide oxidase; covalent flavinylation; eugenol oxidase; flavoenzyme; L-galactono-1,4-lactone dehydrogenase; vanillyl-alcohol oxidase; vitamin C

Abbreviations: AldO, alditol oxidase; ADPS, alkyldihydroxyacetonephosphate synthase; AknOx, aclacinomycin oxidoreductase; BBE, *S*-reticuline oxidase (berberine bridge enzyme); ChitO, chitooligosaccharide oxidase; EUGO, eugenol oxidase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GALDH, L-galactono-1,4-lactone dehydrogenase; HDNO, 6-hydroxy-D-nicotine oxidase; MurB, *N*-acetylenolpyruvyl glucosamine reductase; PCMH, *p*-cresol methylhydroxylase; TbALO, *Trypanosoma brucei* arabinonolactone oxidase; TcGAL, *Trypanosoma cruzi* galactonolactone oxidase; VAO, vanillyl-alcohol oxidase

Introduction

The vanillyl-alcohol oxidase (VAO) flavoprotein family comprises a group of enzymes that share a conserved FAD-binding domain (Fraaije et al., 1998). VAO family members are involved in a wide variety of metabolic processes in all kingdoms of life. A remarkable feature of the FAD-binding module of this protein family is that it favors the covalent attachment of the flavin cofactor. The first VAO family members identified with such a covalent link concerned 6-hydroxy-D-nicotine oxidase (HDNO), involved in nicotine catabolism in *Arthrobacter nicotinovorans* (Möhler et al., 1972; Brandsch et al., 1987) and *p*-cresol methylhydroxylase (PCMH), involved in the microbial detoxification of phenols (McIntire et al., 1981). Only recently, it was found that the isoalloxazine ring of the flavin cofactor can be tethered to the apoprotein via a dual covalent linkage (Huang et al., 2005). All bi-covalent flavoenzymes characterized thus far share a VAO fold.

Covalent flavoenzymes are less widespread than their counterparts containing a dissociable flavin cofactor (Hefti et al., 2003[a]). Several distinct types of covalent flavin binding have been recognized (Mewies et al., 1998; Hefti et al., 2003[a]), but tethering of the 8α-methyl group of the flavin isoalloxazine ring to a histidine residue is most frequently observed. Enzymatic degradation to the level of the aminoacyl riboflavin moiety has been the conventional method to characterize the covalent protein-flavin link (Edmondson and De Francesco, 1991; Decker and Brandsch, 1997). A more sophisticated approach involves the structural characterization of the isolated flavinylated peptide without submitting it to subsequent enzymatic degradation (Halada et al., 2003). Alternatively, the mode of covalent flavinylation can be determined from the three-dimensional protein structure or predicted *in silico* (Fraaije et al., 1998). The importance of the covalent link can be analyzed through the functional characterization of site-directed mutant proteins (Mauch et al., 1989; Fraaije et al., 1999; van den Heuvel et al., 2000; Hassan-Abdallah et al., 2006).

Most evidence obtained thus far suggests that covalent flavinylation is a self-catalytic process, dependent on the primary folding of the polypeptide chain (Brandsch and Bichler, 1991; Mewies et al., 1998). The ability of covalent incorporation of FAD by the apoprotein was first demonstrated for HDNO (Nagursky et al., 1988). The formation of the flavin-protein linkage was promoted by the addition of small organic compounds, e.g. glycerol and glycerol-3-phosphate. For VAO it was shown that covalent flavinylation is not needed for effective binding of the cofactor (Fraaije et al., 1999). This suggests that formation of the histidyl-FAD bond is preceded by noncovalent binding of the cofactor to the folded apoprotein (lock-and-key). It was also discovered that covalent binding of FAD to His422 in VAO requires the presence of an activating nucleophile, His61, in the FAD domain (Fraaije et al., 2000). His422 replacements, prohibiting formation of the histidyl-FAD linkage, showed that

covalent flavinylation increases the redox potential of VAO and thus its oxidation power (Fraaije et al., 1999). For PCMH it was shown that binding of the cytochrome subunit is necessary for the generation of the 8α-*O*-tyrosyl FAD bond (Kim et al., 1995), and that the intermolecular subunit interactions induce small structural changes in the flavin binding pocket that optimize the redox properties of the covalenty bound FAD (Cunane et al., 2005).

In this review we report on the catalytic properties of some newly discovered VAO family members and their mode of flavin binding. Some new information about the aldonolactone oxidoreductase subfamily is presented as well.

New members of the VAO flavoprotein family

With the aid of the 3D-structure and sequence of VAO from *Penicillium simplicissimum* (Mattevi et al., 1997), about 50 different VAO homologs originally were identified (Fraaije et al., 1998). Among these were several characterized flavoenzymes like 6-hydroxy-D-nicotine oxidase, *S*-reticuline oxidase, *p*-cresol methylhydroxylase, D-arabinono-1,4-lactone oxidase, D-lactate dehydrogenasae, cholesterol oxidase, L-gulono-1,4-lactone oxidase, L-galactono-1,4-lactone dehydrogenase, hexose oxidase and alkyldihydroxyacetonephosphate synthase (ADPS) (Table 2.1). UDP-*N*-acetylenolpyruvyl glucosamine reductase (MurB), involved in the biosynthesis of the bacterial cell wall, was also identified as a family member, despite a low sequence similarity. During the last decade several new VAO family members have emerged. These include amongst others alditol oxidase, chitooligosaccharide oxidase, glucooligosaccharide oxidase, lactose oxidase, eugenol dehydrogenase, eugenol oxidase, cytokinin dehydrogenase, aclacinomycin oxidoreductase and Dbv29, a glycopeptide hexose oxidase. The mode of flavin binding of these enzymes is summarized in Table 2.1.

Histidyl-FAD enzymes

Analysis of VAO-type protein sequences has revealed that the target residue for covalent flavinylation is part of a conserved sequence region (Fraaije et al., 1998). The linking histidine is typically found in the N-terminal part of the protein sequence downstream of 3 relatively small residues (e.g. xGGGHx sequence). Examples of such His-FAD containing proteins are HDNO (Brandsch et al., 1987; Koetter and Schulz, 2005), cholesterol oxidase (Coulombe et al., 2001), cytokinin dehydrogenase (Malito et al., 2004; Bae et al., 2007), and alditol oxidase (Heuts et al., 2007[a]). The only exceptions to this rule are VAO and some close homologs (PCMH (Mathews et al., 1991), and eugenol oxidase (Jin et al., 2007)). These enzymes contain a linking residue that is closer to the C-terminus. This suggests that during evolution, at least in two occasions the covalent flavin-protein linkage was formed at the two

sequence regions (loops) that are in close contact with the dimethylbenzyl moiety of the flavin isoalloxazine ring in the VAO fold (Fig. 2.1). The N-terminal loop preferentially binds the flavin via the *N*1 atom of the histidine side chain (Table 2.1). In accordance with this, HDNO contains a 8α-*N*1-histidyl FAD (Koetter and Schulz, 1995), instead of the originally identified 8α-*N*3-histidyl FAD (Möhler et al., 1972). Sequence alignments suggest that D-gluconolactone oxidase might also contain 8α-*N*1-histidyl FAD (Table 2.1). Contrarily, VAO contains an 8α-*N*3-histidyl FAD cofactor.

Table 2.1. (Predicted) FAD-binding mode of VAO family members

Enzyme	EC nummer	Flavin
Histidyl-FAD enzymes		
Vanillyl-alcohol oxidase[1]	1.1.3.38	8α-*N*3-histidyl FAD
Eugenol oxidase[2]	1.1.3.x	8α-*N*3-histidyl FAD[a]
6-hydroxy-D-nicotine oxidase[3]	1.5.3.6	8α-*N*1-histidyl FAD[b]
Cholesterol oxidase[4]	1.1.3.6	8α-*N*1-histidyl FAD
D-arabinono-1,4-lactone oxidase[5]	1.1.3.37	8α-*N*1-histidyl FAD
L-gulono-1,4-lactone oxidase[6]	1.1.3.8	8α-*N*1-histidyl FAD
D-gluconolactone oxidase[7]	1.1.3.x	8α-*N*3-histidyl FAD[c]
Alditol oxidase[8]	1.1.3.x	8α-*N*1-histidyl FAD
Cytokinin dehydrogenase[9]	1.5.99.12	8α-*N*1-histidyl FAD
Cysteinyl-histidyl FAD enzymes		
Glucooligosaccharide oxidase[10]	1.1.3.x	6-*S*-cysteinyl, 8α-*N*1-histidyl FAD
Chitooligosaccharide oxidase[11]	1.1.3.x	6-*S*-cysteinyl, 8α-*N*1-histidyl FAD[d]
Hexose oxidase[12]	1.1.3.5	6-*S*-cysteinyl, 8α-*N*1-histidyl FAD
S-reticuline oxidase[13]	1.21.3.3	6-*S*-cysteinyl, 8α-*N*1-histidyl FAD
Aclacinomycin oxidoreductase[14]	1.1.3.x	6-*S*-cysteinyl, 8α-*N*1-histidyl FAD
Dbv29 (glycopeptide hexose oxidase)[15]	1.1.3.x	6-*S*-cysteinyl, 8α-*N*1-histidyl FMN
Other covalent linkages		
p-cresol methylhydroxylase[16]	1.17.99.1	8α-*O*-tyrosyl FAD
Eugenol hydroxylase[17]	1.17.99.x	8α-*O*-tyrosyl FAD[e]
Non-covalent flavoenzymes		
Alkyldihydroxyacetonephosphate synthase[18]	2.5.1.26	FAD
D-lactate dehydrogenase[19]	1.1.1.28	FAD
L-galactono-1,4-lactone dehydrogenase[20]	1.3.2.3	FAD
UDP-*N*-acetylenolpyruvylglucosamine reductase[21]	1.1.1.158	FAD

[a]Prediction from homology modeling with VAO (Jin et al., 2007), [b]Originally identified as 8α-*N*3-histidyl FAD (Möhler et al., 1972), [c]Prediction from amino acid sequence gives 8α-*N*1-histidyl FAD, [d]Prediction from homology modeling with GOOX (Heuts et al., 2007[b]), [e]Prediction from amino acid sequence gives 8α-*O*-tyrosyl FAD.

References used: 1, (de Jong et al., 1992; Mattevi et al., 1997); 2, (Jin et al., 2007); 3, (Koetter and Schulz, 2005); 4, (Coulombe et al., 2001); 5, (Kenney et al., 1979); 6, (Kenney et al., 1976); 7, (Harada et al., 1979); 8, (Forneris et al., 2008); 9, (Frébortová et al., 2004; Malito et al., 2004); 10, (Huang et al., 2005); 11, (Heuts et al., 2007[b]); 12, (Rand et al., 2006); 13, (Winkler et al., 2006); 14, (Alexeev et al., 2007); 15, (Li et al., 2007); 16, (Mathews et al., 1991); 17, (Priefert et al., 1999); 18, (Razeto et al., 2007); 19, (Dym et al., 2000); 20, (Leferink et al., 2008[b]); 21, (Benson et al., 1995).

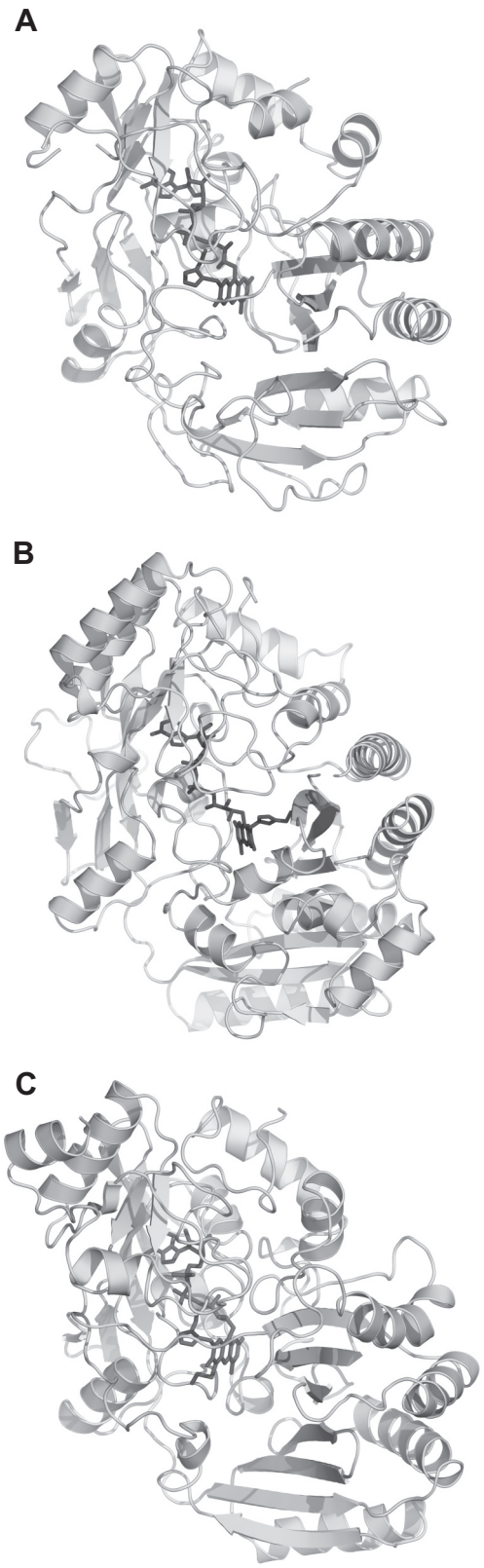


Figure 2.1. Covalent flavin-protein linkages in the VAO fold. (A) Crystal structure of alditol oxidase with 8α-*N*1-histidyl FAD (Forneris et al., 2008). (B) 3D model of eugenol oxidase with 8α-*N*3-histidyl FAD (Jin et al., 2007). (C) 3D model of chitooligosaccharide oxidase with 6-*S*-cysteinyl-8α-*N*1-histidyl FAD (Heuts et al., 2007[b]).

Recently discovered VAO members containing a histidyl-FAD include cytokinin dehydrogenase, alditol oxidase and eugenol oxidase. Cytokinin dehydrogenase (CKX) is involved in the enzymatic degradation of cytokinins (N^6-substituted purine derivatives), which play a major role in growth regulation in plants. The cytokinin dehydrogenase reaction involves the oxidation of the secondary amine group on the side-chain of the adenine ring resulting in cleavage of the side chain (Fig. 2.2A). The enzyme was initially classified as an oxidase but oxygen is only a poor substrate, a range of quinones have been identified as efficient electron acceptors (Frébortová et al., 2004). Based on this, CKX has now been classified as a dehydrogenase. The crystal structure of maize CKX1 complexed with a cytokinin imine suggests that the product prevents the reduced flavin to react with molecular oxygen (Malito et al., 2004; Mattevi, 2006). Typically, plant genomes contain a multitude of CKX genes.

Most VAO-type proteins containing a His-FAD cofactor have been found to act as an oxidase. This correlation has been used to discover novel covalent flavoprotein oxidases by genome database mining. An example of this approach of enzyme discovery is the recent identification of alditol oxidase (AldO)

from *Streptomyces coelicolor* A3 (Heuts et al., 2007[a]). AldO catalyzes the oxidation of the C1 hydroxyl group of preferably alditols into the corresponding aldehydes (Fig. 2.2B).

AldO is closely related to xylitol oxidase and sorbitol oxidase from other *Streptomyces* isolates (Hiraga et al., 1998; Yamashita et al., 2000) and all three oxidases display overlapping substrate specificities. AldO is most active with xylitol and sorbitol, which are converted into D-xylose and D-glucose, respectively (Heuts et al., 2007[a]). The enzyme is an intracellular monomeric protein with a molecular mass of 45 kDa and could be expressed in impressive quantities (350 mg per liter culture) using *Escherichia coli* as expression host (Heuts et al., 2007[a]). The recent elucidation of the crystal structure of AldO confirmed that the FAD cofactor is covalently tethered to a histidine residue (His46) via an 8α-*N*1-histidyl FAD linkage (Forneris et al., 2008). The His46Ala mutant was expressed as the apoprotein in *E. coli* and found to be partially insoluble. This suggests that the covalent flavin-protein interaction is crucial for the structural integrity of AldO (Heuts et al., 2007[a]). Crystal structures of AldO complexed with alditols have revealed the molecular basis for the selective oxidation reactions catalyzed by AldO. By an extensive hydrogen bond network, alditols are positioned with respect to the flavin cofactor in such a way that only one terminal hydroxyl group is oxidized.

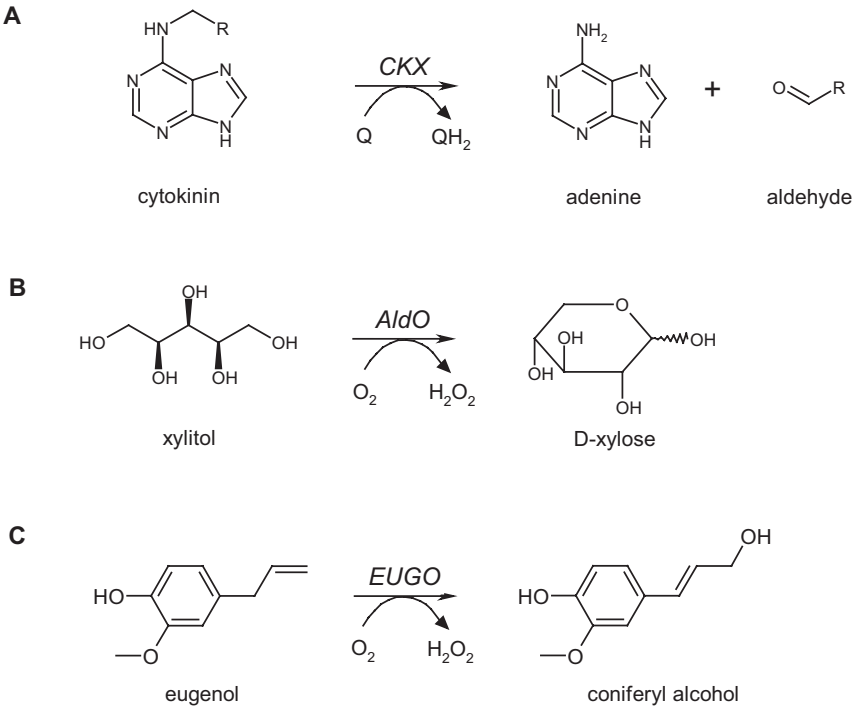


Figure 2.2. Reactions catalyzed by the histidyl-FAD enzymes CKX (A), AldO (B) and EUGO (C).

A detailed kinetic analysis has shown that AldO employs a ternary complex kinetic mechanism for the oxidation of xylitol (K_m = 320 μM, k_{cat} = 13 s^{-1}). Xylitol rapidly reduces the FAD cofactor (k_{red} = 99 s^{-1}) upon which a binary complex is formed between reduced AldO and D-xylose. While complexed with the oxidation product, the flavin cofactor is able to utilize molecular oxygen as electron acceptor (k_{ox} = 1.4 × 10^5 M^{-1} s^{-1}). Besides xylitol and related alditols, AldO also converts other aliphatic alcohols. AldO shows considerable sequence and structural homology with cholesterol oxidase, but does not accept bulky hydrophobic substrates. This can be explained by the narrow binding pocket near the flavin cofactor which determines the substrate specificity.

Another VAO-type histidyl-FAD containing oxidase that was recently discovered by utilizing genome sequence information is eugenol oxidase (Jin et al., 2007). By analyzing the available bacterial genome sequences it was found that the proteome of the actinomycete *Rhodococcus* sp. RHA1 harbors a protein that shows 45% sequence identity to fungal VAO. The presence of a conserved histidine in its C-terminal region suggested that also in this protein, the FAD is covalently linked as in VAO. Indeed, heterologous expression in *E. coli* resulted in overproduction of flavinylated enzyme. Part of the recombinant protein was purified as the apoprotein. Addition of FAD to the purified protein resulted in fully flavinylated protein. This again confirms the autocatalytic mechanism of covalent FAD incorporation. Characterization of the enzyme revealed that it is most active as oxidase with eugenol (K_m = 1.0 μM, k_{cat} = 3.1 s^{-1}) and therefore it was named eugenol oxidase (EUGO) (Fig 2.2C).

VAO and EUGO accept similar substrates, but with significantly different catalytic efficiencies. E.g., while VAO is able to effectively hydroxylate 4-alkylphenols and to deaminate aromatic amines, EUGO is poorly active on these substrates. Another striking difference between VAO and EUGO is their oligomerization state: while VAO forms stable octamers, EUGO is a dimeric enzyme. Inspection of the VAO structure and sequence comparison suggests that VAO has acquired a specific loop region that creates dimer-dimer interactions and thereby stabilizes octamers. This loop region is absent in the EUGO sequence. A nice feature, when compared with VAO, is the fact that EUGO is expressed in large quantities in *E. coli* facilitating production of this novel oxidase.

The substrate specificities of VAO and EUGO have some resemblance to that of the flavocytochromes PCMH (McIntire et al., 1985) and eugenol dehydrogenase (Furukawa et al., 1998). The latter enzyme is also referred to as eugenol hydroxylase (Priefert et al., 1999). Based on sequence comparisons (Kim et al., 1995; Priefert et al., 1999; Brandt et al., 2001) eugenol hydroxylase is predicted to contain 8α-*O*-tyrosyl FAD.

Histidyl-cysteinyl-FAD enzymes

Only very recently it was discovered that in some flavoproteins the flavin cofactor is covalently attached to two amino acid residues. This bi-covalent linkage of FAD was first revealed in 2005 by elucidating the crystal structure of glucooligosaccharide oxidase (Huang et al., 2005). Glucooligosaccharide oxidase preferably oxidizes cellooligosaccharides, that can reach the active site via an open carbohydrate binding groove. In this fungal carbohydrate oxidase the FAD is tethered to Cys130 and His70 via respectively the 6- and 8α-position of the isoalloxazine ring (Fig. 2.3).

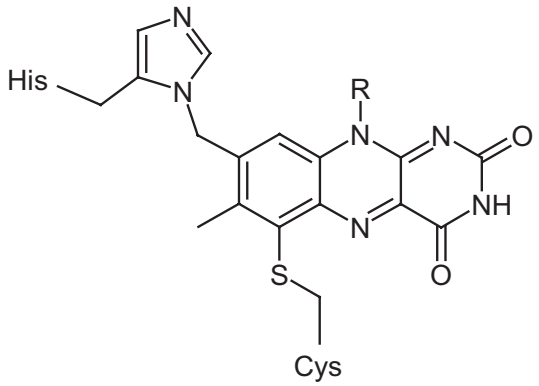


Figure 2.3. Bi-covalent FAD linkage in glucooligosaccharide oxidase: 6-*S*-cysteinyl, 8α-*N*1-histidyl-FAD (Huang et al., 2005).

Based on this finding, it was recognized that also other established covalent flavoproteins may contain a bi-covalent FAD as cofactor. A dual covalent linkage was confirmed for hexose oxidase and *S*-reticuline oxidase (berberine bridge enzyme, BBE) (Rand et al., 2006; Winkler et al., 2006) and probably is also the case for tetrahydrocannabinolic acid synthase, an enzyme involved in controlling marijuana psychoactivity (Sirikantaramas et al., 2004). A bi-covalent FAD cofactor is also predicted for two carbohydrate oxidases from sunflower and tobacco (Carter and Thornburg, 2004; Custers et al., 2004), and for the lactose oxidase from *Microdochium nivale* (Xu et al., 2001). Apart from these known covalent flavoproteins, several new bi-covalent flavoproteins have been described recently: Dbv29, a glycopeptide hexose oxidase (Li et al., 2007), aclacinomycin oxidoreductase (AknOx) (Alexeev et al., 2007), and chitooligosaccharide oxidase (Heuts et al., 2007[b]) (Table 2.1). All above-mentioned bi-covalent flavoproteins represent VAO-type enzymes, again showing that the VAO-fold is favorable for covalent protein-flavin interactions.

The reactions catalyzed by some bi-covalent flavoproteins demonstrate the unusual catalytic power of these enzymes. BBE for example, catalyzes the oxidative cyclization of the *N*-methyl group of *S*-reticuline in the benzophenanthridine alkaloid biosynthesis in plants

(Kutchan and Dittrich, 1995). This reaction involves two steps, the oxidation of the methylene iminium ion and the stereospecific ring closure forming the berberine bridge of *S*-scoulerine (Fig. 2.4A).

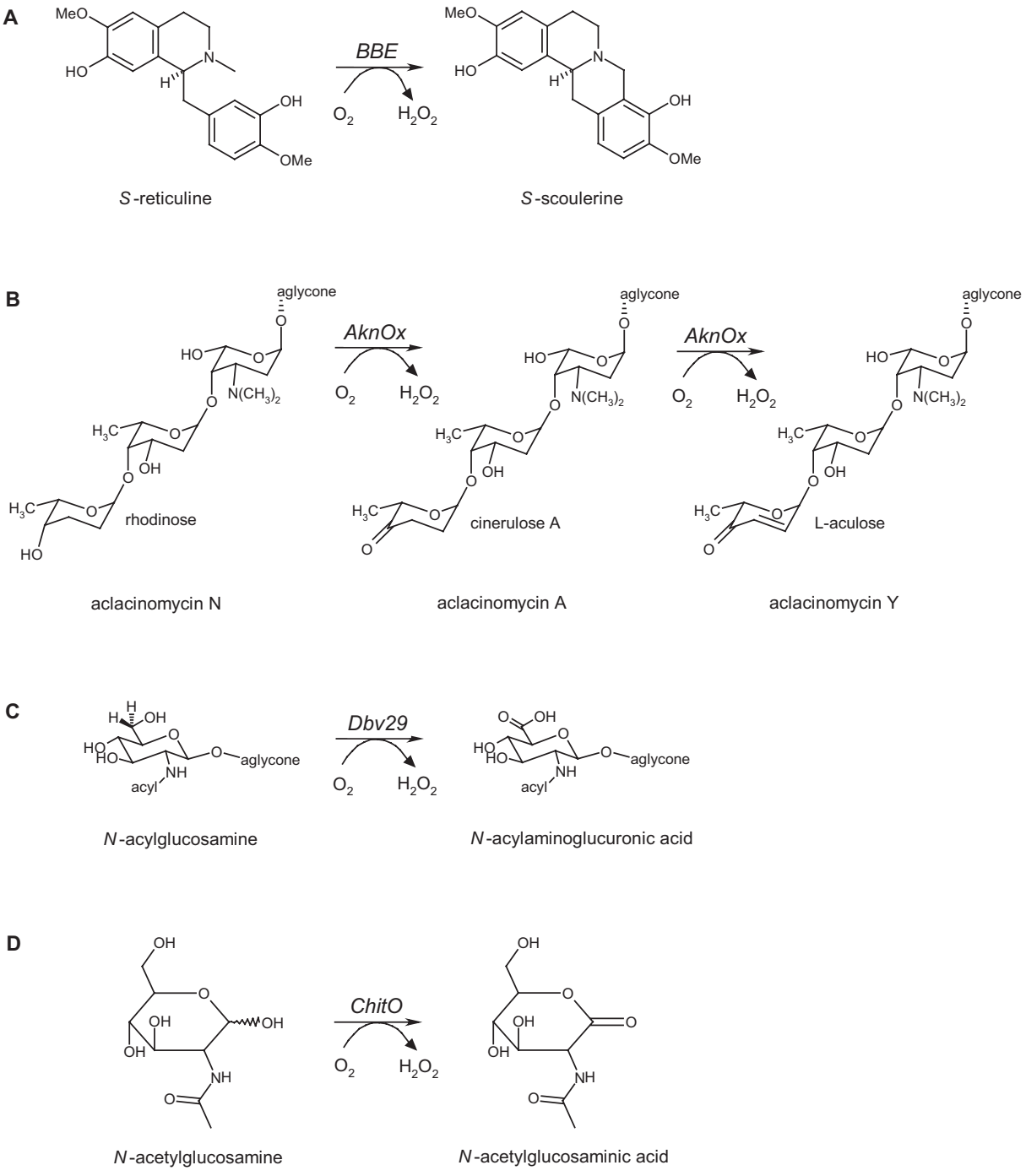


Figure 2.4. Reactions catalyzed by the bi-covalent flavoproteins BBE (A), AknOx (B), Dbv29 (C), and ChitO (D).

Another unusual bi-covalent flavoprotein is AknOx, which catalyzes the last two steps in the biosynthesis of the polyketide antibiotic aclacinomycin in *Streptomyces* species. AknOx uses the same active site to catalyze these two consecutive FAD-dependent reactions but uses two distinct sets of catalytic residues to accomplish this. The first reaction involves the oxidation of the terminal sugar residue rhodinose into cinerulose A, which, in the second reaction, is converted to L-aculose by a desaturation step (Fig. 2.4B) (Alexeev et al., 2007).

The glycopeptide hexose oxidase Dbv29 is the first reported FMN-containing bi-covalent flavoprotein. Dbv29 catalyzes the four-electron oxidation of the *N*-acylglucosaminyl substituent to *N*-acylaminoglucuronic acid during the maturation of the glycopeptide A40926 antibiotic (Fig. 2.4C) (Li et al., 2007).

As part of another genome database mining effort a chitooligosaccharide oxidase (ChitO) from *Fusarium graminearum* was discovered and investigated (Heuts et al., 2007[b]). In contrast to glucooligosaccharide oxidase, ChitO is highly active with *N*-acetylated mono- and oligosaccharides (Fig. 2.4D). Chitotetraose was found to be the best substrate (K_m = 250 μM, k_{cat} = 6.3 s^{-1}). ChitO also converts non-modified saccharides, e.g. glucose, cellobiose and lactose, but with a much lower catalytic efficiency. Based on the sequence similarity with glucooligosaccharide oxidase, a structural model of ChitO was constructed. This enabled the identification of residues that form the chitooligosaccharide-binding pocket. Based on this model, a specific glutamine residue was replaced by an arginine. As predicted, the engineered protein showed a somewhat lower affinity for *N*-acetylated substrates while the catalytic efficiency for non-acetylated carbohydrates improved ~20 fold (Heuts et al., 2007[b]). This confirms that the respective residue is crucial for the recognition of specific classes of oligosaccharides. The availability of recombinant ChitO provides new opportunities to perform selective modifications of (oligo)saccharides.

So far, little is known about the biological significance of bi-covalent flavinylation. Single mutants of Dbv29 (H91A or C151A) showed comparable activity to wild type, while the double mutant retained only 10% activity (Li et al., 2007). From this it was argued that the bi-covalent linkage is required for a proper orientation of the flavin in the active site. The bi-covalent link in *S*-reticuline oxidase was also studied by mutagenic analysis (Winkler et al., 2007). The C166A variant showed an impaired flavin reduction rate of 370-fold and a decrease in redox potential, +53 mV vs. +132 mV for the wild-type enzyme. His104 protein variants could not be expressed in sufficient amounts for biochemical studies. From these data it was concluded that 6-*S*-cysteinylation of the flavin tunes the redox potential. In fact, the redox potential of wild-type *S*-reticuline oxidase is exceptionally high which is in line with the proposed rationale of covalent flavinylation, i.e. increasing the oxidative power of the respective enzyme.

Genomic data indicate that many new bi-covalent flavoenzymes still need to be discovered. Several of these hypothetical enzymes are found, for instance, in *Streptomyces* species (Alexeev et al., 2007) and in plants.

Non-covalently bound FAD enzymes

Within the VAO family also members exist that bind their flavin cofactor non-covalently (Table 2.1). Generally the His residue in the conserved sequence region of the FAD-binding domain is lacking in these enzymes, and they do not react with molecular oxygen. For ADPS, D-lactate dehydrogenase, and MurB the crystal structures have been solved (Benson et al., 1995; Dym et al., 2000; Razeto et al., 2007). ADPS catalyzes ether bond formation in phospholipids, the constituents of eukaryotic cell membranes (Fig. 2.5A). The flavin cofactor is presumed to be involved in this non-redox reaction by trapping the dihydroxyacetonephosphate intermediate via covalent binding (Razeto et al., 2007).

The peripheral membrane protein D-lactate dehydrogenase from *E. coli* catalyzes the oxidation of D-lactate to pyruvate, using quinone as electron acceptor. Besides the FAD-binding domain and the cap-domain, the enzyme also harbors a membrane-binding domain which interacts with the negatively charged phospholipid head groups of the membrane (Dym et al., 2000).

MurB is involved in peptidoglycan biosynthesis in bacteria, it catalyzes the reduction of enolpyruvyl-UDP-*N*-acetylglucosamine to UDP-*N*-acetylmuramic acid using NADPH as electron donor (Fig. 2.5B). The MurB structure consists of three domains, the typical VAO-type FAD-binding domain, and two additional domains comprising the substrate binding site (Benson et al., 1995).

L-Galactono-1,4-lactone dehydrogenase (GALDH) is another VAO family member that binds the FAD in a non-covalent mode. This aldonolactone oxidoreductase is responsible for completing the biosynthesis of vitamin C in plants (Fig. 2.5C).

GALDH has been isolated for the first time from cauliflower mitochondria by Mapson and Breslow in the 1950s (Mapson and Breslow, 1958). Since then the enzyme was isolated from the mitochondria from a number of plants (Mutsuda et al., 1995; Ôba et al., 1995; Imai et al., 1998; Siendones et al., 1999). More recently, recombinant forms have become available for cauliflower, tobacco and *Arabidopsis thaliana* GALDH (Østergaard et al., 1997; Yabuta et al., 2000; Leferink et al., 2008[b]). GALDH homologs in animals (L-gulono-1,4-lactone oxidase), yeast (D-arabinono-1,4-lactone oxidase), and fungi (D-gluconolactone oxidase), are involved in the synthesis of L-ascorbate or its analogs D-erythorbate and D-erythroascorbate (Nishikimi et al., 1976; Huh et al., 1998; Salusjärvi et al., 2004). In contrast to GALDH, these

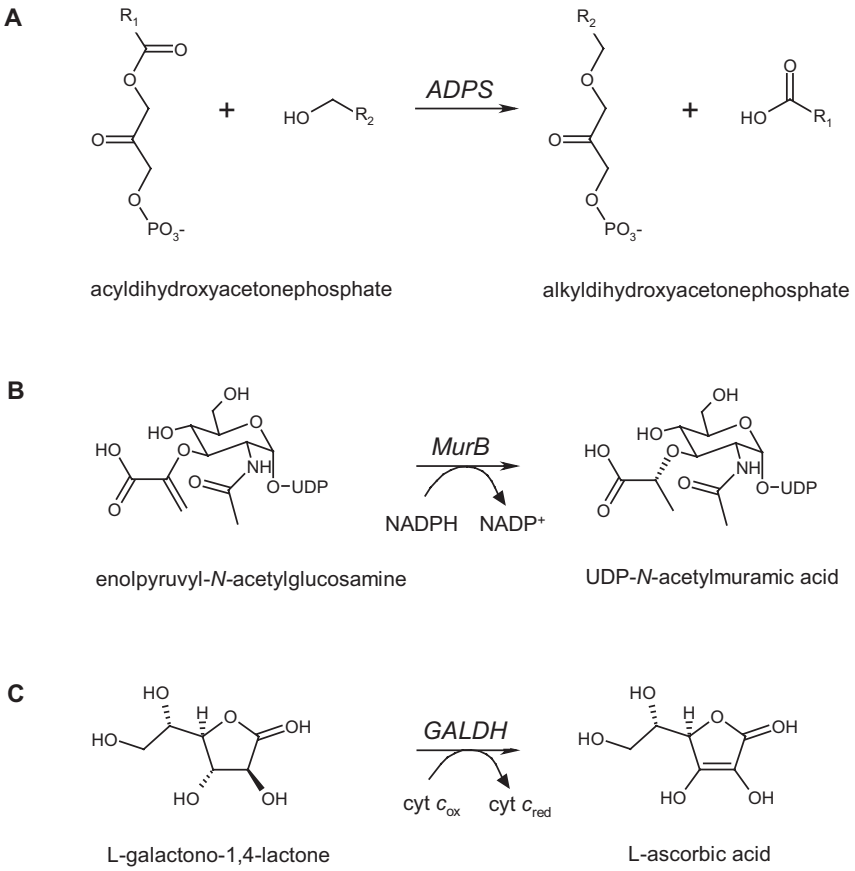


Figure 2.5. Reactions catalyzed by the non-covalent VAO members ADPS (A), MurB (B) and GALDH (C).

oxidases contain a covalently bound FAD (Table 2.1). Recently also a bacterial gulonolactone dehydrogenase (Wolucka and Communi, 2006) and two aldonolactone oxidases from trypanosome parasites (Wilkinson et al., 2005; Logan et al., 2007) have been identified.

The aldonolactone oxidoreductases form a separate clade in the VAO family (Fig. 2.6). The plant GALDH enzymes share about 80-90% sequence identity but they have less than 25% of sequence identity with other aldonolactone oxidoreductases. The highest degree of sequence identity within this sub-family is found in the FAD-binding domain. No crystal structure is available for GALDH or its homologs and little is known about the nature of the active site and the catalytic mechanism. A recurrent feature within the group of aldonolactone oxidoreductases is the sensitivity towards thiol reactive compounds, suggesting the involvement of cysteine residues in catalysis (Mapson and Breslow, 1958; Nakagawa and Asano, 1970; Huh et al., 1994; Ôba et al., 1995; Logan et al., 2007).

GALDH is presumably localized in the mitochondrial intermembrane space associated with mitochondrial complex I (Heazlewood et al., 2003) where it shuttles electrons into the electron transport chain via cytochrome *c* (Millar et al., 2003). Mature GALDH from *A. thaliana* (AtGALDH) can be efficiently produced in *E. coli* when omitting the N-terminal

mitochondrial targeting sequence (Leferink et al., 2008[b]). The monomeric protein shows a high enantio-preference for L-galactono-1,4-lactone (K_m = 0.17 mM, k_{cat} = 134 s^{-1}), though the L-gulono-1,4-lactone isomer is also oxidized at significant rate (K_m = 13.1 mM, k_{cat} = 4 s^{-1}). Thus, a difference in orientation of the 3-hydroxyl group of the substrate is responsible for a 100-fold higher K_m and 2500-fold lower catalytic efficiency.

Most aldonolactone oxidoreductases use molecular oxygen as electron acceptor and contain a covalently bound histidyl-FAD (Table 2.1) (Coulombe et al., 2001; Koetter and Schulz, 2005; Heuts et al., 2007[a]). Plant GALDH lacks this His-residue but contains a Leu instead. Replacement of Leu56 in AtGALDH by His did not result in covalent incorporation of FAD. Instead, FAD was more weakly bound in the mutant than in the wild-type protein (Leferink et al., 2008[b]). Replacing Leu56 with Ala or Cys also yielded variants with loosely bound FAD. Variants L56I and L56F were similar to wild-type AtGALDH, and contained tightly bound FAD. The apo forms of the Leu56 variants could easily be reconstituted by the addition of FAD and their flavin absorption properties were nearly identical to that of the wild-type enzyme. The kinetic parameters of the Leu56 variants were determined and a rough correlation was found between the bulkiness of the residue at position 56 and the Michaelis-constant for L-galactono-1,4-lactone. The absence of a covalent flavin-protein link in the L56H variant might be explained by the absence of an activating base similar to His61 in VAO (Fraaije et al., 2000), which is required for nucleophilic attack. The presence of such an activating residue is not easily predicted due to the lack of structural information for the aldonolactone oxidoreductase subfamily.

The mechanism of L-ascorbate production by AtGALDH involves two half-reactions. In the reductive half-reaction, the oxidized flavin cofactor is reduced to the hydroquinone state by the L-galactono-1,4-lactone substrate. The two-electron reduced enzyme is then re-oxidized in the oxidative half-reaction by cytochrome *c*. This oxidative half-reaction involves two one-electron steps and the transient formation of the red anionic flavin semiquinone (Leferink et al., 2008[b]). The catalytic mechanism of AtGALDH was studied with the stopped-flow technique. The reduction of the flavin by L-galactono-1,4-lactone appears to be the rate limiting step in the catalytic cycle (k_{red} = 750 s^{-1}). The re-oxidation by cytochrome *c* in the oxidative half-reaction occurs relatively fast, with a bimolecular rate constant of 3.6×10^6 M^{-1} s^{-1}, four orders of magnitude faster than the re-oxidation by molecular oxygen ($k_{ox} = 4.4 \times 10^2$ M^{-1} s^{-1}).

Trypanosome parasites also contain an aldonolactone oxidoreductase that harbors a non-covalently bound flavin cofactor and uses cytochrome *c* as electron acceptor. The arabinonolactone oxidase from *Trypanosoma brucei* (TbALO) has a preference for substrates in which the C2 and C3 hydroxyl groups are arranged in the *trans*-configuration, besides

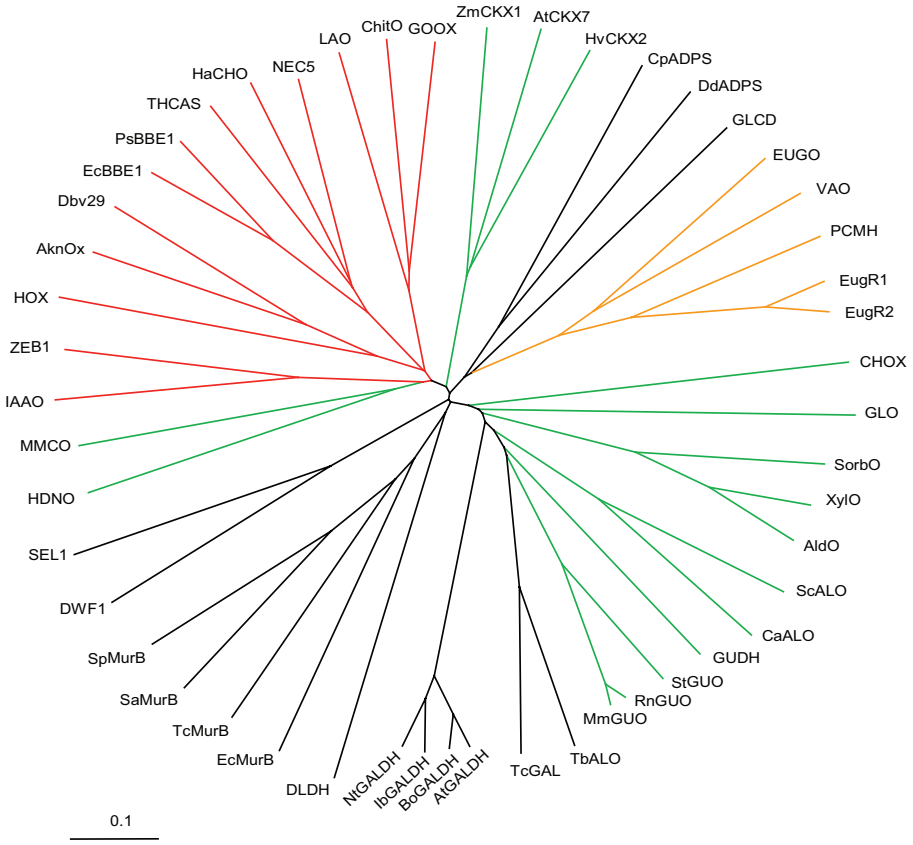


Figure 2.6. Phylogenetic analysis of characterized VAO family members. The phylogenetic tree was constructed from a ClustalX multiple sequence alignment using the neighbor-joining method. (Predicted) covalent flavin linkages are highlighted. Linkages to the N-terminal loop are 8α-*N*1-histidyl in green and 6-*S*-cysteinyl-8α-*N*1-histidyl in red. Covalent linkages to the C-terminal loop are highlighted in orange.

The accession numbers for the sequences used for phylogenetic analysis are: HDNO, P08159; AknOx, Q0PCD7; AldO, Q9ZBU1; *Dictyostelium discoideum* ADPS (DdADPS), O96759; *Cavia porcellus* ADPS (CpADPS), P97275; ChitO, XP_391174; cholesterol oxidase (CHOX), Q7SID9; *Zea mays* CKX 1 (ZmCKX1), Q9T0N8; *A. thaliana* CKX 7 (AtCKX7), Q9FUJ1; *Hordeum vulgare* CKX 2 (HvCKX2), Q8H6F6; *Saccharomyces cerevisiae* arabinonolactone oxidase (ScALO), P54783, *Candida albicans* ALO (CaALO), O93852; Dbv29, Q7WZ62; D-gluconolactone oxidase (GLO), Q671X8; D-lactate dehydrogenase (DLDH), P06149; DWARF1/DIMINUTO (DWF1), Q39085; Seladin-1 (24-dehydrocholesterol reductase, SEL1), Q15392; EUGO, Q9RDU1; *Pseudomonas* sp. HR199 eugenol hydroxylase flavoprotein subunit (EugR1), AAM21269; *Pseudomonas* sp. OPS1 eugenol hydroxylase flavoprotein subunit (EugR2), Q0SBK1; glucooligosaccharide oxidase (GOOX), Q6PW77; glycolate oxidase subunit D (GLCD), P0AEP9; gulonolactone dehydrogenase (GUDH), O06804; hexose oxidase (HOX), P93762; isoamyl alcohol oxidase (IAAO), Q9HGH9; lactose oxidase (LAO), CAI94231-2; AtGALDH, Q8GY16; *Brassica oleracea* GALDH (BoGALDH), O47881; *Ipomoea batatas* GALDH (IbGALDH), Q9ZWJ1; *Nicotiana tabacum* GALDH (NtGALDH), Q9SLW6; *Rattus norvegicus* gulonolactone oxidase (RnGUO), P10867; *Mus musculus* GUO (MmGUO), NP_848862; *Scyliorhinus torazame* GUO (StGUO), Q90YK3; mitomycin radical oxidase (MMCO), P43485; PCMH flavoprotein subunit, P09788; polykethide synthase (ZEB1), Q2VLJ1; *Eschscholzia californica* BBE (EcBBE1), P30986; *Papaver somniferum* BBE (PsBBE1), P93479; sorbitol oxidase (SorbO), P97011; sunflower carbohydrate oxidase (HaCHO), Q8SA59; tetrahydrocannabinolic acid synthase (THCAS), Q8GTB6; tobacco glucose oxidase (NEC5), Q84N20; TbALO, Q57ZU1; TcGAL, Q4DPZ5; *E. coli* MurB (EcMurB), P08373; *Staphylococcus aureus* MurB (SaMurB), P61431; *Streptococcus pneumoniae* MurB (SpMurB), P65466; *Thermus cadophilus* MurB (TcMurB), Q5SJC8; VAO, P56216; xylitol oxidase (XylO), Q9KX73. The bar indicates 10% divergence.

L-galactono-1,4-lactone (K_m = 154 μM, k_{cat} = 21 s^{-1}) it can also oxidize D-arabinono-1,4-lactone (K_m = 55 μM, k_{cat} = 27 s^{-1}) (Wilkinson et al., 2005). Galactonolactone oxidase from *Trypanosoma cruzi* (TcGAL) also utilizes both L-galactono-1,4-lactone (K_m = 161 μM, k_{cat} = 673 s^{-1}) and D-arabinono-1,4-lactone (K_m = 285 μM, k_{cat} = 649 s^{-1}) (Logan et al., 2007). Considering that L-galactono-1,4-lactone is the presumed physiological substrate (Wilkinson et al., 2005; Logan et al., 2007) and that cytochrome *c* is employed as electron acceptor, TbALO and TcGAL should be re-named as galactonolactone dehydrogenases.

Both TcGAL and TbALO are proposed to possess a non-covalently bound FMN as cofactor (Logan et al., 2007). This would be the first members (together with Dbv29 (Li et al., 2007)) of the VAO-family that contain FMN rather than FAD as the prosthetic group. However, comparing the sequences of TcGAL and TbALO with other VAO family members reveals that the residues that normally interact with the pyrophosphate and adenine moiety of the FAD cofactor (Fraaije et al., 1998) are conserved in the trypanosomal enzymes. Therefore, more research is needed to confirm that indeed FMN is bound to these enzymes rather than FAD. Both TbALO and TcGAL lack the His residue in the FAD-binding domain involved in covalent binding of the cofactor, but contain a Lys residue instead (Wilkinson et al., 2005; Logan et al., 2007). Replacement of Lys55 of TcGAL by His or Leu yields mutants that are isolated as apoproteins (Logan et al., 2007). It is not clear whether the isolated apoproteins can be reconstituted to the holo form, as is the case for the AtGALDH Leu56 mutants. Interestingly, a lysine residue in the C-terminal HWXK motif, conserved in all aldonolactone oxidoreductases and some related oxidases, is thought to be involved in catalysis of TcGAL. Replacement of this Lys by Gly (K450G) renders the protein completely inactive, though flavin is still bound (Logan et al., 2007).

Although there are no structural rules that enable prediction of whether or how a flavoenzyme reacts with oxygen (Mattevi, 2006), it is evident that structural data of the aldonolactone oxidoreductase subfamily would be beneficial in future research towards the elucidation of the molecular determinants of (covalent) flavin binding and oxygen reactivity.

Acknowledgments

This research was supported by the Carbohydrate Research Centre Wageningen (CRC-W) and the IBOS (Integration of Biosynthesis and Organic Synthesis) program of the Netherlands Organization for Scientific Research (NWO).

chapter 3

L-Galactono-γ-lactone dehydrogenase from *Arabidopsis thaliana*, a flavoprotein involved in vitamin C biosynthesis

Nicole G. H. Leferink[1], Willy A. M. van den Berg[1], Willem J. H. van Berkel[1]

FEBS Journal (2008) 275(4):713-726

[1] Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

Abstract

L-Galactono-γ-lactone dehydrogenase (GALDH) is a mitochondrial flavoenzyme that catalyzes the final step in the biosynthesis of vitamin C (L-ascorbic acid) in plants. Here we report on the biochemical properties of recombinant *Arabidopsis thaliana* GALDH. AtGALDH oxidizes besides L-galactono-1,4-lactone (K_m = 0.17 mM, k_{cat} = 134 s^{-1}) also L-gulono-1,4-lactone (K_m = 13.1 mM, k_{cat} = 4.0 s^{-1}) using cytochrome *c* as electron acceptor. Aerobic reduction of AtGALDH with the lactone substrate generates the flavin hydroquinone. The two-electron reduced enzyme reacts poorly with molecular oxygen ($k_{ox} = 6 \times 10^2$ M^{-1} s^{-1}). Unlike most flavoprotein dehydrogenases, AtGALDH forms a flavin-N5 sulfite adduct. Anaerobic photoreduction involves the transient stabilization of the anionic flavin semiquinone. Most aldonolactone oxidoreductases contain a histidyl-FAD as covalently bound prosthetic group. AtGALDH lacks the histidine involved in covalent FAD binding, but contains a leucine instead (Leu56). Leu56 replacements did not result in covalent flavinylation but revealed the importance of Leu56 for both FAD-binding and catalysis. The Leu56 variants showed remarkable differences in Michaelis constants for both L-galactono-1,4-lactone and L-gulono-1,4-lactone and released their FAD cofactor more easily than wild-type AtGALDH. This study provides the first biochemical characterization of AtGALDH and some active site variants. The role of GALDH and possible involvement of other aldonolactone oxidoreductases in the biosynthesis of vitamin C in *A. thaliana* are also discussed.

Keywords: *Arabidopsis thaliana*; flavoprotein; L-galactono-1,4-lactone dehydrogenase; site-directed mutagenesis; vitamin C biosynthesis

Abbreviations: ALO, D-arabinono-1,4-lactone oxidase; AtGALDH, *Arabidopsis thaliana* L-galactono-1,4-lactone dehydrogenase; GALDH, L-galactono-1,4-lactone dehydrogenase; GLO, D-gluconolactone oxidase; GSH, reduced glutathione; GUDH, L-gulono-1,4-lactone dehydrogenase; GUO, L-gulono-1,4-lactone oxidase; IPTG, isopropyl thio-β-D-galactopyranoside; Ni-NTA, nickel nitrilotriacetic acid; VAO, vanillyl-alcohol oxidase

Introduction

L-Ascorbic acid (vitamin C) is an important antioxidant, redox buffer and an enzyme cofactor for many organisms. Plants and most animals can synthesize L-ascorbic acid to their own requirements, but humans and other primates have lost this ability during evolution. L-Ascorbic acid is particularly abundant in plants (millimolar concentrations) where it protects cells from oxidative damage resulting from abiotic stresses and pathogens and it is a cofactor for a number of enzymes (Smirnoff and Wheeler, 2000). Fruits and vegetables are the main dietary source of vitamin C for humans.

L-Ascorbic acid and its fungal analogues D-erythroascorbic acid and D-erythorbic acid are produced from hexose sugars. The final step in the biosynthesis of these compounds is catalyzed by so-called sugar-1,4-oxidoreductases or aldonolactone oxidoreductases. These enzymes contain a conserved FAD-binding domain present in the vanillyl-alcohol oxidase (VAO) family of flavoproteins (Fraaije et al., 1998).

In animals, microsomal L-gulono-γ-lactone oxidase (GUO) catalyzes the oxidation of L-gulono-1,4-lactone into L-ascorbate (Linster and van Schaftingen, 2007). Humans are deficient in GUO, the *guo* gene is highly mutated, hence ascorbate is a vitamin for man (Nishikimi et al., 1994). In yeasts D-arabinono-1,4-lactone is converted to D-erythroascorbic acid by a mitochondrial D-arabinono-γ-lactone oxidase (ALO) (Huh et al., 1998) and in fungi extracellular D-gluconolactone oxidase (GLO) produces D-erythorbic acid from D-gluconolactone (Salusjärvi et al., 2004). Recently also a mycobacterial gulonolactone dehydrogenase (Wolucka and Communi, 2006) and two aldonolactone oxidases from trypanosome parasites (Wilkinson et al., 2005; Logan et al., 2007) have been identified. The substrate specificity of the aldonolactone oxidoreductases varies considerably; GUO and ALO, for example, can both oxidize various aldonolactones (Kiuchi et al., 1982; Huh et al., 1994), but plant L-galactono-γ-lactone dehydrogenase (GALDH) is highly specific for L-galactono-1,4-lactone (Ôba et al., 1995; Østergaard et al., 1997; Yabuta et al., 2000).

The biosynthesis of L-ascorbic acid in plants comprises multiple routes (Fig. 3.1), but not all enzymes involved have yet been discovered. The majority of the L-ascorbic acid pool is synthesized via the so-called Smirnoff-Wheeler pathway (Smirnoff and Wheeler, 2000). Recently, the last unknown enzyme from this pathway, responsible for the conversion of GDP-L-galactose into L-galactose-1-phosphate, has been identified (Linster et al., 2007). Part of the L-ascorbic acid pool is synthesized via D-galacturonic acid, a principal component of cell wall pectins (Agius et al., 2003). Also part of the "animal pathway" with L-gulono-1,4-lactone as the final precursor, seems to be operating in plants, but the enzymes involved have not yet been identified (Davey et al., 1999; Wolucka and van Montagu, 2003).

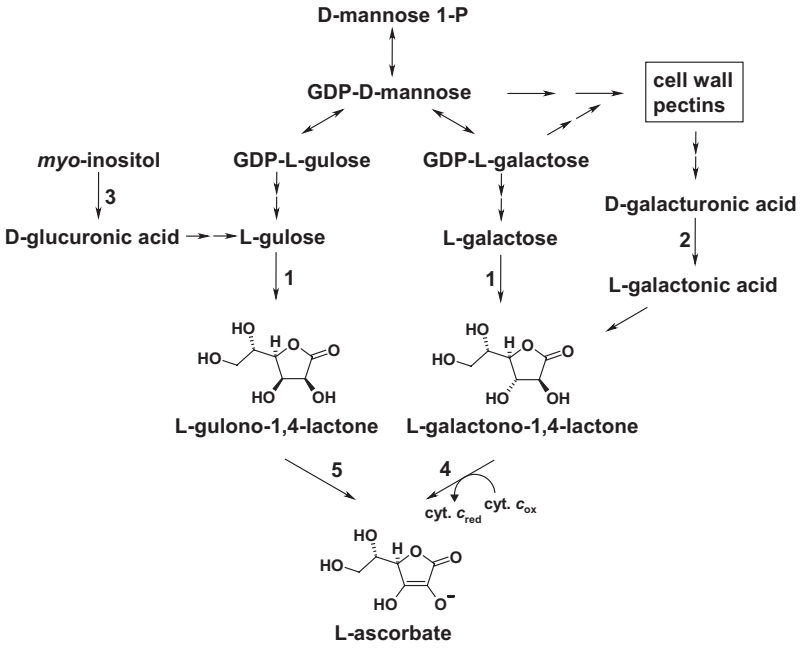


Figure 3.1. Proposed routes towards L-ascorbate biosynthesis in plants (Valpuesta and Botella, 2004; Ishikawa et al., 2006). Oxidoreductases involved: (1) L-galactose dehydrogenase, (2) D-galacturonic acid reductase, (3) *myo*-inositol oxygenase, (4) GALDH, (5) GALDH or an unknown GUO/GUDH.

GALDH (L-galactono-1,4-lactone: ferricytochrome *c*-oxidoreductase; EC 1.3.2.3) catalyzes the oxidation of L-galactono-1,4-lactone to L-ascorbate with the concomitant reduction of cytochrome *c* (Fig. 3.1). GALDH is presumed to be an integral membrane protein of the innermitochondrial membrane where it shuttles electrons into the electron transport chain via cytochrome *c* (Bartoli et al., 2000). GALDH has been extracted from the mitochondria of a number of plants including cauliflower (Mapson and Breslow, 1958), sweet potato (Ôba et al., 1995; Imai et al., 1998), spinach (Mutsuda et al., 1995), and tobacco (Yabuta et al., 2000). GALDH from cauliflower was expressed in yeast (Østergaard et al., 1997) and the enzyme from tobacco has been produced in *Escherichia coli* (Yabuta et al., 2000). GALDH from *Arabidopsis thaliana* has been expressed in *E. coli* as a β-galactosidase fusion protein, but no characterization of the recombinant protein was performed (Tamaoki et al., 2003).

Most aldonolactone oxidoreductases contain a covalently bound FAD, whereas plant GALDH binds the FAD cofactor in a non-covalent manner (Imai et al., 1998; Yabuta et al., 2000). Recently it was proposed that the aldonolactone oxidase from *Trypanosoma cruzi* harbors a non-covalently bound FMN as cofactor (Logan et al., 2007). Though isolated from various sources, aldonolactone oxidoreductases have been poorly characterized. The molecular determinants for the differences in cofactor binding and substrate specificity between these enzymes are unclear, no information is available about the nature of the active

site, and no three-dimensional structure for this group of flavoenzymes is available. In this study, mature GALDH from *A. thaliana* (AtGALDH) was expressed in *E. coli*, and its biochemical properties were investigated. Several AtGALDH variants were constructed to address the role of Leu56 in FAD binding.

Materials and Methods

Chemicals - Ni-NTA agarose was purchased from Qiagen (Valencia, CA, USA), and Bio-Gel P-6DG was from Bio-Rad (Hercules, CA, USA). HiLoad 26/10 Q-Sepharose HP, Superdex 200 HR 10/30, low molecular weight protein marker, pre-stained kaleidoscope protein standards, and the reference proteins catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (BSA) (68 kDa), and ovalbumin (43 kDa) were obtained from Pharmacia Biotech (Uppsala, Sweden). L-Galactono-1,4-lactone, L-gulono-1,4-lactone, D-gulono-1,4-lactone, L-mannono-1,4-lactone, D-galacturonic acid, FAD, FMN, riboflavin, reduced glutathione (GSH), nitroblue tetrazolium, 5-bromo-4-chlor-3-indolylphosphate, bovine heart cytochrome *c*, 1,4-benzoquinone, and phenazine methosulphate were from Sigma-Aldrich (St. Louis, MO, USA). D-Galactono-1,4-lactone was from Koch-Light LTD (Haverhill, Suffolk, UK). L-Ascorbic acid, glucose, and 2,6-dichlorophenolindophenol were from Merck (Darmstadt, Germany). Isopropyl thio-β-D-galactopyranoside (IPTG) and dithiothreitol (DTT) were obtained from MP biomedicals (Irvine, CA, USA). Secondary antibody conjugated to alkaline phosphatase and DNaseI were from Boehringer Mannheim GmbH (Mannheim, Gernamy). Restriction endonucleases, T4-DNA ligase, and dNTPs were purchased from Invitrogen (Carlsbad, CA, USA). Pwo DNA polymerase, glucose oxidase and Pefabloc SC were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Oligonucleotides were synthesized by Eurogentec (Liege, Belgium). The pET23a(+) expression vector and *E. coli* strain BL21(DE3) were from Novagen (San Diego, CA, USA). All other chemicals were from commercial sources and of the purest grade available.

Sequence analysis - The genome of *A. thaliana* was analyzed for the presence of GALDH and related aldonolactone oxidoreductase sequences at www.arabidopsis.org. BLAST-P analysis (www.ncbi.nlm.nih.gov/blast) was performed to find GALDH orthologs in other genomes (Altschul et al., 1997). Multiple sequence alignments were made using the Clustal W software (Thompson et al., 1994). The TargetP (www.cbs.dtu.dk/services/TargetP) and PSORT (www.psort.org) tools were used to predict the subcellular localization of AtGALDH and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) was used to predict the presence of transmembrane regions in the sequence of AtGALDH.

Cloning of AtGALDH cDNA for expression in E. coli - A 1.5 kb DNA fragment encoding mature AtGALDH (amino acids 102-610) was PCR amplified from *A. thaliana* (ecotype Columbia) seedling cDNA, using the oligonucleotides AtGALDH_fw102 (5'-GGA ATT CCA TAT GTA CGC TCC TTT ACC TGA AG-3') and AtGALDH_rv (5'-CCG CTC GAG AGC AGT GGT GGA GAC TG-3'), introducing *Nde*I and *Xho*I restriction sites (underlined), respectively. The amplified fragment was cloned between the *Nde*I and *Xho*I sites of the pET23a(+) expression vector fused to a C-terminal His_6-tag. The resulting construct (pET-AtGALDH-His_6) was verified by automated sequencing of both strands and electroporated to *E. coli* BL21(DE3) cells for recombinant expression.

Site-directed mutagenesis - The AtGALDH mutants L56A, L56C, L56F, L56H, and L56I were constructed using pET-AtGALDH-His_6 as template with the QuikChange II method (Stratagene, La Jolla, CA, USA). The oligonucleotides used are listed in Table 3.1, changed nucleotides are underlined. Successful mutagenesis was confirmed by automated sequencing of both strands. The resulting constructs pET-AtGALDH_L56H-His_6, pET-AtGALDH_L56C-His_6, pET-AtGALDH_L56A-His_6, pET-AtGALDH_L56I-His_6, and pET-AtGALDH_L56F-His_6 were electroporated to *E. coli* BL21(DE3) cells for recombinant expression.

Table 3.1. Oligonucleotides used for the construction of AtGALDH_Leu56 variants. Only sense primers are shown, changed nucleotides are underlined.

Variant	Oligonucleotide sequence (5'→ 3')
L56A	CCCGTTGGATCGGGTGCCTCGCCTAATGGGATTG
L56C	CCCGTTGGATCGGGTTGCTCGCCTAATGGGATTG
L56F	CCCGTTGGATCGGGTTTTTCGCCTAATGGGATTG
L56H	CCCGTTGGATCGGGTCACTCGCCTAATGGGATTG
L56I	CCCGTTGGATCGGGTATTTCGCCTAATGGGATTG

Enzyme production and purification - The Åkta explorer FPLC system (Pharmacia Biotech) was used for all purification steps. For enzyme production *E. coli* BL21(DE3) cells, harboring a pET-AtGALDH plasmid, were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin until an OD_{600} of 0.7 was reached. Expression was induced by the addition of 0.4 mM IPTG and the incubation was continued for 16 h at 37°C. Cells (58 g wet weight) were harvested by centrifugation, resuspended in 60 ml 100 mM potassium phosphate, 1 mM Pefabloc SC, 5 mM GSH, pH 7.4 and subsequently passed twice through a pre-cooled French Pressure cell (SLM Aminco, SLM Instruments, Urbana, IL, USA) at 10,000 PSI. The resulting homogenate was centrifuged at 25,000 × g for 30 min at 4°C to remove cell debris, and the supernatant was applied onto a Ni-NTA agarose column (16 x 50 mm) equilibrated with 50 mM sodium phosphate, 300 mM NaCl, 5 mM GSH, pH 7.4. The column was washed

with two volumes equilibration buffer and with two volumes equilibration buffer containing 20 mM imidazole. The enzyme was eluted with 300 mM imidazole in equilibration buffer. The active fraction was dialyzed at 4°C against 25 mM Tris-HCl, 0.1 mM EDTA, 5 mM GSH, 200 μM FAD, pH 7.4. After removal of insoluble material by centrifugation at 25,000 × g for 30 min at 4°C, the soluble fraction was applied onto a HiLoad 26/10 Q-Sepharose HP column equilibrated with 25 mM Tris-HCl, 5 mM GSH, pH 7.4. After washing with two column volumes of starting buffer, the protein was eluted with a linear gradient of NaCl (0-0.2 M) in the same buffer. Active fractions were pooled and concentrated using the Ni-NTA agarose column (see above). The final preparation was saturated with FAD; excess FAD was removed by size-exclusion chromatography using a Bio-Gel P-6DG column (15 x 130 mm) equilibrated with 20 mM sodium phosphate, 0.1 mM DTT, pH 7.4, and the enzyme was stored at -80°C. Before analysis, the enzyme was freshly incubated with 1 mM DTT.

Protein analysis - SDS-PAGE was performed using 12.5% acrylamide slab gels essentially as described by Laemmli (Laemmli, 1970). Proteins were stained using Coomassie Brilliant Blue R-250. For western blot analysis, the gels were blotted onto a nitrocellulose membrane (Optitran BA-S 85 Reinforced NC, Schleicher & Schuell GmbH, Whatman group, Dassel, Germany) and incubated with rabbit anti-His_6 polyclonal antibodies and a secondary antibody coupled to alkaline phosphatase. Proteins were visualized using nitroblue tetrazolium and 5-bromo-4-chlor-3-indolylphosphate as substrates for alkaline phosphatase detection. Total protein concentrations were estimated using the Bradford protein assay from Bio-Rad with BSA as standard (Bradford, 1976). Analytical gel filtration to investigate the hydrodynamic properties of AtGALDH was performed on a Superdex 200 HR 10/30 column running in 50 mM potassium phosphate and 150 mM potassium chloride, pH 7.0, coupled to the Åkta explorer FPLC system. Gel filtration experiments to study the interaction of AtGALDH with bovine heart cytochrome *c* were performed using the same column running in potassium pyrophosphate, pH 8.8, I = 25 mM. Desalting or buffer exchange of small aliquots of enzyme was performed with Bio-Gel P-6DG columns.

Spectral analysis - Absorption spectra were recorded at 25°C on a Hewlett Packard (Loveland, CO, USA) 8453 diode array spectrophotometer in 50 mM sodium phosphate buffer, pH 7.4. The molar absorption coefficient of protein-bound FAD was determined by recording the absorption spectrum of AtGALDH in the presence and absence of 0.1% (w/v) SDS, assuming a molar absorption coefficient for free FAD of 11.3 mM^{-1} cm^{-1} at 450 nm. Purified enzyme concentrations were routinely determined by measuring the absorbance at 450 nm using the molar absorption coefficient for protein-bound FAD. Spectra were collected and analyzed using the UV-Visible ChemStation software package (Hewlett Packard).

Photoreduction of AtGALDH (11 μM) in the presence of EDTA and 5-deazaflavin was performed in 50 mM sodium phosphate, pH 7.4 as described (Macheroux, 1999). Catalytic amounts of glucose oxidase and 1.5 mM β-D-glucose were added to scavenge final traces of oxygen and catalase was added to remove hydogen peroxide formed during the reaction. Solutions were made anaerobic by alternate evacuation and flushing with oxygen-free argon. Illumination was performed in a 25°C water bath with a 375 W light source (Philips, Eindhoven, The Netherlands) at 15 cm distance. Spectra were taken at regular intervals during illumination until complete reduction was achieved.

Titration of AtGALDH (10 μM) with sodium sulfite was carried out in 50 mM sodium phosphate buffer, pH 7.4. A 1 M sodium sulfite stock solution in 50 mM sodium phosphate buffer, pH 7.4 was freshly prepared before use, suitable dilutions were made in the same buffer before addition to the enzyme solution. Spectra were taken until no further change was observed before the next addition was done. Final sodium sulfite concentrations were 0, 5, 10, 25, 49, 98, 196 and 977 μM. The dissociation constant (K_d) was calculated from the change in the absorbance at 450 nm during the titration using a direct non-linear regression fit to the data with the Igor Pro program (Wavemetrics, Lake Oswego, OR, USA):

$$\Delta A450 = \frac{\Delta A450_{max} \times [\text{sulfite}]}{K_d + [\text{sulfite}]} \qquad \text{(eq. 3.1)}$$

Cofactor determination - The flavin cofactor of AtGALDH was determined by thin layer chromatography (TLC). The cofactor was released from the protein by boiling for 30 min or acid treatment. The protein precipitate was removed by centrifugation and the supernatant was applied together with the reference compounds FAD, FMN and riboflavin onto a TLC plate (Baker-flex Silica Gel 1B2, J.T. Baker Inc., Phillipsburg, NY, USA). Butanol:acetic acid:water (5:3:3) was used as mobile phase.

Activity measurements - GALDH activity was routinely assayed by following the reduction of cytochrome *c* at 550 nm at 25°C on a Hewlett Packard 8453 diode array spectrophotometer. Initial velocity values were calculated using a molar difference absorption coefficient (Δε) of 21 mM^{-1} cm^{-1} for reduced minus oxidized cytochrome *c*. Because DTT interferes with the reaction, it was removed from the enzyme solution by Bio-Gel P-6DG gel filtration immediately prior to use. For activity measurements enzyme preparations were diluted in assay buffer containing 1 mg/ml BSA. The standard assay mixture (1 ml) contained assay buffer with pH 8.8 and an ionic strength of 25 mM, 1 mM L-galactono-1,4-lactone, and 50 μM cytochrome *c*; the reaction was started by the addition of enzyme. One unit of enzyme activity (U) is defined as the amount of enzyme that oxidizes 1 μmol of L-galactono-1,4-lactone per min, which is equivalent to the reduction of 2 μmol of cytochrome *c* (Ôba et al.,

1995). The optimal pH for activity of AtGALDH was determined using 25 mM Hepes, Taps, and Ches buffers with varying pH (pH 7 – 9.5) and adjusted to an ionic strength of 25 mM with NaCl.

The kinetic parameters K_m and V_{max} were calculated from multiple measurements with various substrate concentrations using a direct non-linear regression fit to the data. The activity of AtGALDH with L-gulono-1,4-lactone followed Michaelis-Menten kinetics, K_m and V_{max} values of wild-type GALDH for L-gulono-1,4-lactone were calculated using the Michaelis-Menten equation. The K_m and V_{max} values of wild-type GALDH for L-galactono-1,4-lactone were calculated using an equation which includes substrate inhibition:

$$V = \frac{V_{app} \times [S]}{K_m + [S] + \left(\frac{[S]^2}{K_i} \right)} \qquad \text{(eq. 3.2)}$$

The turnover number (k_{cat}, s^{-1}) was calculated using:

$$k_{cat} = \frac{V_{max} \times 58.8\ \text{kDa}}{60} \qquad \text{(eq. 3.3)}$$

The activity of AtGALDH with other electron acceptors was also determined from initial rate experiments. In all cases DTT can interfere with the reaction, so it was removed from the enzyme stock solution by Bio-Gel P-6DG gel filtration immediately prior to use. All reactions were performed with 1 mM L-galactono-1,4-lactone as substrate in assay buffer with pH 8.8 and an ionic strength of 25 mM at 25°C. The activity with 2,6-dichlorophenolindophenol was measured at 600 nm (ε_{600} = 22 mM^{-1} cm^{-1}), the activity with phenazine methosulfate was measured in the presence of the mediator 2,6-dichlorophenolindophenol at 600 nm, the activity with potassium ferricyanide was measured at 420 nm (ε_{420} = 1 mM^{-1} cm^{-1}), and the activity with 1,4-benzoquinone was measured at 290 nm (ε_{290} = 2.3 mM^{-1} cm^{-1}). The reactivity with molecular oxygen was determined with a polarographic oxygen uptake assay using a Clark electrode.

Enzyme stability - The thermal stability of AtGALDH was determined at 52°C. Enzyme preparations were diluted in 50 mM sodium phosphate, pH 7.4 to a final concentration of 1 μM and incubated at the indicated temperatures in the presence or absence of 10 μM FAD. The time-dependent loss of activity was followed by the standard assay procedure; aliquots were withdrawn from the incubation mixtures at intervals and assayed for residual enzyme activity. To discriminate between global and local unfolding, at the end of the heating period, the enzyme activity was also measured in the presence of 10 μM FAD.

Apoprotein preparation - The ease of cofactor release was examined by on-column washing with chaotropic salts (Hefti et al., 2003[b]). To this end, the His-tagged proteins (ca. 2.5 mg) were bound to a 1 ml Ni-affinity gravity-flow column (HisGraviTrap, GE Healthcare Bioscience AB, Uppsala, Sweden) in the presence of 50 mM sodium phosphate, 300 mM NaCl, pH 7.4; the column was washed with 10 column volumes of the same buffer containing 2 M KBr and subsequently with buffer containing 2 M KBr and 2 M urea. (Apo)protein was collected by elution with 300 mM imidazole in buffer. The collected fractions were analyzed for GALDH activity in the absence and presence of FAD.

Product analysis - To analyze the products of the enzymatic oxidation of L-galactono-1,4-lactone and L-gulono-1,4-lactone by AtGALDH, 1 mM solutions of these compounds were incubated for 2 h at 25°C with the recombinant enzyme (10 μg) in air saturated assay buffer (0.24 mM O_2). Before analysis the incubation mixtures were centrifuged for 10 min at maximal speed in a standard tabletop microfuge. The supernatants were analyzed by HPLC using a Waters (Milford, MA, USA) 600 controller with a Waters In Line Degasser coupled to a Waters 996 photo-diode array detector. Separation was performed at room temperature on an Alltima C_{18} column (150 x 4.6 mm, 5 μm particle size; Alltech Associates, Deerfield, IL, USA). The column was equilibrated with 0.1% trifluoroacetic acid, 5% acetonitrile in water, elution was performed with a linear gradient of 5 to 100% acetonitrile in 20 min. Chromatograms were recorded at 254 nm. L-Ascorbic acid, L-galactono-1,4-lactone and L-gulono-1,4-lactone served as references. System control and data collection and analysis was performed using the Millenium[32] software package (Waters). Due to its hydrophilic nature, L-ascorbate was not retarded on the column under these conditions and eluted in the flow-through with a retention time of ca. 2 min.

Results

Sequence analysis - Genome analysis revealed that *A. thaliana* contains one gene (At3g47930) coding for GALDH. The full-length AtGALDH protein contains 610 amino acids with a theoretical molecular mass of 68,496 Da. Multiple sequence alignment showed that AtGALDH shares about 80-90% sequence identity with GALDH proteins from other plants. Less than 25% sequence identity and about 30-40% sequence similarity was found with other aldonolactone oxidoreductases. The highest degree of sequence conservation was found in the FAD-binding domain (Fig. 3.2). From the alignment it is clear that plant GALDH's lack the histidine residue involved in covalent flavinylation in GUO, ALO, and GLO; but contain a leucine residue instead (Leu56 in mature AtGALDH), indicating that the flavin cofactor is non-covalently bound to the protein.

Full-length AtGALDH contains a mitochondrial target sequence with a putative FR/YA cleavage site (Fig. 3.2). An identical cleavage site is present in the sequences of GALDH from cauliflower, sweet potato, and tobacco (Østergaard et al., 1997; Imai et al., 1998; Yabuta et al., 2000). N-terminal sequence analysis of GALDH isolated from cauliflower mitochondria showed that the mature protein starts exactly at the tyrosine of the predicted cleavage site (Østergaard et al., 1997). Though plant GALDH's were previously identified as integral membrane proteins of the inner mitochondrial membrane (Siendones et al., 1999; Bartoli et al., 2000), we did not find any transmembrane regions in the sequence of mature AtGALDH.

Cloning and functional expression of AtGALDH in E. coli - A 1.5 kb DNA fragment encoding mature AtGALDH was PCR amplified from an *A. thaliana* seedling cDNA library. The amplified fragment was cloned into the pET23a vector under the control of the strong T7 promoter. An in-frame fusion at the 3' end was made with a fragment encoding a His_6-tag on the vector. The resulting ORF encodes a 511 residues long polypeptide, comprising mature AtGALDH, two extra residues (Leu and Glu) and the His_6-tag.

Mature AtGALDH-His_6, with a predicted molecular mass of 58,763 Da, was expressed in *E. coli* BL21(DE3) cells as soluble cytoplasmic protein. Highest levels of expression were found after 16 h of induction with 0.4 mM IPTG at 37°C. Expression of the recombinant His_6-tagged protein was confirmed by Western blot analysis with a polyclonal rabbit anti-His_6-antibody and by the presence of GALDH activity in the cell extract of IPTG induced *E. coli* BL21(DE3) - pET-AtGALDH-His_6 cells. The recombinant protein was purified to apparent homogeneity by two successive chromatographic steps (Fig. 3.3). About 210 mg of recombinant AtGALDH protein could be purified from a 12 L batch culture containing 58 g of cells (wet weight). The final preparation had a specific activity of 76 U mg^{-1} (Table 3.2). This 'as isolated' activity increased by a factor of about 1.4 when the enzyme was treated with 1 mM DTT (*vide infra*). Recombinant AtGALDH migrated in SDS-PAGE as a single band with an apparent molecular mass of around 55 kDa (Fig. 3.3). This value is in fair agreement with the calculated molecular mass (58.8 kDa). The relative molecular mass of recombinant AtGALDH was estimated to be 56 kDa by analytical size-exclusion chromatography, which indicates a monomeric structure (data not shown).

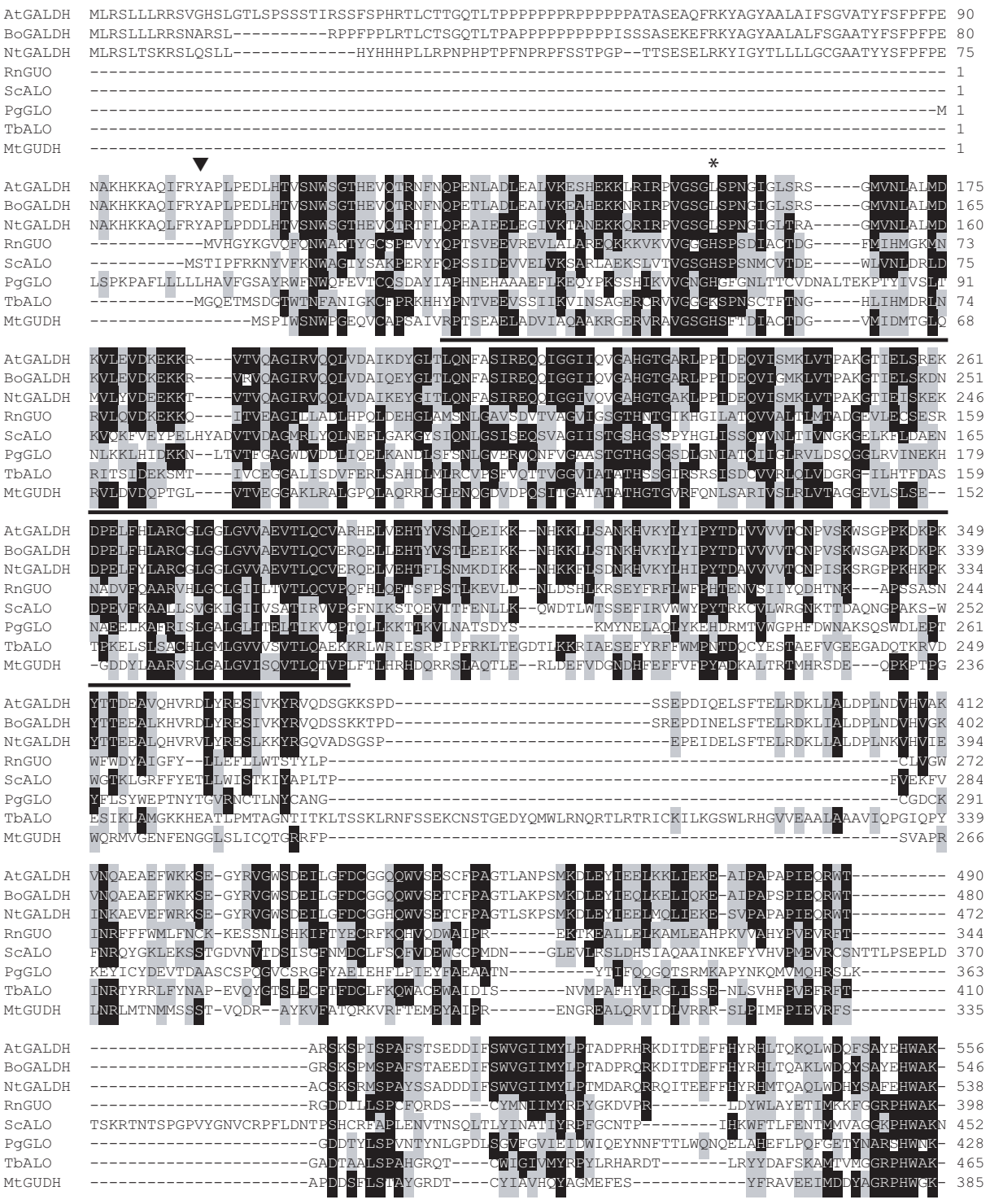

AtGALDH
BoGALDH
NtGALDH
RnGUO
ScALO
PgGLO
TbALO
MtGUDH

Figure 3.2. (Previous page). Multiple sequence alignment of the full length amino acid sequence of AtGALDH with several aldonolactone oxidoreductases. The accession numbers [NCBI Entrez Protein Database] used for the multiple sequence alignment are: BoGALDH: cauliflower GALDH (CAB09796), NtGALDH: tobacco GALDH (BAA87934), RnGUO: rat GUO (P10867), ScALO: *Saccharomyces cerevisiae* ALO (P54783), PgGLO: *Penicillium griseoroseum* GLO (AAT80870), TbALO: *Trypanosoma brucei* ALO (AAX79383), MtGUDH: *Mycobacterium tuberculosis* GUDH (CAB09342). Alignment was done using the program ClustalW. Amino acid residue numbers are shown on the right. Identical residues are shaded in black, similar residues are shaded in grey. The arrow (▼) indicates the putative cleavage site of the mitochondrial targeting sequence in plant GALDH (FR/YA). The asterisk (*) marks the histidine residue involved in covalent binding of the FAD cofactor in GUO, ALO and GLO. The FAD-binding domain (Fraaije et al., 1998) is underlined.

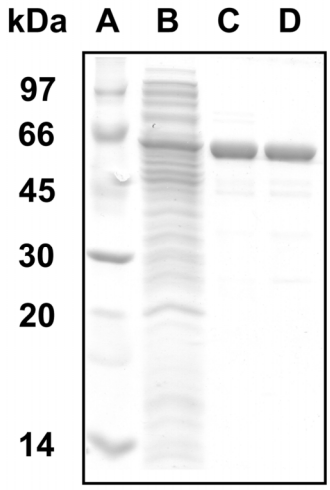


Figure 3.3. SDS-PAGE analysis of the purification of recombinant AtGALDH. Lane A: low molecular weight marker; lane B: cell extract; lane C: Ni-NTA pool; lane D: Q-Sepharose pool.

Table 3.2. Purification of AtGALDH expressed in *E. coli*.

Step	Protein (mg)	Activity (U)	Spec. activity (U mg^{-1})	Yield (%)
Cell extract	3469	25947	7	100
Ni-NTA agarose	401	24878	62	96
Q-Sepharose	214	16365	76[a]	63

[a]As isolated.

Spectral properties of AtGALDH - Recombinant AtGALDH showed a typical flavoprotein absorption spectrum with maxima at 276 nm, 375 nm and 450 nm and a shoulder at 475 nm (Fig. 3.4A, solid line). The molar absorption coefficient of the protein-bound flavin was determined to be 12.9 mM^{-1} cm^{-1} at 450 nm. The A_{276}/A_{450} ratio of the FAD-saturated protein preparation was 8.15. The redox active flavin cofactor could be released from the protein by boiling or acid treatment, confirming the non-covalent binding mode already predicted from the amino acid sequence. The released cofactor was identified as FAD by TLC.

Aerobic incubation of the protein with excess L-galactono-1,4-lactone resulted in a rapid bleaching of the yellow color and a completely two-electron reduced flavin spectrum, indicating that the FAD cofactor participates in the electron-transfer reaction (Fig. 3.4A, dotted line). Because cytochrome *c* is a one-electron acceptor, the re-oxidation of AtGALDH by cytochrome *c* involves two consecutive one-electron transfer steps involving a flavin semiquinone intermediate. In an attempt to identify the nature of this radical species, the protein was artificially reduced by photoreduction in the presence of EDTA and 5-deazaflavin (Fig. 3.4B). During the first part of the reduction an absorption peak appears around 390 nm, which is indicative for the formation of the red anionic flavin semiquinone. Reduction proceeds until the fully reduced flavin hydroquinone state is obtained. Exposing the two-electron reduced protein to air readily resulted in the re-appearance of the fully oxidized spectrum.

The stabilization of the red anionic form of the flavin semiquinone intermediate together with the formation of a flavin N5-sulfite adduct are properties commonly associated with flavoprotein oxidases, and are indicative for the presence of a positive charge near the flavin N1 locus (Lederer, 1978; Fraaije and Mattevi, 2000). The formation of such a flavin-sulfite adduct results in bleaching of the yellow color (Massey et al., 1969). AtGALDH readily reacted with sodium sulfite with a dissociation constant (K_d) of 18 μM for the flavin-sulfite complex (Fig. 3.4C). Addition of excess L-galactono-1,4-lactone (4 mM) to the AtGALDH-sulfite complex yielded the spectrum of the reduced enzyme (cf. Fig. 3.4A), demonstrating that the reaction with sulfite is reversible.

Catalytic properties of AtGALDH - Recombinant AtGALDH was highly active with its natural substrate L-galactono-1,4-lactone and its electron acceptor cytochrome *c* (Table 3.3). The L-gulono-1,4-lactone isomer was also oxidized at significant rate (Table 3.3). AtGALDH was inhibited by the L-galactono-1,4-lactone substrate at concentrations above 2 mM (Fig. 3.5A; K_i = 16.4 mM). No substrate inhibition was found with L-gulono-1,4-lactone at concentrations up to 100 mM (Fig. 3.5B). The substrate analogues D-galactono-1,4-lactone, D-gulono-1,4-lactone, L-mannono-1,4-lactone and D-galacturonic acid were no substrates for AtGALDH and did not inhibit the oxidation of L-galactono-1,4-lactone.

The product of the AtGALDH mediated oxidation of L-galactono-1,4-lactone and L-gulono-1,4-lactone was analyzed by HPLC. Because the presumed product L-ascorbate can reduce cytochrome *c*, resulting in the formation of dehydroascorbic acid, which is hydrolyzed to 2,3-diketo-L-gulonic acid at the pH of the reaction, the reaction was performed without the addition of cytochrome *c*. Although the reaction with oxygen occurs slowly, after several hours of incubation enough product was generated to perform the analysis. The products of the reaction of AtGALDH with both L-galactono-1,4-lactone and L-gulono-1,4-lactone eluted

with the same retention time as the L-ascorbic acid reference and showed identical spectral properties (results not shown).

AtGALDH was also active with the artificial electron acceptors phenazine methosulfate and 1,4-benzoquinone (Table 3.3). The reaction with molecular oxygen (aerated buffer) proceeded very slow with a bi-molecular rate constant (k_{ox}) of 6×10^2 M^{-1} s^{-1}. 2,6-Dichlorophenolindophenol and potassium ferricyanide were no electron acceptors for recombinant AtGALDH.

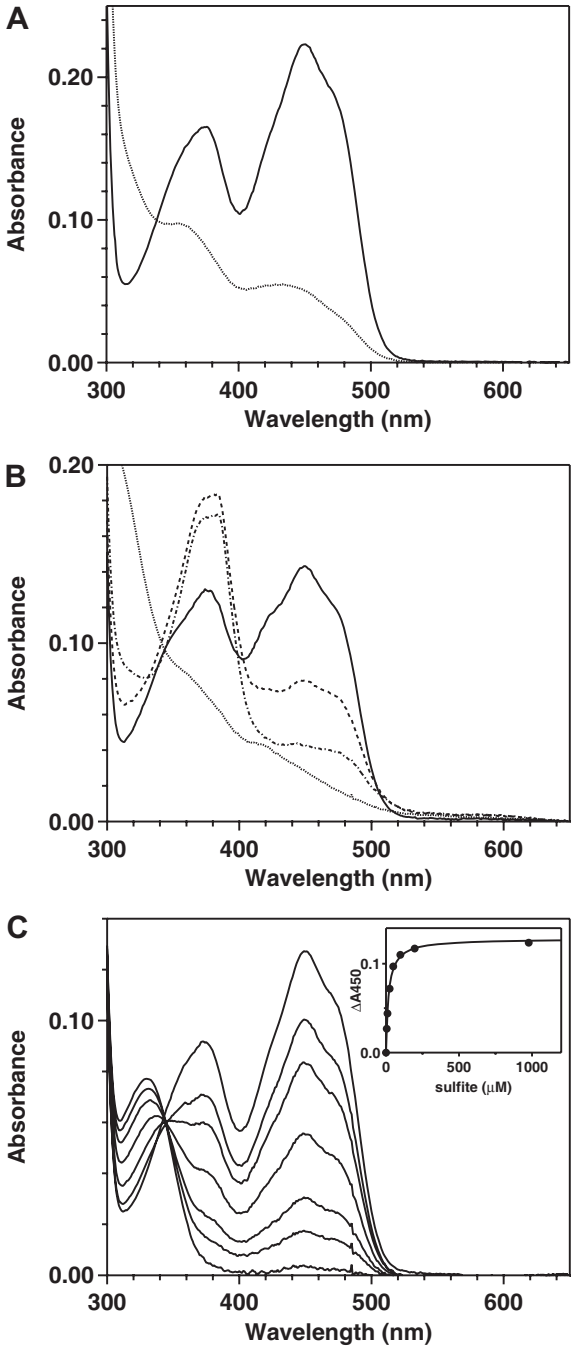


Figure 3.4. Spectral properties of recombinant AtGALDH. (A) Aerobic reduction with excess substrate. The reaction mixture contained 50 mM sodium phosphate, pH 7.4, 20 μM AtGALDH and 1 mM L-galactono-1,4-lactone and was incubated at 25°C. Spectra were taken before (solid line) and after the addition of L-galactono-1,4-lactone. Complete reduction was achieved 4 min after the addition of the substrate (dotted line). (B) Anaerobic photoreduction in the presence of EDTA and 5-deazariboflavin. The reaction mixture contained 50 mM sodium phosphate, pH 7.4, 11 μM AtGALDH, 1 mM EDTA, and 7 μM 5-deazaflavin. Spectra were taken at regular intervals before illumination (solid line), and at regular intervals during illumination until complete reduction was achieved after 15 min (dotted line). The dashed line and the dashed-dotted line represent the intermediate spectra observed during the reduction after 1 min and 2 min of illumination, respectively. Spectra were corrected for 5-deazaflavin absorption. (C) Titration of AtGALDH with sodium sulfite. The reaction was carried out with 10 μM AtGALDH in 50 mM sodium phosphate buffer, pH 7.4. Spectra are shown after the addition of 0, 5, 10, 25, 49, 98, 196 and 977 μM sulfite (final concentrations) until no further changes were observed. Spectra were corrected for changes in the reaction volume during the experiment. The inset shows the absorbance difference at 450 nm during the titration, from which a dissociation constant (K_d) for the enzyme-sulfite complex of 18 μM was calculated.

Table 3.3. Steady-state kinetic parameters of AtGALDH. Apparent kinetic constants were determined at 25°C in assay buffer, pH 8.8 (*I* = 25 mM). *Substrate concentrations varied between 5 μM and 5 mM for L-galactono-1,4-lactone and between 0.5 and 100 mM for L-gulono-1,4-lactone, with a constant cytochrome *c* concentration of 50 μM. Values are presented as mean ± SD of three experiments. #Electron acceptor concentrations varied between 1 μM and 200 μM for cytochrome *c*, 1 μM and 500 μM for phenazine methosulfate and between 10 μM and 2.3 mM for 1,4-benzoquinone, with a constant L-galactono-1,4-lactone concentration of 1 mM. Values are presented as mean ± SD of two experiments.

	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m (mM^{-1} s^{-1})
Substrate*			
L-galactono-1,4-lactone	0.17 ± 0.01	134 ± 5	7.7×10^2
L-gulono-1,4-lactone	13.1 ± 2.8	4.0 ± 0.2	3.1×10^{-1}
Electron acceptor#			
Cytochrome *c*	0.034 ± 0.002	151 ± 1	4.4×10^3
Phenazine methosulfate	0.026 ± 0.004	64 ± 3	2.4×10^3
1,4-Benzoquinone	0.280 ± 0.05	108 ± 12	3.9×10^2

AtGALDH displayed a broad pH optimum for activity with cytochrome *c* between pH 8 and 9.5 with a maximum around pH 8.8 (Fig. 3.5C). The activity of AtGALDH with cytochrome *c* was highly dependent on the ionic strength of the solution. Maximal activity was at *I* = 25 mM and respectively 75 %, 30% and 10% of the maximal activity was found at *I* = 5 mM, *I* = 100 mM, and *I* = 200 mM. No specific inhibition by cations or anions was observed. The theoretical p*I* of the recombinant AtGALDH-His_6 is 6.8. No interaction between AtGALDH and cytochrome *c* (p*I* = 10-10.5) was observed during analytical gel filtration at pH 8.8, either in the absence or presence of L-galactono-1,4-lactone (data not shown).

Recombinant AtGALDH appeared to be very stable under storage conditions; long term storage (> 12 months) at -80°C resulted in a 30-50% loss of activity, which could be completely restored upon incubation with the reducing agent DTT. Recombinant AtGALDH was relatively stable when incubated at elevated temperatures, with a half-life of 20 min at 52°C. In the presence of excess FAD, the half-life at 52°C was increased to 115 min, suggesting that the holo form of the enzyme is more thermostable than the apo form. Both local and global unfolding play a role in the thermoinactivation process. This is concluded from the fact that in both incubations, 10 ± 4 % of enzyme activity was recovered at the end of the heating process when excess FAD was included in the assay mixture.

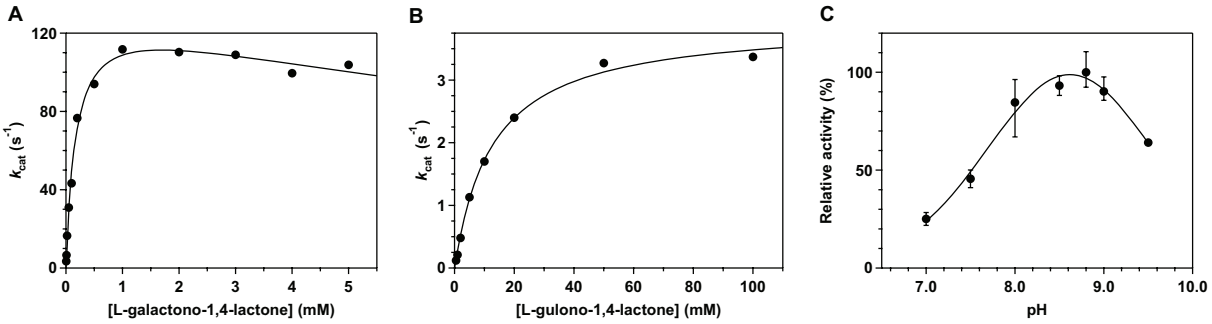


Figure 3.5. Activity of recombinant AtGALDH. (A) Michaelis-Menten kinetics of the AtGALDH-mediated oxidation of L-galactono-1,4-lactone. (B) Michaelis-Menten kinetics of the AtGALDH-mediated oxidation of L-gulono-1,4-lactone. (C) AtGALDH activity as a function of pH. Activities were measured in 25 mM Hepes (pH 7-8), Taps (pH 8-9), and Ches (pH 9-9.5) buffers with a constant ionic strength of 25 mM adjusted with NaCl containing 1 mM L-galactono-1,4-lactone and 50 μM cytochrome *c* at 25°C.

Properties AtGALDH Leu56 mutants - In order to find out more about the role of Leu56 in the FAD binding site, several AtGALDH Leu56 variants were constructed (see Materials and Methods section). The L56A, L56C and L56H variants were expressed and purified in essentially the same way as wild-type AtGALDH-His_6 with similar yields (see Materials and Methods). The L56I and L56F variants were purified in a single gravity-flow Ni-affinity chromatography step with comparable yields and purities as the other variants.

All AtGALDH Leu56 variants contained non-covalently bound FAD. The FAD cofactor was partially released during the purification procedure, a phenomenon hardly observed with the wild-type enzyme. The holo forms of the Leu56 variants could easily be reconstituted by the addition of FAD and their flavin absorption properties were nearly identical to the wild-type enzyme.

The Leu56 variants showed interesting catalytic properties. The L56I variant displayed a higher turnover rate with the L-galactono-1,4-lactone substrate than wild-type AtGALDH and the L56F variant, 240 s^{-1} vs. 134 and 126 s^{-1}, respectively. The other Leu56 variants were all considerably less active than the wild-type enzyme and showed remarkable differences in apparent Michaelis constants for the L-galactono-1,4-lactone substrate (Table 3.4). L56H as well as L56I and L56F showed a relatively low K_m, in the same range as wild-type AtGALDH, whereas the L56C and L56A variants had rather high K_m values in the millimolar range. A similar trend in K_m values was found for the L-gulono-1,4-lactone substrate. As for wild-type AtGALDH, molecular oxygen could not serve as efficient electron acceptor for the mutant enzymes.

Table 3.4. Steady-state kinetic parameters of AtGALDH variants. Apparent kinetic constants were determined at 25°C in assay buffer, pH 8.8 (*I* = 25 mM) with L-galactono-1,4-lactone concentrations varying between 10 μM and 10 mM and a constant cytochrome *c* concentration of 50 μM. Values are presented as mean ± SD of at least two experiments.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m (mM^{-1} s^{-1})
Wild-type	0.17 ± 0.01	134 ± 5	7.7×10^2
L56I	0.32 ± 0.01	240 ± 12	7.5×10^2
L56H	0.12 ± 0.01	32 ± 1	2.6×10^2
L56F	0.56 ± 0.02	126 ± 1	2.3×10^2
L56C	0.99 ± 0.05	76 ± 3	7.8×10^1
L56A	1.7 ± 0.05	45 ± 2	2.6×10^1

As noted above, the FAD cofactor is more loosely bound in the Leu56 variants than in wild-type AtGALDH. Cofactor binding was analyzed in more detail by nickel-affinity chromatography (Hefti et al., 2003[b]). Washing the immobilized proteins with chaotropic salts resulted in elution of the flavin for all Leu56 variants, but was least for wild-type AtGALDH as judged by the presence of the yellow color. The (apo)proteins were subsequently eluted from the column with buffer containing 300 mM imidazole and tested for activity. In the absence of FAD in the assay mixtures, wild-type AtGALDH and the L56F and L56I variants still contained respectively 60, 50 and 40% of their original activity, whereas the other variants had lost 80-90% of their activity. All Leu56 variants regained most of their activity (60-90%) in the presence of FAD, whereas the activity of variant L56C was restored to less than 30%. The L56C variant is rather unstable without its cofactor bound, and irreversibly forms aggregates after elution from the affinity column. It is clear that under the conditions applied, FAD is most firmly bound in the wild-type enzyme and in the variants in which Leu56 is replaced by (large) hydrophobic residues. Replacing Leu56 with a polar or less bulky residue results in easier loss of FAD, indicating that the interaction of Leu56 with the cofactor is of hydrophobic nature and may also involve a steric effect.

The thermal stability of variant L56H was examined in more detail. This variant, with a half-life of 8 min at 52°C, appeared somewhat less thermostable than wild-type AtGALDH. Addition of FAD during the incubation increased the half-life of L56H at 52°C to 46 min.

Discussion

In this report, we present for the first time a detailed study of the biochemical properties of recombinant AtGALDH and some active site variants. In contrast with an earlier report (Wolucka and van Montagu, 2003), AtGALDH is not strictly specific for L-galactono-1,4-lactone. The enzyme oxidizes L-gulono-1,4-lactone at significant rate, but the catalytic efficiency for the gulonolactone isomer is relatively low. For GALDH from sweet potato and

tobacco it was stated that these enzymes also oxidize the gulonolactone isomer (Ôba et al., 1995; Yabuta et al., 2000), but no kinetic parameters were reported. From our results we conclude that AtGALDH shows a high enantiopreference for L-galactono-1,4-lactone and that a difference in orientation of the 3-hydroxyl group of the substrate is responsible for a 100-fold higher K_m and 3000-fold lower catalytic efficiency.

The main precursor of L-ascorbate in plants is L-galactono-1,4-lactone (Smirnoff and Wheeler, 2000). It has been demonstrated that plants can also produce L-ascorbate via L-gulono-1,4-lactone, but the enzymes involved are unknown. Arabidopsis cell suspensions can synthesize and accumulate L-ascorbate from the precursor L-gulono-1,4-lactone (Davey et al., 1999). Furthermore, L-gulono-1,4-lactone oxidase/dehydrogenase activity has been demonstrated in hypocotyl homogenates of kidney beans (Siendones et al., 1999) and in cytosolic and mitochondrial fractions from Arabidopsis cell suspensions (Davey et al., 1999) and potato tubers (Wolucka and van Montagu, 2003). These data suggest the existence of differently localized isozymes that can produce vitamin C from either L-galactono- or L-gulono-1,4-lactone. Bartoli and co-workers predicted that GALDH from sweet potato tubers is an integral membrane protein with three transmembrane regions (Bartoli et al., 2000). We did not find any transmembrane regions in the sequence of mature AtGALDH. In agreement with this, the enzyme was expressed in soluble form in *E. coli*. This leaves the possibility that the observed gulonolactone oxidizing capability of AtGALDH is of significance *in vivo.*

A recent study on the RNAi silencing of GALDH from tomato revealed the importance of GALDH for plant and fruit growth. A severe reduction in GALDH activity can be lethal to the plant. Interestingly, the total ascorbate content remained unchanged in the GALDH silenced plants. As possible explanations the reduction in ascorbate turnover and the activation of alternative ascorbate biosynthesis pathways were proposed (Alhagdow et al., 2007). Although the gulonolactone activity of AtGALDH might be of physiological relevance it cannot be excluded that other aldonolactone oxidoreductases with different subcellular localizations are responsible for the observed gulonolactone activity *in vivo*. It has been proposed that members of a putative subfamily of VAO-like flavoproteins might be responsible for the conversion of L-gulono-1,4-lactone into L-ascorbate (Wolucka and van Montagu, 2003). Sequence analysis of the predicted gene products suggest that they are targeted to different subcellular locations.

So far no information was available about the thermal stability of GALDH enzymes. AtGALDH appeared to be a rather stable enzyme, though at elevated temperatures it looses its FAD cofactor. The strong increase in thermal stability in the presence of excess FAD indicates that the cofactor protects the enzyme from irreversible unfolding or aggregation. Covalent flavinylation has also been associated with improving flavoprotein stability, a covalent flavin-protein link is presumed to have a similar stabilizing effect as a disulfide

bridge (Mewies et al., 1998). Nevertheless, several aldonolactone oxidoreductases with a covalently bound FAD are less stable than AtGALDH. ALO from *C. albicans* completely lost activity within 1 min at 50°C (Huh et al., 1994). GLO from *P. cyaneo-fulvum* (renamed *P. griseoroseum*) quickly lost its activity above 45°C (Salusjärvi et al., 2004), and also rat GUO readily lost its activity at elevated temperatures; 90% of the activity was lost after 10 min incubation at 49°C (Eliceiri et al., 1969). The thermal stability of AtGALDH is more comparable with that of GUDH from *Gluconobacter oxydans* (Sugisawa et al., 1995) and *M. tuberculosis* (Wolucka and Communi, 2006). These enzymes lost about 50% of their activity after 5 min incubation at 55 and 60°C, respectively. The absence of a covalent flavin link could provide GALDH with a greater conformational flexibility which may be needed for cross-talk with cytochrome *c*.

The mechanism of L-ascorbate production by AtGALDH involves two half-reactions. In the reductive half-reaction, the oxidized flavin cofactor is converted to the hydroquinone state by the L-galactono-1,4-lactone substrate. The two-electron reduced enzyme is then re-oxidized in the oxidative half-reaction by cytochrome *c*. This half-reaction involves two subsequent one-electron steps and the formation of a flavin semiquinone radical. Spectral analysis revealed that AtGALDH is able to form the red anionic flavin semiquinone, which was visualized by artificial photoreduction of the protein and is characterized by a strong absorbance around 390 nm. AtGALDH also readily reacted with sulfite, resulting in the formation of a flavin N5 sulfite adduct, with a K_d of 18 μM for the enzyme-sulfite complex. The stabilization of the red anionic semiquinone and the formation of a flavin N5 sulfite adduct are properties commonly associated with flavoprotein oxidases (Massey et al., 1969). However, AtGALDH is not the only exception to this rule. Flavocytochrome b_2 also stabilizes the red anionic semiquinone and a flavin N5 sulfite adduct, and is poorly active with oxygen (Lederer, 1978; Ould Boubacar et al., 2007). In flavocytochrome b_2 an Arg residue is involved in both catalysis and the stabilization of the N5 sulfite adduct (Mowat et al., 2000). A similar situation is observed in adenosine-5'-phophosulfate reductase, another flavoprotein for which a crystal structure of the enzyme-sulfite complex is known (Schiffer et al., 2006). Both flavocytochrome b_2 and adenosine-5'-phophosulfate reductase do bind a negatively charged substrate. Therefore, it will be of interest to see if a positively charged residue is present in the active site of AtGALDH and related enzymes.

Many aldonolactone oxidoreductases contain a covalently bound FAD cofactor. Possible advantages of such a mode of flavin binding are saturation of the active site with cofactor in flavin deficient environments, anchoring of the isoalloxazine ring, and modulating the redox properties (Mewies et al., 1998; Fraaije et al., 1999). AtGALDH lacks the histidine involved in covalent attachment of the FAD cofactor, but contains a leucine (Leu56) at this position. Replacement of Leu56 into His in AtGALDH showed that the presence of a histidine at this

position does not initiate covalent binding of the cofactor. Covalent coupling of the FAD cofactor presumably is an autocatalytic process, requiring a pre-organized binding site (Fraaije et al., 2000). Covalent flavinylation commonly requires a base-assisted attack of the FAD cofactor, resulting in a flavoquinone methide intermediate and subsequent formation of the covalent link (Mewies et al., 1998). Mutagenesis studies in VAO revealed that the histidine residue involved in covalent cofactor binding (His422) is activated by a neighboring base (His61) for attack of the C8α position of the isoalloxazine ring thus forming the covalent bond (Fraaije et al., 2000). Covalent flavinylation in AtGALDH-L56H might thus require nucleophilic activation of His56. The prediction of such an activating base in the sequence of AtGALDH is hampered by the lack of structural information for GALDH and related aldonolactone oxidoreductases.

Leu56 replacements of AtGALDH established that Leu56 plays an important role in binding of the non-covalently bound FAD cofactor and in catalysis. Variants with a bulky hydrophobic residue at position 56 bind the cofactor more tightly than variants containing small and/or polar residues. The catalytic and FAD-binding properties of the Leu56 variants are not easily explained but possibly reflect subtle changes in the protein-FAD interaction rather than a direct interaction of residue 56 with the substrate.

In conclusion, we have described for the first time the biochemical properties of recombinant AtGALDH and some active site variants. The results obtained provide a good framework for further structure-function relationship studies aimed at identifying important residues involved in catalysis and flavin binding.

Acknowledgements

We are grateful to Yu Lu and Daan Binnewijzend for experimental contributions. We thank Marco Fraaije for critically reading the manuscript. This research was supported by a grant from the Carbohydrate Research Centre Wageningen (CRC-W).

chapter 4

Functional assignment of Glu386 and Arg388 in the active site of L-galactono-γ-lactone dehydrogenase

Nicole G. H. Leferink[1], Mac Donald F. Jose[1], Willy A. M. van den Berg[1], Willem J. H. van Berkel[1]

[1] Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

Abstract

The flavoprotein L-galactono-γ-lactone dehydrogenase (GALDH) catalyzes the terminal step of vitamin C biosynthesis in plants, the oxidation of L-galactono-1,4-lactone into L-ascorbate with the concomitant reduction of cytochrome *c*. No three-dimensional structure is available for GALDH or related aldonolactone oxidoreductases. Here we identified two essential active site residues, Glu386 and Arg388, in GALDH from *Arabidopsis thaliana*. Glu386 and Arg388 variants, including E386D, E386A, R388K, and R388A, have very high K_m values for L-galactono-1,4-lactone and low turnover rates. The E386D mutant shows, in contrast to wild-type GALDH, a preference for L-gulono-1,4-lactone. The Arg388 mutants highlight the importance of a positive charge near the isoalloxazine moiety of the flavin. Removal of this charge in the R388A variant yields a nearly inactive protein that is unable to stabilize negative charges in the active site.

Keywords: L-galactono-1,4-lactone dehydrogenase (EC 1.3.2.3); vitamin C; flavoprotein, VAO family; site-directed mutagenesis

Abbreviations: AldO, alditol oxidase; CO, cholesterol oxidase; GALDH, L-galactono-γ-lactone dehydrogenase; VAO, vanillyl-alcohol oxidase

Introduction

L-Ascorbate (vitamin C) is an important antioxidant, redox buffer and enzyme cofactor for many organisms. Plants and most animals can synthesize ascorbate to meet their daily requirements, while humans and other primates have lost this ability during evolution. Ascorbate is particularly abundant in plants, the main dietary source of vitamin C for humans. The terminal step of ascorbate biosynthesis in plants is catalyzed by the flavoenzyme L-galactono-γ-lactone dehydrogenase (GALDH; L-galactono-1,4-lactone: ferricytochrome *c*-oxidoreductase; EC 1.3.2.3). GALDH is localized in the mitochondrial intermembrane space where it catalyzes the oxidation of L-galactono-1,4-lactone into L-ascorbate with the concomitant reduction of cytochrome *c* (Bartoli et al., 2000; Heazlewood et al., 2003). Besides producing ascorbate, GALDH is also required for the proper functioning of plant mitochondria and for the correct assembly of plant respiratory complex I (Alhagdow et al., 2007; Pineau et al., 2008).

GALDH and related aldonolactone oxidoreductases involved in vitamin C biosynthesis belong to the vanillyl-alcohol oxidase (VAO) flavoprotein family. Members of this family share a two-domain folding topology, comprising a conserved N-terminal FAD-binding domain and a less conserved C-terminal cap domain that determines the substrate specificity. The active site is located at the interface of the domains (Fraaije et al., 1998; Leferink et al., 2008[a]).

Aldonolactone oxidoreductases have been isolated from various sources, including several plants, animals and fungi, but are relatively poorly characterized (Mapson and Breslow, 1958; Nishikimi et al., 1976; Kiuchi et al., 1982; Huh et al., 1994; Ôba et al., 1995; Østergaard et al., 1997; Huh et al., 1998; Imai et al., 1998; Yabuta et al., 2000). No three-dimensional structure is known for these enzymes, hence little information is available about the nature of the active site and the reaction mechanism. Previously we showed that recombinant GALDH from *Arabidopsis thaliana* contains a non-covalently bound FAD as prosthetic group and has a strong preference for L-galactono-1,4-lactone as substrate (Leferink et al., 2008[b]). Furthermore, GALDH reacts poorly with molecular oxygen, which distinguishes the enzyme from its animal and yeast homologues, that act as true oxidases (Leferink et al., 2009).

Inspection of the crystal structures of alditol oxidase (AldO) (Forneris et al., 2008) and cholesterol oxidase (CO) (Coulombe et al., 2001) revealed that both these VAO-family members contain a Lys residue and a Glu-Arg pair in the active site that are conserved among aldonolactone oxidoreductases. The Lys and Arg residues are located at similar positions in the AldO and CO structures (Fig. 4.1A and B), and are likely involved in substrate activation (Forneris et al., 2008). Glu475 has been proposed to function as a base for proton abstraction in CO (Coulombe et al., 2001), whereas in AldO, Glu320 is involved in extensive hydrogen

bonding with the polyol substrate (Forneris et al., 2008). Removal of the positive charge of Arg477 in CO resulted in a dramatic loss of activity (Piubelli et al., 2008). Furthermore, the Glu-Arg pair of CO is located at the bottom of a tunnel that extends from the surface to the active site, and is implicated to function as a gate to control oxygen access (Coulombe et al., 2001; Piubelli et al., 2008). The active site Lys is part of a recently identified C-terminal sequence motif (HWXK) specific for aldonolactone oxidoreductases and some closely related VAO-members including AldO (Fig. 4.1C). Replacement of this Lys by Gly in *Trypanosoma cruzi* galactonolactone oxidoreductase (TcGAL) resulted in an inactive holoprotein, indicative for the important role of this residue in TcGAL catalysis (Logan et al., 2007). In line with this observation, the homologous Lys375 in AldO hydrogen bonds with the C1 hydroxyl group of the substrate, the site of oxidation (Forneris et al., 2008).

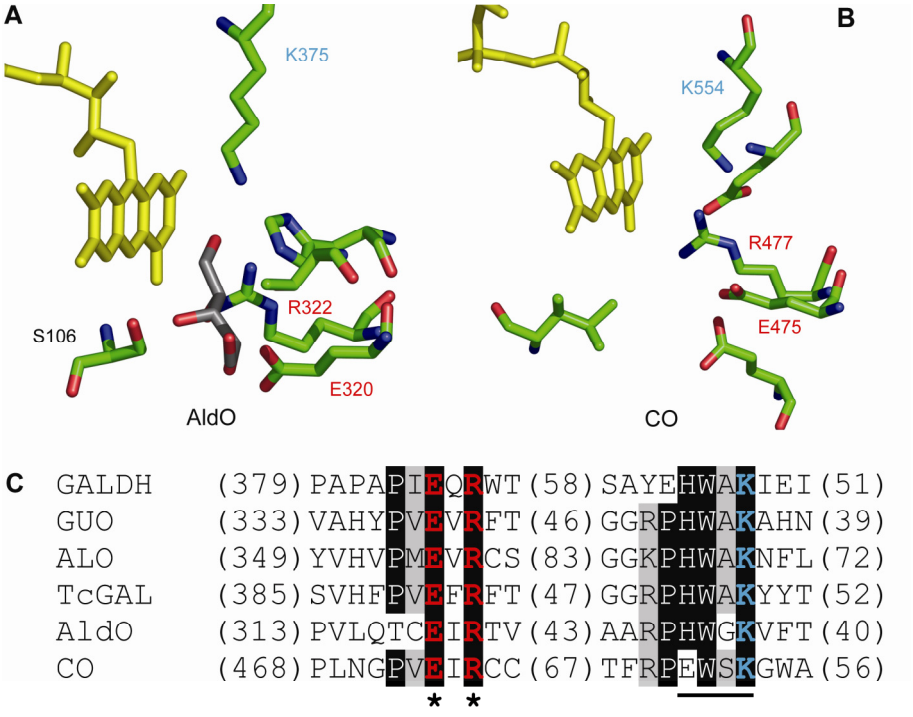


Figure 4.1. Comparison of putative active site residues of GALDH with related VAO-family members. A) Crystal structure of the active site of AldO with bound xylitol (pdb, 2VFS). B) Crystal structure of the active site of CO (pdb, 1I19). Residues conserved in GALDH are indicated in red and blue. C) Clustal W multiple sequence alignment of part of the active site region of several aldonolactone oxidoreductases with related VAO-family members. Identical residues are shaded in black, similar residues are shaded in grey. The conserved Arg-Glu pair is indicated in red and marked with asterisks (*) and the C-terminal HWXK sequence motif is underlined. The number of residues present at the termini and in gaps in the sequence are indicated in parentheses. Amino acid sequences used are: GALDH, Q8GY16; GUO, P10867; ALO, P54783; TcGAL, Q4DPZ5; AldO, Q9ZBU1; CO, Q7SID9.

Here we studied the role of the Glu-Arg pair (Glu386 and Arg388) in the active site of GALDH from *A. thaliana* by site-directed mutagenesis. Biochemical analysis of the generated mutants revealed that both residues are essential for efficient catalysis. Removal of the positive charge of Arg388 results in a nearly inactive GALDH variant that is unable to stabilize negative charges in the active site. Moreover, a GALDH variant with a preference for L-gulono-1,4-lactone was created by the Glu386Asp mutation.

Materials and Methods

Materials - L-Galactono-1,4-lactone was synthesized from D-galacturonic acid by borohydride reduction according to the method described by Mapson and Breslow (Mapson and Breslow, 1958). The crude product was purified on a silica column (particle size 40-63 μm) running in ethyl acetate/methanol (6:4). The purified product was re-crystallized from ethanol. All other chemicals were from commercial sources and of the purest grade available.

Cloning and site-directed mutagenesis - The cDNA encoding mature GALDH from *A. thaliana* has been cloned before into the pET23a(+) vector (Novagen, San Diego, CA, USA) to yield pET-GALDH-His_6 (Leferink et al., 2008[b]). The various GALDH active site mutants were constructed using pET-GALDH-His_6 as template using the QuikChange II protocol (Stratagene, La Jolla, CA, USA). The oligonucleotides used are listed in Table 4.1. Successful mutagenesis was confirmed by automated sequencing. The resulting constructs were electroporated to *E. coli* BL21(DE3) cells (Novagen) for recombinant expression.

Table 4.1. Oligonucleotides used for the construction of the various GALDH variants. Only sense primers are shown, changed codons are underlined

Variant	Oligonucleotide sequence (5' to 3')
E386A	GCACCTGCTCCAATAGCGCAGCGATGGACAGC
E386Q	GCACCTGCTCCAATACAGCAGCGATGGACAGC
E386D	GCACCTGCTCCAATAGATCAGCGATGGACAGC
R388A	GCTCCAATAGAGCAGGCATGGACAGCTCG
R388H	GCTCCAATAGAGCAGCATTGGACAGCTCG
R388K	GCTCCAATAGAGCAGAAATGGACAGCTCG

Enzyme production and purification - *E. coli* BL21(DE3) cells, harboring a pET-GALDH plasmid, were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin until an OD_{600} of 0.7 was reached. GALDH expression was induced by addition of 0.4 mM isopropyl-thio-β-D-galactopyranoside and the incubation was continued for 16 h at 37°C. The His_6-tagged GALDH variants were purified essentially as described before (Leferink et al., 2008[b]).

Spectral analysis - Absorption spectra were recorded at 25°C on a Hewlett Packard (Loveland, CO, USA) 8453 diode array spectrophotometer in 50 mM sodium phosphate, pH 7.4. Spectra were collected and analyzed using the UV-Visible ChemStation software package (Hewlett Packard). The molar absorption coefficients for the mutant proteins were determined by recording absorption spectra in the presence and absence of 0.1% (w/v) SDS, assuming a molar absorption coefficient for free FAD of 11.3 mM^{-1} cm^{-1} at 450 nm. Enzyme concentrations were routinely determined by measuring the absorbance at 450 nm using a molar absorption coefficient of 12.9 mM^{-1} cm^{-1} for wild-type GALDH (Leferink et al., 2008[b]). Photoreduction of GALDH variants (10-20 μM) in the presence of EDTA and 5-deazaflavin was performed in 50 mM sodium phosphate, pH 7.4 as described (Macheroux, 1999). Catalytic amounts of glucose oxidase and 10 mM β-D-glucose were added to scavenge final traces of oxygen and catalase was added to remove hydrogen peroxide formed during the reaction. Solutions were made anaerobic by alternate evacuation and flushing with oxygen-free argon. Illumination was performed in a 25°C water bath with a 375 W light source (Philips, Eindhoven, The Netherlands) at 15 cm distance. Spectra were taken at regular intervals during illumination until complete reduction was achieved. Titration of GALDH variants (10 μM) with sodium sulfite was carried out in 50 mM sodium phosphate, pH 7.4. A 1 M sodium sulfite stock solution in 50 mM sodium phosphate, pH 7.4 was freshly prepared before use, suitable dilutions were made in the same buffer before addition to the enzyme solution. Spectra were taken until no further change was observed before the next addition was done. The dissociation constant (K_d) for the enzyme-sulfite complex was calculated from the change in absorbance at 450 nm (Leferink et al., 2008[b]).

Activity measurements - GALDH activity was routinely assayed by following the reduction of cytochrome *c* at 550 nm (Leferink et al., 2008[b]). Because dithiothreitol interferes with the reaction, it was removed from the enzyme solution by Bio-Gel P-6DG (Bio-Rad, Hercules, CA, USA) gel filtration immediately prior to use. Initial velocities were calculated using a molar difference absorption coefficient ($\Delta\varepsilon_{550}$) of 21 mM^{-1} cm^{-1} for reduced minus oxidized cytochrome *c*. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of L-galactono-1,4-lactone per min, which is equivalent to the reduction of 2 μmol of cytochrome *c* (Ôba et al., 1995).

Results

Spectral properties of GALDH mutants - To evaluate the role of Glu386 and Arg388 in GALDH catalysis, mutant proteins were created in which Glu386 was replaced by Asp, Gln or Ala and Arg388 was replaced by Lys, His or Ala. All six GALDH variants were expressed

as soluble holoproteins in *E. coli*, and could be purified in similar yields as wild-type GALDH. The specific activities of the purified mutant proteins were all less than 1% of the wild-type enzyme, which has a specific activity of 76 U mg^{-1} (Leferink et al., 2008[b]).

Four mutants E386D, E386A, R388K, and R388A were selected for further biochemical characterization. The UV/VIS absorption spectral properties of the Glu386 mutants were very similar to that of wild-type GALDH (Fig. 4.2A). The molar absorption coefficients of protein-bound flavin were determined to be 13.6 mM^{-1} cm^{-1} at 451 nm for E386D and 13.1 mM^{-1} cm^{-1} at 452 nm for E386A. Small perturbations were observed in the absorption spectra of both Arg388 mutants (Fig. 4.2B). Compared to wild-type GALDH, the absorption maximum at 375 nm is about 7 nm blue-shifted in both mutants, and the spectrum of R388A shows a more pronounced shoulder at 466 nm, similar to the CO variant R477A (Piubelli et al., 2008). The molar absorption coefficients were determined to be 13.6 mM^{-1} cm^{-1} at 450 nm for R388K and 13.5 mM^{-1} cm^{-1} at 450 nm for R388A.

Anaerobic photoreduction - Previously, we established that wild-type GALDH stabilizes the red anionic flavin semiquinone during anaerobic photoreduction (Leferink et al., 2008[b]). This property is indicative for the presence of a positive charge near the flavin. The ability of the GALDH active site mutants to stabilize the red anionic flavin semiquinone was determined by anaerobic photoreduction. Both Glu386 mutants stabilize maximal amounts of the red anionic semiquinone, similar to wild-type GALDH. Figure 4.2C shows the spectral changes of E386D during photoreduction. The R388K mutant partially stabilizes the red semiquinone (Fig. 4.2D), while the R388A undergoes complete reduction without the stabilization of a semiquinone species (not shown). In all cases full re-oxidation was observed upon aeration.

Sulfite adduct formation - The ability to form a flavin N5 sulfite adduct is characteristic for many flavoprotein oxidases, and correlates with the ability of the protein to stabilize the red anionic semiquinone. Although wild-type GALDH reacts poorly with molecular oxygen, it readily forms a covalent flavin N5-sulfite adduct with a K_d of 18 μM (Leferink et al., 2008[b]). The ability of the GALDH active site variants to form a sulfite adduct was investigated. Mutation of the Glu386 residue did not have a significant effect on the formation of the sulfite adduct. Both E386D (Fig. 4.2E) and E386A (not shown) stabilize the sulfite adduct with a similar K_d for the enzyme-sulfite complex as wild-type GALDH (14 and 5 μM, respectively). Mutation of Arg388, however, did have a clear effect on the formation of the sulfite adduct. Replacing Arg388 by Lys gave a K_d of 0.44 mM for the enzyme-sulfite complex, a 25-fold increase (Fig. 4.2F). Removal of the positive charge of Arg388 (R388A) has an even more drastic effect on the stabilization of the flavin-sulfite adduct, resulting in a K_d of > 20 mM, a more than 1000-fold increase compared to the wild-type enzyme (not shown).

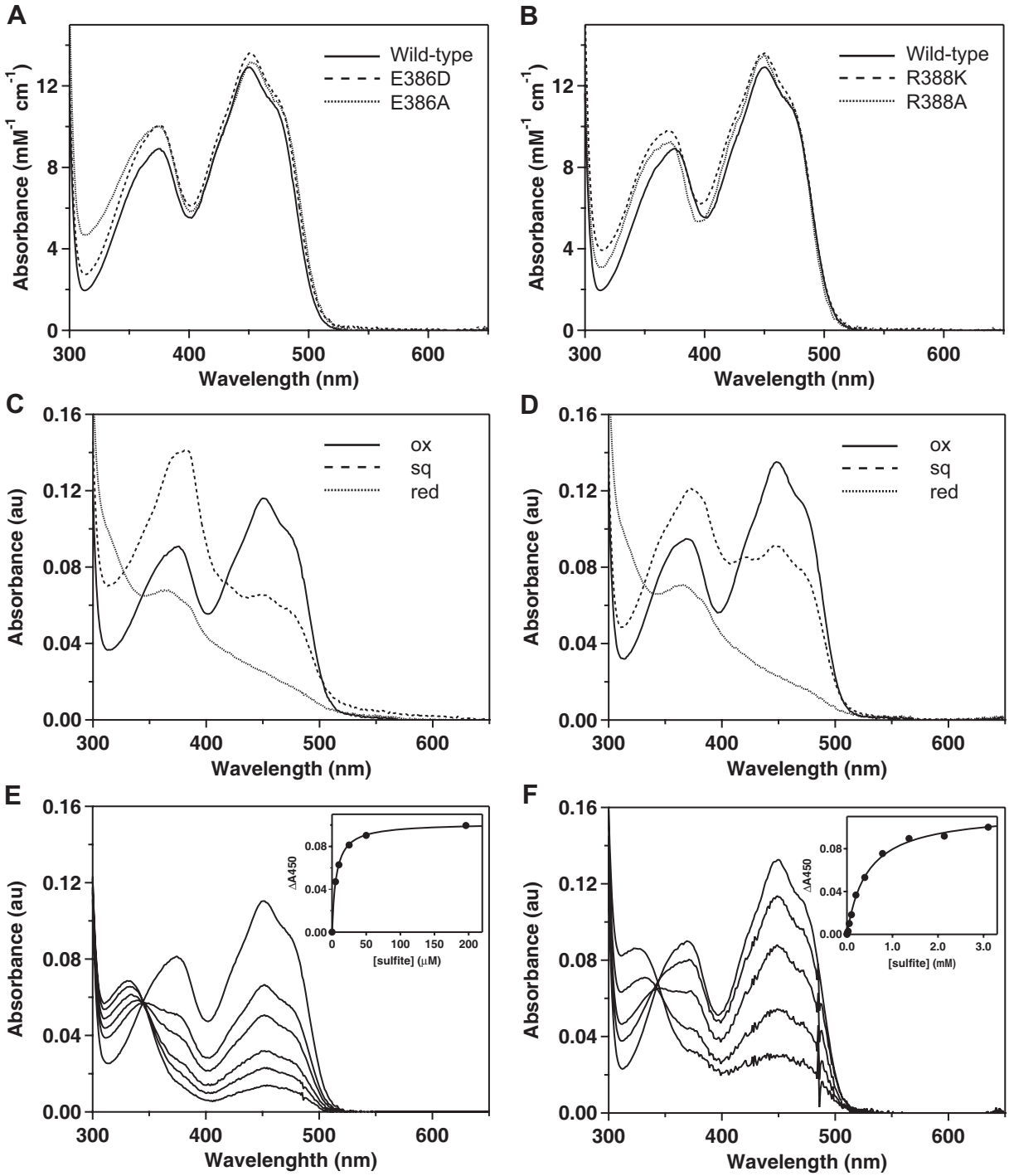


Figure 4.2. Spectral properties of GALDH variants. Top: Optical spectra of oxidized Glu386 (A) and Arg388 (B) variants compared to wild-type GALDH. Middle: Anaerobic photoreduction of GALDH E386D (C) and R388K (D). The solid lines represent the spectra taken before illumination (ox), the dashed lines the maximum amount of semiquinone (sq) observed, and the dotted lines represent fully reduced enzyme (red). Bottom: Titration of GALDH E386D (E) and R388K (F) with sodium sulfite. Spectra were recorded when no further changes were observed after each addition of sulfite. For clarity, only selected spectra are shown. The insets show the absorbance differences at 450 nm during the titration. All spectra were recorded in 50 mM sodium phosphate, pH 7.4.

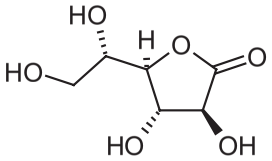
Catalytic properties - The steady-state catalytic properties of the various GALDH active site variants were determined for L-galactono-1,4-lactone, L-gulono-1,4-lactone and D-arabinono-1,4-lactone, using cytochrome *c* as electron acceptor. All GALDH variants were considerably less active than the wild-type enzyme (Table 4.2). The Glu386 variants show about a 100-fold increase in the Michaelis constant for the L-galactono-1,4-lactone substrate accompanied by a significant reduction in catalytic turnover. Interestingly, the E386D variant shows a preference for L-gulono-1,4-lactone over L-galactono-1,4-lactone, exhibiting a higher turnover number with L-gulono-1,4-lactone than the wild-type enzyme. Wild-type GALDH shows considerable activity with D-arabinono-1,4-lactone (k_{cat} = 51 s^{-1}) but has a rather low affinity for this substrate (K_m = 10.2 mM), suggesting that the length and stereochemistry of the side-chain is important for proper binding and aligning of the substrate lactone. For the active site mutants activity with D-arabinono-1,4-lactone could only be detected with E386D, but again the mutant is far less efficient than the wild-type enzyme. The Arg388 variants show a severe reduction in affinity for L-galactono-1,4-lactone, reflected by a nearly 1000-fold increase in the K_m value. The R388K variant shows considerable turnover, but removal of the positive charge in the R388A variant almost completely abolishes enzyme activity. Oxidation of L-gulono-1,4-lactone and D-arabinono-1,4-lactone by the Arg388 variants could not be detected, even at substrate concentrations up to 100 mM.

Discussion

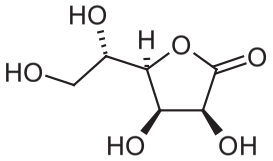
The flavoprotein GALDH completes the biosynthesis of vitamin C in plants. As a member of the VAO flavoprotein family, the enzyme has a two-domain folding topology with a conserved N-terminal FAD-binding domain and a C-terminal cap-domain. The active site is located at the interface of the two-domains. Due to the lack of a crystal structure for GALDH and related aldonolactone oxidoreductases, little is known about the nature of their active sites. The large variation in substrates between VAO family members (Leferink et al., 2008[a]) causes little conservation in the cap-domain, hampering the modeling of the substrate binding pocket of GALDH. Here we identified two GALDH active site residues in the cap-domain, Glu386 and Arg388, that are involved in substrate binding and catalysis. This Glu-Arg pair is conserved in other aldonolactone oxidoreductases as well as in AldO and CO, two related VAO-members with a known crystal structure. Both residues are located at similar positions in AldO and CO (Fig. 4.1), and interact with the substrate and the flavin through the formation of hydrogen bonds (Coulombe et al., 2001; Forneris et al., 2008).

Single Glu386 replacements drastically decrease the affinity of GALDH for L-galactono-1,4-lactone. Removal of Glu386 does not influence the electrostatics around the flavin, but does have a large effect on k_{cat}. Glu386 could therefore act as catalytic base, similar to Glu475

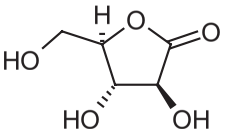
Tabel 4.2. Steady-state kinetic parameters of GALDH variants. Apparent kinetic constants were determined at 25°C in assay buffer (pH 8.8) (*I* = 25 mM) with varying aldonolactone concentrations (10 μM – 100 mM) and a constant cytochrome *c* concentration (50 μM). Values are the mean ± SD of at least two experiments



L-galactono-1,4-lactone



L-gulono-1,4-lactone



D-arabinono-1,4-lactone

Variant	K_m (mM)	k_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})
Wild-type	0.17 ± 0.01[a]	134 ± 5[a]	770[a]	13.1 ± 2.8[a]	4.0 ± 0.2[a]	0.31[a]	10.2 ± 0.8	51 ± 2	5.0
E386D	12.1 ± 1.3	4.7 ± 0.1	0.39	18.7 ± 1.1	10.4 ± 1.0	0.56	107 ± 8	1.2 ± 0.5	0.011
E386A	17.9 ± 3.1	1.4 ± 0.1	0.078	12.4 ± 2.8	0.22 ± 0.01	0.018	nd	nd	nd
R388K	123 ± 3	48.5 ± 0.8	0.40	nd	nd	nd	nd	nd	nd
R388A	113 ± 25	0.7 ± 0.5	0.006	nd	nd	nd	nd	nd	nd

[a] Data from ref. (Leferink et al., 2008[b])

nd = not detectable

in CO (Coulombe et al., 2001). Glu320 in AldO does also not directly interact with the FAD-cofactor, but forms hydrogen bonds with two hydroxyl groups of the substrate (Forneris et al., 2008). Replacing Glu386 with Asp in GALDH results in a gulonolactone dehydrogenase variant that is more active with L-gulono-1,4-lactone than the wild-type enzyme. Presumably the shorter side chain of residue 386 provides a better geometry for the interaction with the L-gulono-1,4-lactone substrate, which has a different stereochemical configuration of the hydroxyl substituent at C3 than L-galactono-1,4-lactone. A difference in catalytic efficiency as a result of a stereochemically unfavorably substrate conformation has also been reported for the AldO substrates sorbitol and mannitol (Forneris et al., 2008).

Wild-type GALDH exhibits a high K_m (10.2 mM) for the D-arabinono-1,4-lactone substrate, which has the same stereochemical configuration at C2 and C3 as L-galactono-1,4-lactone but a shorter side chain. This suggests that all hydroxyl groups are involved in substrate binding, participating in a well defined hydrogen-bonding network as was proposed for AldO (Forneris et al., 2008). Previously we identified a Cys residue (Cys340) that is also involved in substrate binding. This Cys is strictly conserved in aldonolactone oxidoreductases and not in other related VAO-members (Leferink et al., 2008[c]). Together with the present findings this might explain the narrow substrate specificity of GALDH.

Single replacements of Arg388 also have a dramatic effect on GALDH catalysis. Whereas R388K shows a reasonable turnover rate, removal of the charge results in a protein that is hardly active ($k_{cat} < 1\ s^{-1}$). Arg388 critically interacts with the substrate, replacement by either Lys or Ala gives a thousand-fold increase in the Michaelis constant for L-galactono-1,4-lactone. The corresponding Arg322 in AldO is involved in hydrogen bonding with substrate C1, the site of oxidation, and flavin C4=O, suggesting a similar critical role (Forneris et al., 2008).

GALDH can stabilize negative charges in the active site (Leferink et al., 2008[b]). Replacement of Arg388 by Ala results in a variant that is unable to stabilize the anionic flavosemiquinone and the flavin-sulfite adduct. The fact that the positive charge of Arg388 is important for the stabilization of the anionic flavosemiquinone suggests that it is located near the N1-C2 locus of the isoalloxazine ring of the flavin. A positive charge in this region will also stabilize the anionic form of the two-electron reduced flavin, which generally has a low pK_a (Müller, 1991), and increase the flavin redox potential (Ghisla and Massey, 1989). Interestingly, the non-covalently bound FAD of GALDH has a relatively high redox potential (-44 mV) (Leferink et al., 2009).

The dissociation constants for the enzyme-sulfite adducts of the Arg388 variants are directly related to the presence of a positive charge in close proximity of the flavin. The ability of a flavoprotein to form a flavin-sulfite adduct is not related to its oxidase activity, as previously proposed (Massey et al., 1969), but to the nature of the active site, i.e. the presence

of positive charges. The crystal structure of AldO in complex with sodium sulfite was determined (Forneris et al., 2008). The sulfite-adduct is stabilized by Arg322 and Lys375 in AldO, corresponding to Arg388 and Lys455 in GALDH. The adduct is formed on the *si*-face of the flavin, the same side as the substrate binding pocket, while the reaction with molecular oxygen presumably takes place on the *re*-face of the flavin in VAO-members (Leferink et al., 2009).

In conclusion, we have shown that Glu386 and Arg388 of GALDH are required for optimal catalysis. Glu386 might be involved in substrate activation and Arg388 seems crucial for the stabilization of negative charge that is generated as a result of flavin reduction.

Acknowledgements

We are grateful to Dr. Gerben Visser and Dr. Carel Weijers (Laboratory of Organic Chemistry, Wageningen University) for help with the synthesis of L-galactono-1,4-lactone. This research was supported by a grant from the Carbohydrate Research Centre Wageningen (CRC-W).

chapter 5

Interaction of L-galactono-γ-lactone dehydrogenase with cytochrome *c*

Nicole G. H. Leferink[1]*, Qamar Bashir[2]*, Marcellus Ubbink[2],
Willem J. H. van Berkel[1]

[1] Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
[2] Gorlaeus Laboratories, Leiden Institute of Chemistry, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands
* these authors contributed equally

Abstract

L-Galactono-γ-lactone dehydrogenase (GALDH) catalyzes the terminal step of vitamin C biosynthesis in plant mitochondria, the oxidation of L-galactono-1,4-lactone into L-ascorbate with the concomitant reduction of cytochrome *c* (Cyt *c*). Electron transfer between GALDH and Cyt *c* presumably involves the formation of a complex between the two redox partners. A surface engineering strategy was followed to identify the role of charged residues on the surface of *Arabidopsis thaliana* GALDH. In total three patches of Lys and Glu residues in close proximity were identified and mutated to Ala. Using heteronuclear nuclear magnetic resonance (NMR) spectroscopy transient complex formation was demonstrated between *A. thaliana* GALDH and a 9-fold surface mutant with isotopically enriched ferric yeast iso-1-Cyt *c*. Chemical shift perturbations for ^{1}H and ^{15}N nuclei of ferric yeast iso-1-Cyt *c*, arising from the interactions with unlabelled GALDH, were used to map the interacting surface of yeast iso-1-Cyt *c*. Based on the chemical shift perturbations, the residues on yeast iso-1-Cyt *c* involved in the interaction with GALDH have been highlighted and the dissociation constant of the complex has been calculated. The interaction with Cyt *c* remains intact after removing 9 charged residues at the GALDH protein surface. It is concluded that the complex is transient and of a dynamic nature.

Keywords: L-galactono-γ-lactone dehydrogenase; cytochrome *c*; NMR; electron transfer; surface engineering; vitamin C

Abbreviations: Cyt, cytochrome; DTT, dithiothreitol; GALDH, L-galactono-γ-lactone dehydrogenase; HSQC, heteronuclear single-quantum coherence; NMR, nuclear magnetic resonance; VAO, vanillyl-alcohol oxidase

Introduction

The mitochondrial flavoenzyme L-galactono-γ-lactone dehydrogenase (GALDH; L-galactono-1,4-lactone: ferricytochrome *c*-oxidoreductase, EC 1.3.2.3) completes vitamin C (L-ascorbate) biosynthesis in plants. GALDH is a so-called aldonolactone oxidoreductase belonging to the vanillyl-alcohol oxidase (VAO) flavoprotein family (Fraaije et al., 1998; Leferink et al., 2008[a]). Members of this family share a conserved FAD-binding domain and a less conserved cap-domain, the latter domain determines the substrate specificity. Most VAO-members are hydrogen peroxide producing oxidases, plant GALDH, however, exclusively uses cytochrome *c* (Cyt *c*) as electron acceptor (Leferink et al., 2008[b]). GALDH is localized in the mitochondrial intermembrane space where it is involved, besides from producing L-ascorbate, in shuttling electrons into the respiratory chain via Cyt *c* and the assembly of respiratory complex I (Bartoli et al., 2000; Alhagdow et al., 2007; Pineau et al., 2008). The highly soluble Cyt *c* is a strongly conserved heme-containing component of the electron transport chain, where it transfers electrons between the membrane bound Cyt *c* reductase and Cyt *c* oxidase complexes (Saraste, 1999).

Electron transfer between GALDH and Cyt *c* presumably involves a physical interaction between both redox partners. Due to the charged nature of Cyt *c* (p*I* ~9-11) electrostatic interactions are thought to play an important role in complex formation. Cyt *c* contains a patch of positively charged residues surrounding the heme edge, the part of the surface where the heme cofactor is most accessible (Ubbink and Bendall, 1997). No crystal structure is available for GALDH or its homologues, so no information is available about possible interaction sites on the protein surface. The GALDH sequence, however, contains an above average number of charged residues, mostly Glu and Lys, which could be involved in surface interactions. Large, surface exposed hydrophilic residues often occur in patches of 2-3 residues in close proximity (Baud and Karlin, 1999) and their replacement by Ala has been shown to improve protein crystal quality (Derewenda, 2004).

Previously, we were unable to detect any interaction between *Arabidopsis thaliana* GALDH and bovine heart Cyt *c* by size-exclusion chromatography (Leferink et al., 2008[b]). This raised the possibility of transient interactions (Crowley and Carrondo, 2004), which are likely influenced by the surface properties of both proteins. To gain insight into this matter, NMR chemical shift perturbation analyses were performed on wild-type GALDH and a GALDH 9-fold surface mutant using ^{15}N-labelled ferric yeast iso-1 Cyt *c*. The residues on yeast iso-1 Cyt *c* involved in the interaction with GALDH and the 9-fold surface mutant have been highlighted and the dissociation constants of the complexes have been calculated.

Materials and Methods

Chemicals - FAD, L-galactono-1,4-lactone, and bovine heart cytochrome *c* were from Sigma-Aldrich (St Louis, MO, USA). Pwo DNA polymerase and mini-Complete protease inhibitor was from Roche Molecular Biochemicals (Mannheim, Germany). Restriction endonucleases, T4-DNA ligase and dNTPs were purchased from Invitrogen (Carlsbad, CA, USA). Oligonucleotides were synthesized by Eurogentec (Liege, Belgium). The pET23a(+) expression vector and *E. coli* strain BL21(DE3) were from Novagen (San Diego, CA, USA). All other chemicals were from commercial sources and of the purest grade available.

Cloning and site-directed mutagenesis - The cDNA encoding mature GALDH (accession nr. At3g47930), has been cloned previously into the pET23a(+) vector to yield pET-GALDH-His_6 (Leferink et al., 2008[b]). The GALDH surface mutants were constructed using the QuickChangeII method (Stratagene, La Jolla, CA, USA) in the AtGALDH background without His_6-tag. The native GALDH gene was amplified from the pET-GALDH-His_6 (Leferink et al., 2008[b]) vector using the oligonucleotides AtGALDH_fw102 (5'-GGA ATT CCA TAT GTA CGC TCC TTT ACC TGA AG-3') introducing a *Nde*I restriction site (underlined), and AtGALDH_rv (5'-CCG GAA TTC TTA AGC AGT GGT GGAGAC-3'), introducing a TAA stop-codon and *Eco*RI restriction site (underlined). The amplified fragment was cloned between the *Nde*I and *Eco*RI sites of the pET23a(+) expression vector. The resulting pET-GALDH2 construct was verified by automated sequencing of both strands. The GALDH surface variants were constructed using pET-GALDH2 as template. The oligonucleotides used are listed in Table 5.1. The 9-fold surface mutant was constructed by three rounds of mutagenesis. Successful mutagenesis was confirmed by restriction enzyme digestions and automated sequencing. The resulting constructs were electroporated to *E. coli* BL21(DE3) cells for recombinant expression.

Table 5.1. Oligonucleotides used for the construction of the GALDH surface variants. Only sense primers are shown, changed nucleotides are in small case, introduced restriction sites are underlined.

Variant	Oligonucleotide sequence (5' to 3')	Site
E44A-K45A-K46A	GTTAAGGAATCTCATgcGgccgcGTTAAGGATTCGTCC	*Not*I
E83A-K84A-K85A	CTAGAGGTGGATAAAGcGgcGgctcGaGTTACGGTGCAGG	*Xho*I
K245A-K247A-K249A	GGAGTGGGCCACCTgcaGACgcACCAgcGTACACTACAGATGAGG	*Pst*I

Enzyme production and purification - Wild-type GALDH-His_6 was expressed and purified essentially as described (Leferink et al., 2008[b]). For production of the GALDH surface mutants, *E. coli* BL21(DE3) cells, harbouring a pET-GALDH2 mutant plasmid were grown in LB medium supplemented with 100 μg/ml ampicillin until the OD_{600} reached ~0.6.

Expression was induced by addition of 0.2 mM isopropyl-thio-β-D-galactopyranoside and the incubation was continued for 16 h at 37°C. The cells were harvested by centrifugation and resuspended in 25 mM Tris-HCl, pH 7.4, supplemented with 1 tablet of mini-Complete protease inhibitor (Roche Molecular Biochemicals), 10 mM $MgCl_2$ and DNase I (pH 7.4) and subsequently passed twice through a pre-cooled French Pressure cell (SLM Aminco, SLM Instruments, Urbana, IL, USA) at 10 000 psi. The resulting homogenate was centrifuged at 25 000 *g* for 30 min at 4°C to remove cell debris and the supernatant was subsequently loaded onto a HiLoad 26/10 Q-Sepharose FF column (GE Healthcare, Uppsala, Sweden) equilibrated with 25 mM Tris-HCl, pH 7.4. Proteins were eluted with a linear gradient of 0-0.2 M NaCl in the same buffer. Active fractions were pooled and dialyzed o/n against 25 mM sodium phosphate, pH 6.5. After removal of insoluble material, the supernatant was loaded onto a Ceramic hydroxyapatite XK 26/11 (GE Healthcare) column equilibrated with the same buffer and the proteins were eluted with a gradient of 25-500 mM sodium phosphate, pH 6.5. Active fractions were pooled and concentrated. The concentrated fraction was treated with dithiothreitol (DTT) and saturated with FAD. Excess FAD was removed by passing the enzyme solution through Biogel P-6DG (Bio-Rad) equilibrated with 20 mM NaPi, 0.1 mM DTT, pH 7.4. The final preparation was stored at -80°C.

Protein analysis - SDS-PAGE was performed using 12.5% acrylamide slab gels essentially as described by Laemmli (Laemmli, 1970). Proteins were stained using Coomassie Brilliant Blue R-250. Total protein concentrations were estimated using the Bradford protein assay from Bio-Rad (Hercules, CA, USA) with BSA as standard. Desalting or buffer exchange of small aliquots of enzyme was performed with Bio-Gel P-6DG columns (Bio-Rad).

Spectral analysis - Absorption spectra were recorded at 25°C on a Hewlett Packard (Loveland, CO, USA) 8453 diode array spectrophotometer in 50 mM sodium phosphate, pH 7.4. Enzyme concentrations were routinely determined by measuring the absorbance at 450 nm using a molar absorption coefficient of 12.9 mM^{-1} cm^{-1} for wild-type GALDH (Leferink et al., 2008[b]). The molar absorption coefficients of the surface mutants were determined by recording absorption spectra in the presence and absence of 0.1% (w/v) SDS, assuming a molar absorption coefficient for free FAD of 11.3 mM^{-1} cm^{-1} at 450 nm. Spectra were collected and analyzed using the UV-Visible ChemStation software package (Hewlett Packard).

Activity measurements - GALDH activity was routinely assayed by following the reduction of cytochrome *c* at 550 nm (Leferink et al., 2008[b]). Initial rates were calculated using a molar difference absorption coefficient ($\Delta\varepsilon_{550}$) of 21 mM^{-1} cm^{-1} for reduced minus oxidized

cytochrome *c*. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of L-galactono-1,4-lactone per min, which is equivalent to the reduction of 2 μmol of cytochrome *c* (Ôba et al., 1995).

Preparation of NMR samples - The isotopically-enriched ^{15}N Cyt *c* was produced in *E. coli* and purified as reported previously (Pollock et al., 1998; Morar et al., 1999). The protein concentration of ^{15}N Cyt *c* was determined from the absorbance peak at 410 nm (ε = 106.1 $mM^{-1}cm^{-1}$) (Margoliash and Frohwirt, 1959). The NMR samples contained 70 nmoles of the unlabelled GALDH and varying amounts of the ^{15}N labelled Cyt *c* in 20 mM sodium phosphate pH 7.4, 6% D_2O for lock, and 0.1 mM $CH_3CO^{15}NH_2$ as internal reference. The pH of the samples was checked before and after each titration step and adjusted, if necessary, with small aliquots of 0.1 M NaOH or 0.1 M HCl solutions. For the NMR titrations, microlitre aliquots of the stock solution of the ^{15}N labelled protein (1.57 mM of ^{15}N labelled ferric Cyt *c*) were added to the sample containing 0.5 ml of the unlabelled partner protein with an initial concentration of 0.14 mM. The titration consisted of 13 experimental points with 0, 20, 35, 50, 75, 100, 125, 150, 175, 200, 225, 250, and 300 nmoles of the ^{15}N labeled Cyt *c*.

NMR experiments - The NMR experiments were performed at 303 K on a Bruker DMX600 spectrometer equipped with TCI-Z-GRAD cryoprobe (Bruker, Karlsruhe, Germany). For each titration, 1D and 2D [^{15}N, ^{1}H] HSQC spectra were acquired. The spectral widths (in Hz) for 2D [^{15}N, ^{1}H] HSQC spectra were 1520.45 and 8090.61. All 2D spectra were acquired with 2048 and 160 complex points in the ^{1}H and ^{15}N dimensions, respectively. Data processing of the 1D ^{1}H and 2D [^{15}N, ^{1}H] HSQC spectra were performed in XWINNMR (Bruker) and AZARA 2.7 (http://www.bio.cam.ac.uk/azara/), respectively. The amides not observed in the present work were A3, E21, H26, V28, N31, H33, G34, H39, G45, S47, N56, G77, M80, G83, G84, K86, K87 and E88 for ferric Cyt *c*. Chemical shift perturbations of ^{15}N and ^{1}H nuclei were analysed by overlaying the spectrum of bound ^{15}N labelled protein with that of the free protein in Ansig-for-Windows (Helgstrand et al., 2000). Chemical shift titration curves were analysed with a two-parameter non-linear least square fit using a one site binding model:

$$\Delta v^{i} = \tfrac{1}{2}\Delta v^{0}\left[A - \sqrt{A^{2} - \frac{4}{R}}\right] \qquad \text{(eq. 5.1)}$$

$$A = 1 + \tfrac{1}{R} + \frac{P_0 R + C_0}{R P_0 C_0 K_A} \qquad \text{(eq. 5.2)}$$

where P_0 is the starting concentration P in the tube, C_0 is the stock concentration of C, R is the ratio of the total concentrations of C and P at step i in the titration, Δv^i is the chemical shift change at step i, Δv^0 is the maximal change in the chemical shift and K_A is the association constant. In the fits Δv^i and R were the dependent and independent variables, respectively, and Δv^0 and K_A the fitted parameters.

Results

Properties GALDH surface mutants - Wild-type GALDH readily forms crystals that diffract to about 3.5 Å resolution (NGH Leferink, E Carpanelli, A Mattevi & WJH van Berkel, unpublished results). A general strategy to improve crystal quality involves the removal of large surface exposed hydrophilic residues (Derewenda, 2004). The GALDH sequence contains an above average number of Lys and Glu residues (9.1% and 7.2% respectively, vs. 5.8% and 6.1% respectively, on average, (Derewenda and Vekilov, 2006)), covering the whole sequence. Surface exposed patches of Lys and Glu residues were assigned based on their predicted position in a 3D homology model of GALDH (Fig. 5.1). Three patches of Glu and/or Lys residues were mutated into Ala resulting in three triple mutants (E44A-K45A-K46A, FAD domain; E83A-K84A-K85A, FAD domain; K245A-K247A-K249A, cap domain) and one 9-fold surface mutant in which all mutations were combined.

All four GALDH surface variants were expressed as holoproteins and could be purified in similar quantities. During anion-exchange chromatography, a clear difference in elution behaviour was observed, confirming the surface exposed nature of the mutated residues. The triple mutants E44A-K45A-K46A (predicted pI = 6.6) and E83A-K84A-K85A (predicted pI = 6.6) eluted earlier than the wild-type enzyme (predicted pI = 6.7), while K245A-K247A-K249A (predicted pI = 6.4) and the 9-fold surface mutant (predicted pI = 6.1) were more retarded applying the same chromatographic conditions.

The purified surface mutants exhibited nearly identical optical properties for protein-bound FAD as wild-type GALDH. The molar absorption coefficients at 450 nm were respectively 13.5 ± 0.03 mM^{-1} cm^{-1} for E44A-K45A-K46A, 12.8 ± 0.05 mM^{-1} cm^{-1} for E83A-K84A-K85A, 13.5 ± 0.03 mM^{-1} cm^{-1} for K245A-K247A-K249A and 13.2 ± 0.05 mM^{-1} cm^{-1} for the 9-fold surface mutant. These values are in the same range as the value of 12.9 ± 0.02 mM^{-1} cm^{-1} reported for wild-type GALDH (Leferink et al., 2008[b]). The specific activity of the enzyme was fully retained after the surface mutations and the steady-state kinetic properties of the 9-fold surface mutant were nearly identical to wild-type GALDH (Table 5.2). These results show that the removal of hydrophilic residues at the protein surface does not influence the proper folding of GALDH. However, the GALDH surface mutants are prone

to precipitation upon thawing from storage conditions, particularly the 9-fold surface mutant, but they could be re-dissolved upon incubation at 37°C without loss of activity.

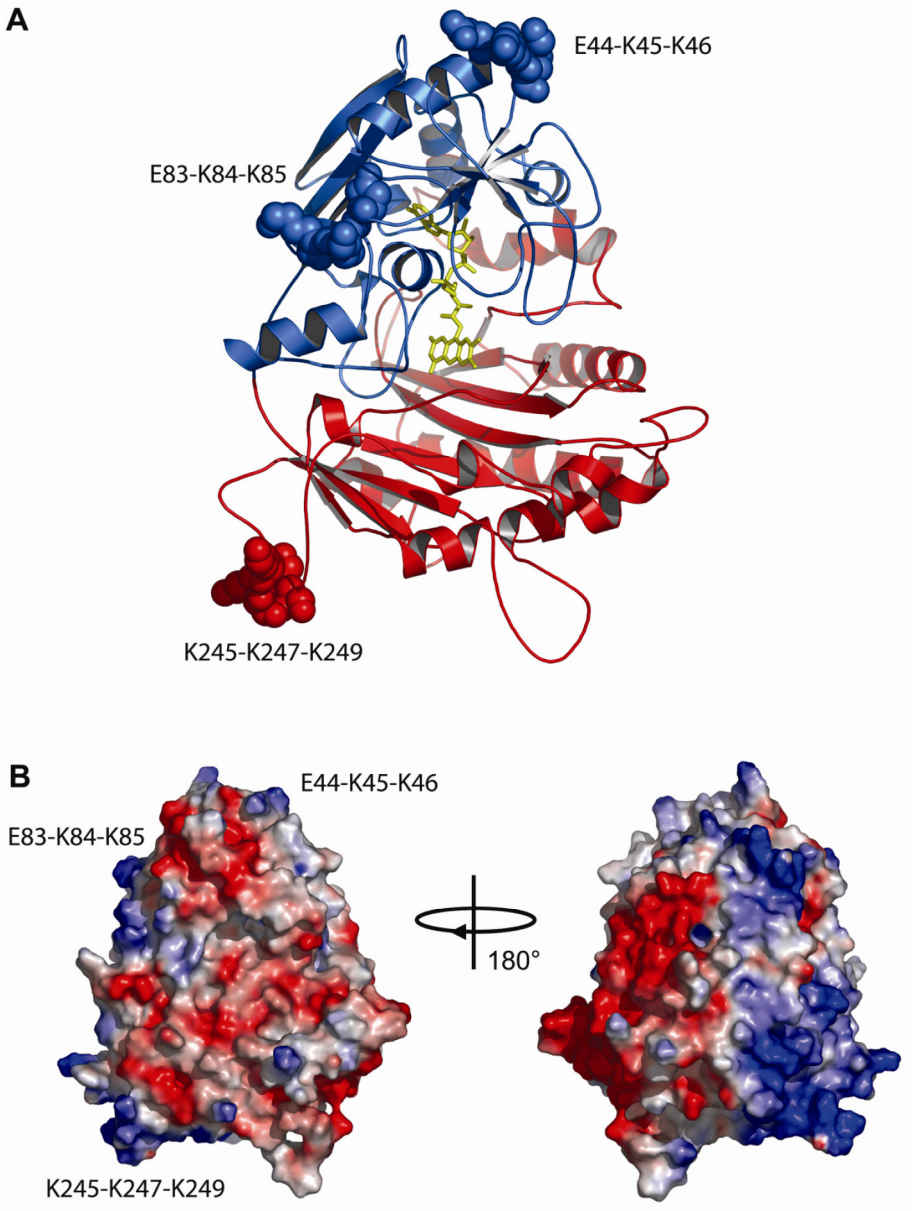


Figure 5.1. Prediction of the GALDH surface. A) Ribbon diagram of a 3D model of GALDH with the predicted patches of surface exposed residues. The predicted FAD-binding domain is in blue and the cap-domain in red. The mutated patches of surface residues are represented as spheres, the FAD-cofactor is represented in yellow sticks. B) Electrostatic surface model of GALDH. Electrostatic potentials were calculated in PyMol (www.pymol.org) using the vacuum electrostatics method. A positive surface potential is represented in blue, a negative surface potential is in red. The GALDH homology model was prepared with cholesterol oxidase (pdb 1I19; (Coulombe et al., 2001)) and alditol oxidase (pdb 2VFS; (Forneris et al., 2008)) as template structures using the programme Modeller (Sali and Blundell, 1993; Eswar et al., 2003).

The surface mutations did not significantly improve the crystal quality of GALDH. Because of its severely different iso-electric point, the 9-fold surface mutant was selected for the interaction studies with ^{15}N labeled Cyt *c*.

Table 5.2. Kinetic parameters of GALDH variants for L-galactono-1,4-lactone using bovine heart Cyt *c* (50 μM) as electron acceptor. Data are presented as the mean ± SD of at least two experiments.

GALDH variant	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m (mM^{-1} s^{-1})
Wild-type[a]	0.17 ± 0.05	134 ± 5	770
9-fold mutant	0.18 ± 0.01	132 ± 11	690

[a] Data obtained from (Leferink et al., 2008[b]).

NMR chemical shift perturbation analyses - The interaction of GALDH and the 9-fold surface mutant with ferric yeast iso-1 Cyt *c* was studied in reverse titration experiments, varying the concentration of ^{15}N labeled Cyt *c*. Complex formation was evidenced by an increase in line-width for all peaks in the NMR spectrum, as well as chemical shift perturbations of certain resonances. Chemical shift perturbations for the amide resonances were monitored in a series of 2D [^{15}N, ^{1}H] HSQC spectra. The chemical shift perturbations in [^{15}N] dimension ($\Delta\delta^N$) were more significant than those in [^{1}H] dimension ($\Delta\delta^H$). The $\Delta\delta^N$ values for Cyt *c* (75 nmoles) in the presence of 70 nmoles of wild-type GALDH and the GALDH 9-fold surface mutant are presented in Figure 5.2. Thr12 showed the largest chemical shift perturbation. The chemical shift perturbations for this residue were plotted against the Cyt *c*/GALDH ratio and the titration curves were fitted to a 1:1 binding model (Fig. 5.3). From these titration curves, the values of K_d and $\Delta\delta_{max}$ were calculated, yielding 50 ± 10 μM and 0.75 ± 0.04 ppm, respectively, for wild-type GALDH and 44 ± 10 μM and 0.93 ± 0.05 ppm for the 9-fold surface mutant. In case of the 9-fold surface mutant some extra peaks were observed in the spectra due to protein precipitation.

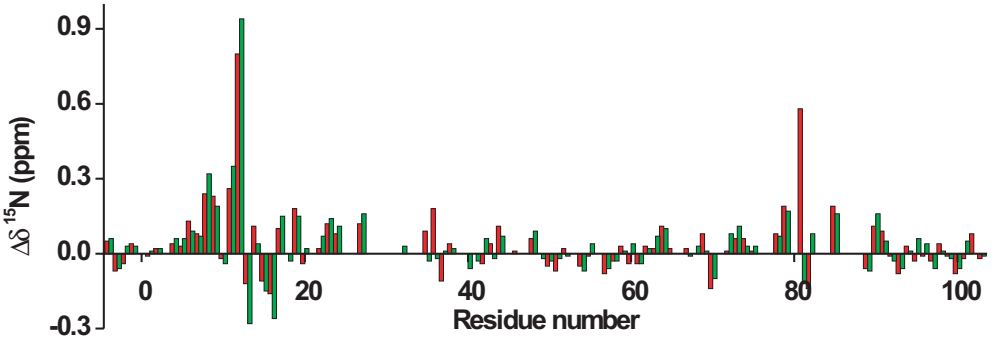


Figure 5.2. Amide chemical shift perturbations ($\Delta\delta^N$) for the Cyt *c* - GALDH complexes. Chemical shift perturbations caused by wild-type GALDH are in green, and chemical shift perturbations caused by the GALDH 9-fold surface mutant are in red.

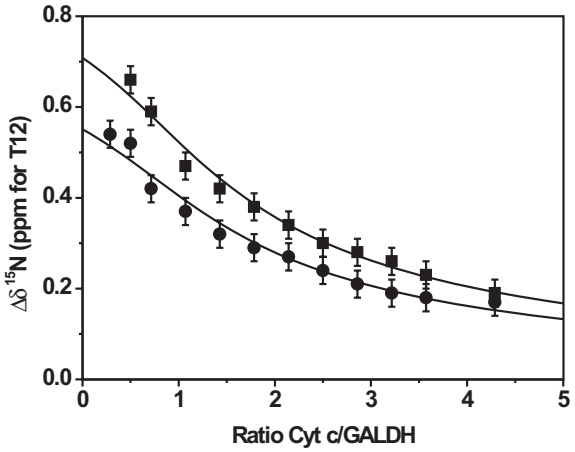


Figure 5.3. Titration profile of ferric Cyt *c* Thr12 resonances. The binding curve of wild-type GALDH and cyt *c* is represented by the closed circles (●), and the binding curve of the GALDH 9-fold surface mutant and cyt *c* is represented by the closed squares (■). Each curve represents the best fit to a 1:1 binding model.

For all titrations the magnitude of the line broadening in the bound form was the same for both shifted and unaffected peaks, and a single set of amide peaks in the 2D [^{15}N, ^{1}H] HSQC spectrum was observed. This indicates that the GALDH – Cyt *c* complex is in fast exchange on the NMR time scale ($k_{off} > 125\ s^{-1}$).

Discussion

GALDH is one of the few members of the VAO-family that uses Cyt *c* as electron acceptor rather than molecular oxygen (Leferink et al., 2008[a]). Related aldonolactone oxidoreductases are hydrogen peroxide producing oxidases that are unable to transfer electrons to Cyt *c*. Most aldonolactone oxidoreductases contain a covalently linked FAD (Kenney et al., 1976; Harada et al., 1979; Kenney et al., 1979). The absence of a covalent flavin link could provide GALDH with a greater conformational flexibility which may be needed for cross-talk with Cyt *c* (Leferink et al., 2008[b]). Here we studied the interaction of GALDH from *A. thaliana* with Cyt *c* in more detail and found that the interaction with Cyt *c* remains intact after removing 9 charged residues at the GALDH protein surface. No crystal structure is available for the aldonolactone oxidoreductases, hence little information is available about the structural properties of GALDH. Surface engineering is a tool that is used to improve protein crystal quality (Derewenda, 2004). Using this strategy three patches of Lys/Glu residues were identified on the surface of GALDH and replaced by Ala. Steady-state kinetic analysis of the 9-fold surface mutants for the galactonolactone substrate gave K_m and k_{cat} values that are highly similar compared to wild-type GALDH, indicating that the mutations did not affect the active site architecture and folding of the protein. The surface exposed nature of the mutations

was confirmed by anion-exchange chromatography. The crystallization properties of GALDH were not significantly improved by the introduced surface mutations, presumably due to their tendency to form aggregates. Already small disturbances in the homogeneity of the protein preparation may have negative effects on crystal growth and diffraction quality (Niesen et al., 2008).

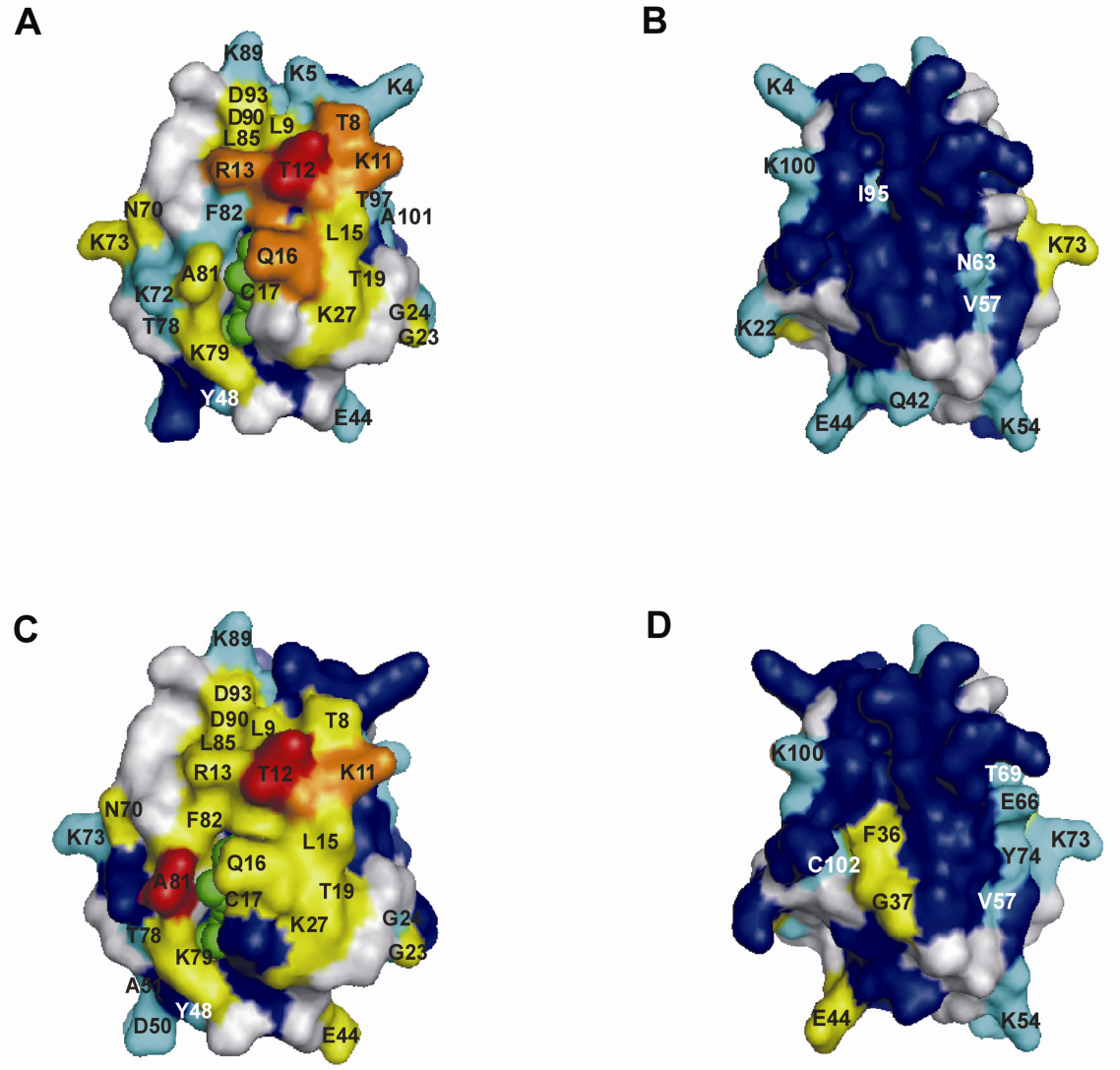


Figure 5.4. Map of chemical shift perturbations of Cyt *c* upon binding with GALDH. Surface representations of Cyt *c* (PDB entry 1YCC); the residues are coloured according to $\Delta\delta^N$ experienced upon binding of ferric Cyt *c* ($\Delta\delta^N$ > 0.5 ppm in red, $\Delta\delta^N$ > 0.25 ppm in orange, $\Delta\delta^N$ > 0.10 ppm in yellow, $\Delta\delta^N$ > 0.05 ppm in cyan and $\Delta\delta^N$ < 0.05 ppm in blue). Unassigned and proline residues are shown in grey, and the heme is colored green. A) and (B) represent the front side and back side of Cyt *c* in complex with wild-type GALDH, (C) and (D) represent the front side and back side of Cyt *c* in complex with 9-fold surface mutant of GALDH.

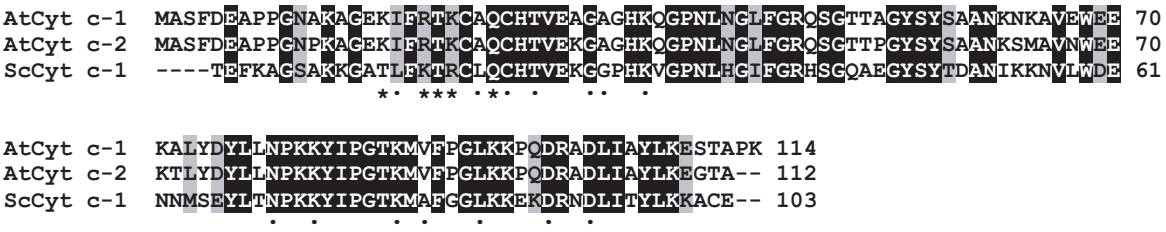


Figure 5.5. Clustal W multiple sequence alignment of *Arabidopsis* Cyt *c* isozymes and yeast iso-1 Cyt *c* (ScCyt *c*-1). Accession numbers used AtCyt *c*-1, At1g22840; AtCyt *c*-2, At4g10040; and ScCyt *c*-1, P00044. Identical residues are shaded in black, similar residues are shaded in grey. Residues of ScCyt *c*-1 showing the largest chemical shift perturbations (>0.25 ppm) are marked with an asterisk (*) and residues showing moderate chemical shift perturbations (>0.10 ppm) are marked with a dot. N.B. the numbering of yeast iso-1 Cyt *c* starts at -5.

The interactions of GALDH and the 9-fold surface mutant were studied by NMR chemical shift perturbation analyses using ^{15}N-labelled ferric yeast iso-1 Cyt *c*. The Cyt *c* residues involved in the interaction with GALDH are confined to a single surface patch on the front side of the molecule (Fig. 5.4). Only a few residues (Phe36 and Gly37 in case of the 9-fold surface mutant) on the back side are affected as well which may represent transmitted effects due to binding on the front side. Remarkably, wild-type GALDH and the 9-fold surface mutant show a similar binding constant for Cyt *c*. The binding map for the 9-fold surface mutant is very similar to wild-type GALDH, although there are subtle differences suggesting that in the complex with the 9-fold mutant Cyt *c* assumes a less dynamic, more well-defined orientation relative to GALDH than in complex with wild-type GALDH. The removal of 7 positive charges on the GALDH surface might result in less electrostatic repulsion between GALDH and Cyt *c*. Interestingly, binding maps of Cyt *c* in the complexes with GALDH (this work), Cyt b_5 (Volkov et al., 2005) and the non-physiological partner adrenodoxin (Worrall et al., 2003) are strikingly similar. Moreover, chemical shift mapping studies of Cyt *c* in the complexes with bovine Cyt b_5 (Volkov et al., 2005), yeast Cyt *c* peroxidase (Worrall et al., 2001), cyanobacterial Cyt *f* (Crowley et al., 2002), pea plastocyanin (Ubbink and Bendall, 1997), and GALDH (this work) indicate that Thr12 (Gln12 in horse heart Cyt *c* used in ref. (Ubbink and Bendall, 1997)) and Gln16 give the biggest binding shifts. This finding suggests that Cyt *c* employs a conserved set of surface-exposed residues for the interactions with a variety of proteins. There are several buried groups whose chemical shift perturbations cannot be explained by the direct interaction with a partner protein. Most likely, these are caused by transmittance of the binding effects from the surface of the protein to its core via covalent and hydrogen bonds (Ubbink and Bendall, 1997). The size of $\Delta\delta$ observed for the Cyt *c* - GALDH complex is small which can be explained by multiple fast-exchanging protein - protein orientations within the complex. For multiple, fast-exchanging, isoenergetic protein - protein configurations, the observed $\Delta\delta$ would be averaged over all orientations. This suggests that Cyt *c* and GALDH adopt different relative orientations within the complex, rather than form a

single, well defined structure. The residues of yeast iso-1 Cyt *c* showing the highest chemical shift perturbations (> 0.25 ppm) are conserved among both *Arabidopsis* Cyt *c* paralogs, including Thr12 (Fig. 5.5), suggesting that the interaction of GALDH and its physiological electron acceptors involves a similar mechanism.

In conclusion, we demonstrated that GALDH forms a transient complex with Cyt *c* and the removal of 9 charged surface residues has little effect on the formation and dynamics of the complex indicating that a defined binding or docking site for Cyt *c* is absent from GALDH.

Acknowledgements

This research was supported by a grant from the Carbohydrate Research Centre Wageningen to WJHB (CRC-W). QB was supported by a fellowship from the Higher Education Commission of Pakistan. We thank Elena Carpanelli and Andrea Mattevi (University of Pavia, Italy) for protein crystallization and Adrie Westphal (Laboratory of Biochemistry, Wageningen University) for protein homology modelling.

chapter 6

Galactonolactone dehydrogenase requires a redox-sensitive thiol for optimal production of vitamin C

Nicole G. H. Leferink[1], Esther van Duijn[2,3], Arjan Barendregt[2,3], Albert J. R. Heck[2,3], Willem J. H. van Berkel[1]

[1] Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
[2] Biomolecular Mass Spectrometry and Proteomics Group, Bijvoet Centre for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands
[3] Netherlands Proteomics Centre

Abstract

The mitochondrial flavoenzyme L-galactono-γ-lactone dehydrogenase (GALDH) catalyzes the ultimate step of vitamin C biosynthesis in plants. We found that recombinant GALDH from *Arabidopsis thaliana* is inactivated by hydrogen peroxide due to selective oxidation of Cys340, located in the cap domain. Electrospray ionization mass spectrometry (ESI-MS) revealed that the partial reversible oxidative modification of Cys340 involves the sequential formation of sulfenic, sulfinic and sulfonic acid states. *S*-glutathionylation of the sulfenic acid switches off GALDH activity and protects the enzyme against oxidative damage *in vitro*. C340A and C340S GALDH variants are insensitive towards thiol oxidation, but exhibit a poor affinity for L-galactono-1,4-lactone. Cys340 is buried beneath the protein surface and its estimated pK_a of 6.5 suggests the involvement of the thiolate anion in substrate recognition. The indispensability of a redox-sensitive thiol provides a rationale why GALDH was designed by Nature as a dehydrogenase and not, like related aldonolactone oxidoreductases, as an oxidase.

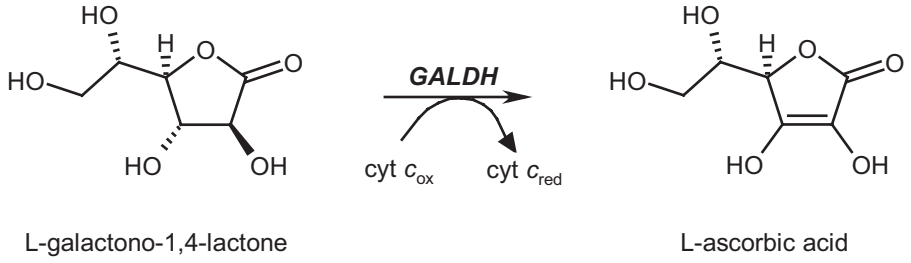
Keywords: cysteine modification; flavoprotein; dehydrogenase; glutathionylation; oxidative stress; vitamin C

Abbreviations: ALO, D-arabinono-γ-lactone oxidase; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; GALDH, L-galactono-γ-lactone dehydrogenase; GLO, D-gluconolactone oxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GUO, L-gulono-γ-lactone oxidase; 3-maleimido PROXYL, 3-maleimido-2,2,5,5-tetramethyl-1-pyrolidinyloxyl; NEM, *N*-ethylmaleimide; TNB, 5-thio-2-nitrobenzoate; VAO, vanillyl-alcohol oxidase

Introduction

L-Ascorbate (vitamin C) is the most consumed vitamin on earth. It is a multifunctional antioxidant that is particularly abundant in plants where it can reach millimolar concentrations, representing over 10% of the soluble carbohydrate content. L-Ascorbate is a cofactor for a number of enzymes and it is a major constituent of the intracellular redox buffer. Its main function in plants is to scavenge reducing equivalents originating from respiration and photosynthetic activity, protecting proteins, unsaturated fatty acids, and DNA from irreversible oxidative damage (Smirnoff and Wheeler, 2000).

The terminal step of L-ascorbate biosynthesis in plants is catalyzed by the mitochondrial flavoenzyme L-galactono-γ-lactone dehydrogenase (GALDH; L-galactono-1,4-lactone: ferricytochrome *c*-oxidoreductase; EC 1.3.2.3). GALDH mediates the two-electron oxidation of L-galactono-1,4-lactone into L-ascorbic acid with the concomitant reduction of cytochrome *c* (scheme 6.1):



Scheme 6.1. Reaction catalyzed by GALDH

Besides from producing L-ascorbate, the exploitation of the electron transport chain by GALDH is important for the proper functioning of plant mitochondria (Alhagdow et al., 2007). Furthermore, it has been reported that GALDH is required for the correct assembly of respiratory complex I (Pineau et al., 2008).

GALDH and other aldonolactone oxidoreductases are two-domain proteins with a conserved vanillyl-alcohol oxidase (VAO)-type FAD domain (Fraaije et al., 1998; Leferink et al., 2008[a]). Most aldonolactone oxidoreductases are hydrogen peroxide-producing oxidases containing covalently bound FAD, while GALDH reacts poorly with molecular oxygen and contains non-covalently bound FAD (Leferink et al., 2008[b]). Aldonolactone oxidoreductases have been isolated from various sources, but they are not well characterized. No crystal structure is available, and little is known about the nature of the active site and the catalytic mechanism. Several aldonolactone oxidoreductases, including GALDH from plants (Mapson and Breslow, 1958; Ôba et al., 1995; Østergaard et al., 1997; Imai et al., 1998; Yabuta et al., 2000), L-gulono-γ-lactone oxidase (GUO) from animals (Nishikimi, 1979), D-arabinono-γ-

lactone oxidase (ALO) from yeast (Huh et al., 1994), and trypanosomal aldonolactone oxidoreductases (Logan et al., 2007), are sensitive towards inactivation by thiol modifying agents. In line with this, we previously found that recombinant GALDH from *Arabidopsis thaliana* is slowly inactivated during storage and that the activity can be completely restored by treatment with the reducing agent dithiothreitol (DTT) (Leferink et al., 2008[b]).

So far nothing is known about the nature of the thiol inactivation, and the effect of oxidants on the activity of aldonolactone oxidoreductases has not been studied before. Here we investigated the susceptibility of GALDH to oxidative stress and identified a critical cysteine in the substrate binding site that makes the enzyme vulnerable towards irreversible inactivation.

Materials and Methods

Mutagenesis, expression and purification of GALDH variants - The GALDH variants C340S, and C340A, were constructed using pET-GALDH-His_6 (Leferink et al., 2008[b]) as template with the QuikChange II method (Stratagene, La Jolla, CA, USA). The following oligonucleotides (Eurogentec, Liege, Belgium) were used (only sense primers are shown, changed nucleotides are underlined): 5'-GAA ATT CTG GGC TTT GAC T<u>C</u>T GGT GGT CAG CAG TG-3' (C340S) and 5'-GAA ATT CTG GGC TTT GAC <u>GC</u>T GGT GGT CAG CAG TG-3' (C340A). For enzyme production *E. coli* BL21(DE3) cells, harboring a pET-GALDH plasmid, were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin until an OD_{600} of 0.7 was reached. Expression was induced by addition of 0.4 mM isopropyl-thio-β-D-galactopyranoside and the incubation was continued for 16 h at 37°C. The recombinant proteins were purified essentially as described before (Leferink et al., 2008[b]).

Spectral analysis - Absorption spectra were recorded at 25°C on a Hewlett Packard (Loveland, CO, USA) 8453 diode array spectrophotometer in 50 mM sodium phosphate, pH 7.4. Enzyme concentrations were routinely determined by measuring the absorbance at 450 nm using a molar absorption coefficient of 12.9 mM^{-1} cm^{-1} for wild-type GALDH (Leferink et al., 2008[b]). The molar absorption coefficients of C340S and C340A were determined by recording absorption spectra in the presence and absence of 0.1% (w/v) SDS, assuming a molar absorption coefficient for free FAD of 11.3 mM^{-1} cm^{-1} at 450 nm.

Activity measurements - GALDH activity was routinely assayed by following the reduction of cytochrome *c* at 550 nm (Leferink et al., 2008[b]). Initial velocities were calculated using a molar difference absorption coefficient ($\Delta\varepsilon_{550}$) of 21 mM^{-1} cm^{-1} for reduced minus oxidized cytochrome *c*. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1

μmol of L-galactono-1,4-lactone per min, which is equivalent to the reduction of 2 μmol of cytochrome *c* (Ôba et al., 1995).

Cysteine modification - All thiol modifications were carried out with purified enzyme preparations freshly incubated with 1 mM DTT and 200 μM FAD for 15 min at room temperature. Excess reagents were removed by Bio-Gel P-6DG (Bio-Rad, Hercules, CA, USA) gel filtration in 50 mM sodium phosphate, pH 7.4. The estimation of sulfhydryl groups of native and unfolded GALDH was performed according to the method of Ellman (Ellman, 1959) with the modifications of Habeeb (Habeeb, 1972). The assay was performed on 2 μM GALDH in 100 mM sodium phosphate, 0.5 mM EDTA, pH 8.0 with a 25-times molar excess of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (Merck, Darmstadt, Germany). The time-dependent release of 5-thio-2-nitrobenzoate anion (TNB) was measured at 412 nm (ε_{412} TNB = 13.6 mM^{-1} cm^{-1}). Recombinant GALDH was labeled with *N*-ethylmaleimide (NEM, Sigma-Aldrich, St Louis, MO, USA) or 3-maleimido-2,2,5,5-tetramethyl-1-pyrolidinyloxyl (3-maleimido-PROXYL, Syva, Palo Alto, CA, USA) by incubating 100 μM enzyme with 1 mM maleimide for 15 min at room temperature. The reaction was stopped by the addition of 5 mM DTT; excess reagents were removed by Bio-Gel P-6DG gel filtration in 50 mM sodium phosphate, pH 7.4. The time-dependent inactivation of GALDH by the different thiol modifying agents was performed at room temperature. The incubation mixtures contained 2 μM enzyme, and 50 μM DTNB, 50 μM NEM or 5 mM hydrogen peroxide. The pH-dependence of the hydrogen peroxide inactivation was performed at 25°C in 50 mM (Bis) Tris-HCl buffers, pH 6.0 – 8.8. Aliquots were withdrawn at intervals and assayed for residual enzyme activity using the standard assay procedure. The pK_a of Cys340 was calculated by fitting the inactivation data to equation 6.1:

$$y = a + \frac{b - a}{1 + e^{\frac{-\mathrm{pH} - \mathrm{pKa}}{k}}} \qquad \text{(eq. 6.1)}$$

Where *y* is the observed half-life of inactivation, *a* the calculated half-life at high pH, *b* the calculated half-life at low pH, and *k* the rate of inactivation.

Electron paramagnetic resonance (EPR) - EPR spectra of wild type GALDH and the C340A mutant, labeled with the 3-maleimido-PROXYL spin-label, were recorded on a X-band Bruker Elexsys E-500 ESR system equipped with a super-high-Q cavity ER 4122SHQE in combination with a SuperX X-Band Microwave Bridge type ER 049X. The concentration of spin-labeled enzyme was about 30 μM. Spectra were obtained at room temperature using 50 μl glass capillaries inserted in a quartz EPR tube. Spectra were recorded with a scan range of

10 mT, a modulation amplitude of 0.2 mT, a time constant of 10.24 ms, a scan time of 42 seconds and a microwave power of 5 mW. Up to 50 spectra were accumulated to improve the signal-to-noise ratio. All spectra were analyzed using the Xepr software (Bruker).

Mass spectrometry - For the identification of hydrogen peroxide induced modifications, 100 μM GALDH or C340A in 50 mM sodium phosphate, pH 7.4 was incubated with 5 mM hydrogen peroxide for 1 or 3 h at room temperature. *S*-glutathionylated GALDH was prepared by incubation of DTNB inactivated GALDH with 5 mM reduced glutathione (GSH), or by incubation of wild-type GALDH with 5 mM hydrogen peroxide and 5 mM GSH in 50 mM sodium phosphate, pH 7.4 for 3 h at room temperature. Excess reagents were removed by pouring over a Bio-Gel P-6DG column equilibrated with 50 mM ammonium acetate, pH 6.8. The protein samples were analyzed by mass spectrometry, as control untreated wild-type and C340A GALDH in 50 mM ammonium acetate, pH 6.8, were also analyzed. All mass spectrometric analyses were performed at protein concentrations of 2 μM under denaturing conditions to allow accurate molecular mass determinations. A solution of 5% formic acid was used to denature the protein samples.

Mass spectrometry measurements were performed in positive ion mode using an electrospray ionization time-of-flight (ESI-ToF) instrument (LCT, Waters, UK) equipped with a Z-spray nano-electrospray ionization source. Needles were made from borosilicate glass capillaries (Kwik-Fil, World Precision Instruments, Sarasota, FL) on a P-97 puller (Sutter Instruments, Novato, USA), coated with a thin gold layer by using an Edwards Scancoat (Edwards Laboratories, Milpitas, USA) six Pirani 501 sputter coater. Mass spectra were recorded with a capillary voltage of 1.2 kV and cone voltage of 70 V. The source pressure was raised to 6.8 mbar (Tahallah et al., 2001), and the pressure in the ToF was $1 \cdot 10^{-6}$ mbar. All spectra were mass calibrated by using an aqueous solution of cesium iodide (25 mg/ml).

Results

Assignment of the critical cysteine - Previously we established that recombinant GALDH from *A. thaliana* is slowly inactivated during storage and that DTT restores the activity (Leferink et al., 2008[b]). In line with this observation, GALDH was readily inactivated by the thiol-modifying agents DTNB (Fig. 6.1A) and NEM (Fig. 6.1B). Pre-incubation of the enzyme with L-galactono-1,4-lactone protected the enzyme from thiol modification, increasing the half-life of inactivation by one order of magnitude (Fig. 6.1A and 6.1B).

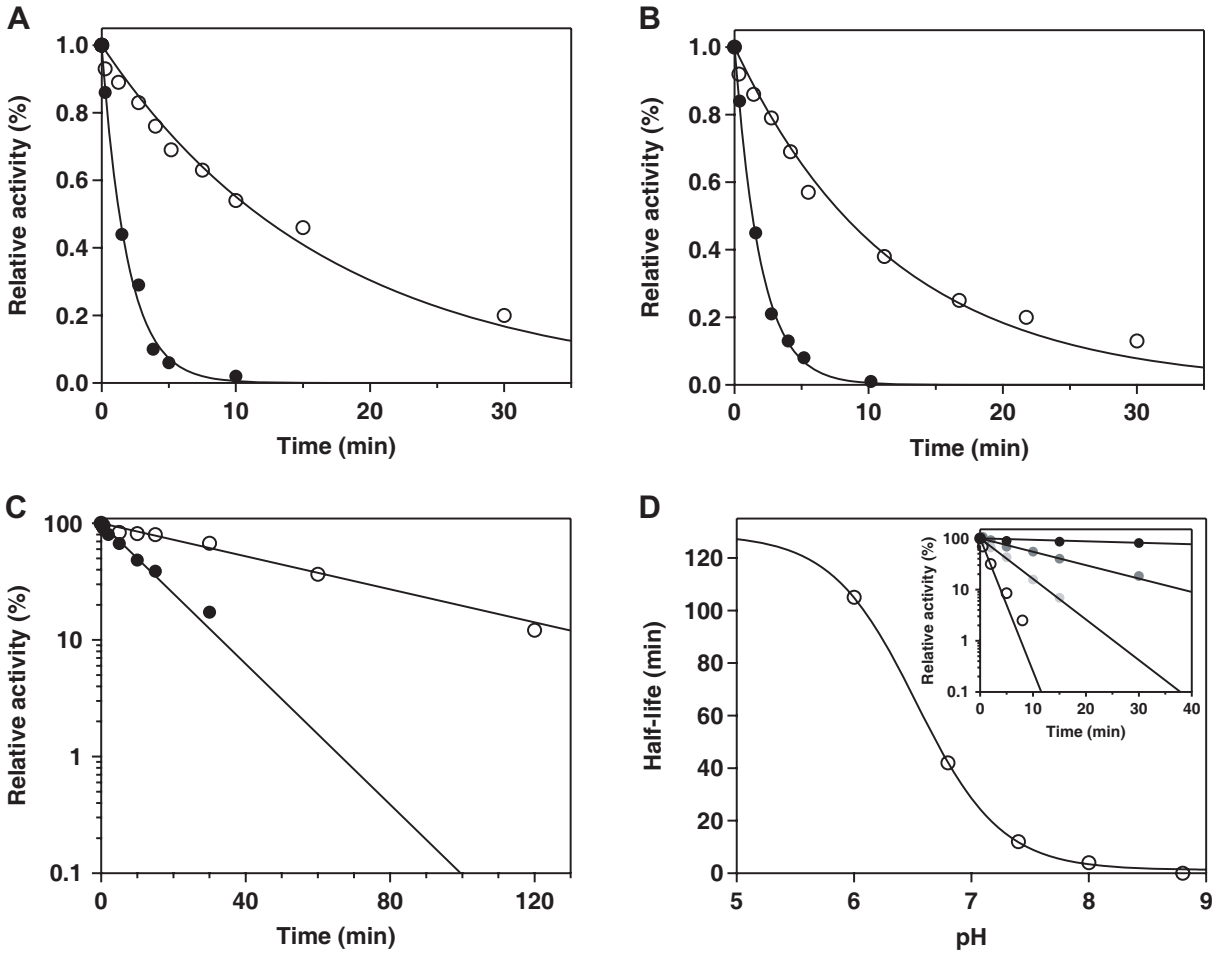


Figure 6.1. Time-dependent inactivation of GALDH with thiol-modifying agents. GALDH (2 μM) was incubated with 50 μM DTNB (A), 50 μM NEM (B) or 5 mM hydrogen peroxide (C) in 50 mM sodium phosphate, pH 7.4 in the presence (open circles) or absence (closed circles) of 1 mM L-galactono-1,4-lactone. D) Half-life of inactivation of GALDH (2 μM) by hydrogen peroxide (5 mM) as a function of pH. The inset shows the time-dependent inactivation of GALDH (2 μM) by hydrogen peroxide (5 mM) in 50 mM Bis-Tris-Cl, pH 6.0 (black circles), 50 mM Tris-Cl, pH 7.4 (dark gray circles), 50 mM Tris-Cl, pH 8.0 (light gray circles), and 50 mM Tris-Cl, pH 8.8 (open circles) For clarity, only selected traces are shown.

The number of reactive sulfhydryl groups in GALDH was estimated with the method of Ellman (Ellman, 1959). DTNB analysis of unfolded GALDH yielded a total number of five cysteines per polypeptide chain, consistent with the amino acid sequence of mature GALDH (Table 6.1). DTNB analysis of native GALDH revealed only one reactive cysteine (Table 6.1). DTNB analysis of NEM-inactivated GALDH yielded no response, indicating that both thiol-modifying reagents react with the same accessible cysteine.

To identify the critical cysteine residue, the amino acid sequence of GALDH was compared with other aldonolactone oxidoreductases. Interestingly, only one cysteine residue, Cys340 in recombinant GALDH, is conserved among the aldonolactone oxidoreductases that are sensitive towards inactivation by thiol modifying agents. D-Gluconolactone oxidase from

Table 6.1. Thiol content of native and unfolded GALDH variants as determined by DTNB analysis. Data are presented as the mean ± SD of two experiments.

GALDH	Thiol groups (mol/mol FAD)	
	Native	Unfolded
Wild-type	0.8 ± 0.1	4.9 ± 0.5
NEM-GALDH	0.2 ± 0.1	3.8 ± 0.4
C340S	0.2 ± 0.1	3.8 ± 0.1
C340A	0.1 ± 0.05	3.6 ± 0.1

Penicillium griseoroseum (PgGLO), which is resistant to inactivation by these compounds (Takahashi et al., 1976), lacks this cysteine, as well as the related VAO family members alditol oxidase, xylitol oxidase and sorbitol oxidase (Fig. 6.2). The conserved cysteine is located in the C-terminal cap domain, which determines the substrate specificity in VAO-type flavoproteins (Fraaije et al., 1998).

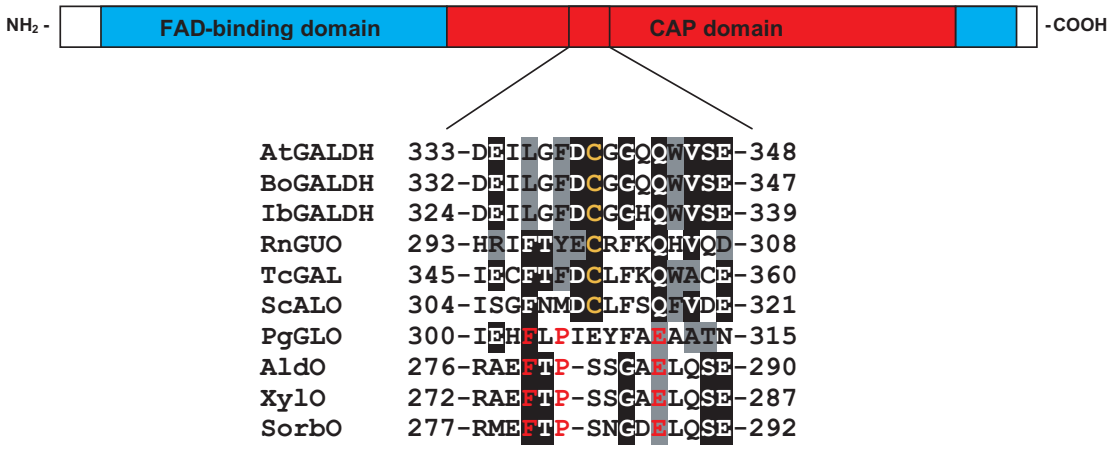


Figure 6.2. Sequence comparison of GALDH and related VAO-members. Upper panel: Schematic representation of the primary structure of mature GALDH with the FAD-binding domain in blue and the cap-domain in red. Lower panel: Sequence alignment around the conserved Cys. The accession numbers used are: *A. thaliana* GALDH (AtGALDH), Q8GY16; *Brassica oleracea* GALDH (BoGALDH), O47881; *Ipomoea batatas* GALDH (IbGALDH), Q9ZWJ1; *Rattus norvegicus* L-gulono-1,4-lactone oxidase (RnGUO), P10867; *Trypanosoma cruzi* galactonolactone oxidase (TcGAL), Q4DPZ5; *Saccharomyces cerevisiae* D-arabinono-1,4-lactone oxidase (ScALO), P54783; *Penicillium griseoroseum* D-gluconolactone oxidase (PgGLO), Q671X8; alditol oxidase (AldO), Q9ZBU1; xylitol oxidase (XylO), Q9KX73; sorbitol oxidase (SorbO), P7011. Identical residues are shaded in black, similar residues are shaded in gray. The conserved Cys is indicated in orange, conserved residues lining the active site entrance in AldO are marked in red.

To confirm Cys340 as the target of modification, two GALDH variants were created by site-directed mutagenesis in which Cys340 was replaced by a serine residue (C340S) or an alanine residue (C340A). Both mutant proteins were expressed as holoproteins and could be purified in similar quantities as the wild-type protein (Leferink et al., 2008[b]). DTNB analysis

showed that native C340S and C340A contained no surface accessible cysteine residues (Table 6.1). Both C340S and C340A are also insensitive towards inactivation by DTNB. These data establish that Cys340 is the target reactive cysteine in wild-type GALDH.

Cys340 is located in or near the active site - To investigate the role of Cys340 in GALDH catalysis, the biochemical properties of the C340S and C340A variants were studied in more detail. Both mutant proteins have flavin absorption characteristics similar to wild-type GALDH (not shown). The molar absorption coefficient (ε_{450}) of the protein-bound flavin was determined to be 13.2 and 13.0 mM^{-1} cm^{-1} for C340S and C340A, respectively, similar to the value of 12.9 mM^{-1} cm^{-1} reported for wild-type GALDH (Leferink et al., 2008[b]). Steady-state kinetic analysis revealed that the Michaelis-constant for L-galactono-1,4-lactone is 30-50 fold higher for C340S and C340A than for wild-type GALDH (Table 6.2). However, the turnover rate is only slightly lower in the Cys340 variants (Table 6.2). The high K_m and k_{cat} of the mutant proteins suggest that Cys340 is involved in substrate binding rather than in catalysis. This is in agreement with the fact that the substrate protects the enzyme from inactivation by DTNB and NEM (Fig. 6.1A and 6.1B).

Table 6.2. Kinetic parameters of GALDH variants with L-galactono-1,4-lactone. Data are presented as the mean ± SD of at least two experiments.

GALDH	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m (mM^{-1} s^{-1})
Wild-type[a]	0.17 ± 0.01	134 ± 5	770
C340S	5.0 ± 0.5	59 ± 2	12
C340A	9.0 ± 0.6	79 ± 3	9

[a] Data obtained from (Leferink et al., 2008[b]).

To probe the micro-environment around Cys340, wild-type GALDH was modified with the maleimide spin-label 3-maleimido-PROXYL via Cys340 (Fig. 6.3A). The flavin absorption properties of the spin-labeled protein were identical to the unlabeled protein (Fig. 6.3B), suggesting that the spin-label is not located in close vicinity of the isoalloxazine moiety of FAD. EPR analysis of the modified enzyme revealed a highly immobile spin-label (Fig. 6.3C), indicating that Cys340 has a buried location and is not surface exposed. Maleimide spin-labeling of the C340A variant revealed the covalent incorporation of a minor amount of highly mobile spin-label (Fig. 6.3C). The spin-labeling of C340A most likely originates from a slow reaction with surface lysines or other less accessible cysteines and does not result in any loss of activity. This confirms that the reaction of GALDH with 3-maleimido-PROXYL is highly specific for Cys340.

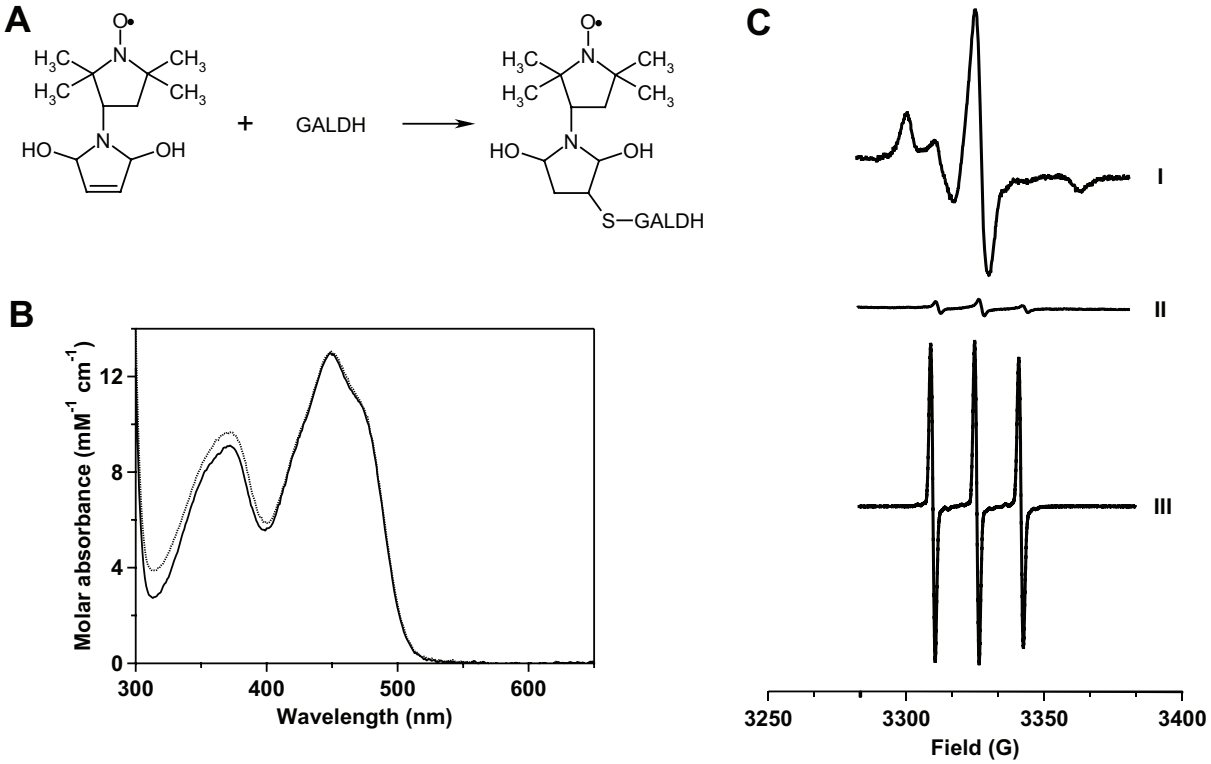
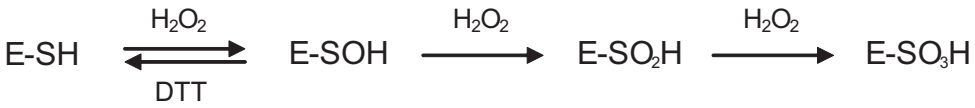


Figure 6.3. Spin-labeling and EPR of wild-type GALDH and C340A. A) Spin-labeling reaction of wild-type GALDH with 3-maleimido-PROXYL via Cys340. B) Absorption spectra of wild-type GALDH (solid line) and C340A (dotted line) labeled with 3-maleimido-PROXYL recorded in 50 mM sodium phosphate, pH 7.4. C) EPR spectra of wild-type GALDH modified with the 3-maleimido-PROXYL spin label (I), GALDH C340A modified with the 3-maleimido-PROXYL spin label (II) and free spin-label (III). A sample of 30 μM spin-labeled enzyme or free spin-label in 50 mM sodium phosphate, pH 7.4 was used.

Susceptibility of GALDH to oxidative stress - The requirement of Cys340 for optimal catalysis might explain the sensitivity of GALDH towards (reversible) inactivation during long term storage at low temperature (Leferink et al., 2008[b]). Because this inactivation is likely caused by cysteine oxidation, the effect of oxidative stress on the enzyme activity was examined in further detail. Fig. 6.1C shows that at room temperature pH 7.4, GALDH is completely inactivated within 1 h by incubation with 5 mM hydrogen peroxide. In agreement with the thiol modifications reported above, the time-dependent inactivation of GALDH by hydrogen peroxide is retarded in the presence of L-galactono-1,4-lactone (Fig 6.1C). L-Ascorbate protects the enzyme from fast inactivation in a similar fashion as L-galactono-1,4-lactone, implying that the product can bind to the oxidized enzyme. The peroxide inactivation of wild-type GALDH is strongly pH-dependent (Fig 6.1D). From the pH-dependence of enzyme inactivation a pK_a value of 6.5 was estimated for Cys340, which is significantly lower than the pK_a of 8.3 for free cysteine in solution.

Treatment of the peroxide-inactivated enzyme with DTT restores only part of the initial enzyme activity, indicating that irreversible oxidative damage has occurred. The partial

reversible inactivation of GALDH with hydrogen peroxide is consistent with the following reaction sequence (van Berkel and Müller, 1987; Poole et al., 2004) (Scheme 6.2),



Scheme 6.2. Oxidation states of cysteine, and their reversibility by DTT.

where E-SH, E-SOH, E-SO_2H and E-SO_3H represent the sulfhydryl, sulfenic, sulfinic and sulfonic acid form of Cys340, respectively. The sulfenic acid is generally unstable and readily reacts with other thiols to form (mixed) disulfides or it can undergo further oxidation to the sulfinic or sulfonic acid (Poole et al., 2004; Jacob et al., 2006).

The oxidation state of Cys340 resulting from the reaction of GALDH with hydrogen peroxide was determined by ESI-MS (Table 6.3). For this purpose, wild-type GALDH and the C340A variant were treated with 5 mM hydrogen peroxide for 1 h (partial inactivation) or 3 h (complete inactivation). The measured mass of denatured wild-type GALDH (Fig. 6.4A, top panel) is 58832.64 ± 8.69 Da. The mass spectrometric data show that after 1 h of incubation with hydrogen peroxide a species with a mass increase of 32 Da is observed (Fig. 6.4A, middle panel). After 3 h of incubation with peroxide, only a species with a mass increase of 48 Da is present (Fig. 6.4A, bottom panel). The inset shows the separation of the three different GALDH species by ESI-MS, zoomed in on charge state 54+. The measured mass of denatured C340A is 58800.78 ± 7.21 Da, as expected, the C340A mutant did not show any mass increase upon treatment with peroxide (Table 6.3), confirming that Cys340 is the only site of oxidation.

Wild-type GALDH treated with peroxide for 1 h regained nearly 50% of its activity after DTT treatment. However, ESI-MS of the DTT-treated wild-type sample did not show any reversibility of the +32 Da species (Table 6.3). The inability to detect a +16 Da species indicates that the sulfenic acid is indeed highly reactive, and not stable. In line with the abovementioned scheme (Scheme 6.2), the obtained results show that Cys340 in GALDH is readily oxidized to the sulfinic and sulfonic acid stage, and that the oxidation is irreversible.

S-*Glutathionylation of GALDH* - Reduced glutathione (GSH; γ-L-glutamyl-L-cysteinyl-glycine) is the most prevalent low molecular weight thiol present in cells (millimolar range). GSH is often associated with the protection of surface exposed protein thiols against over-oxidation. Glutathionylated proteins are also of clinical relevance, glutathionyl hemoglobin is, for example, used as a marker for oxidative stress in human blood, underlining the growing

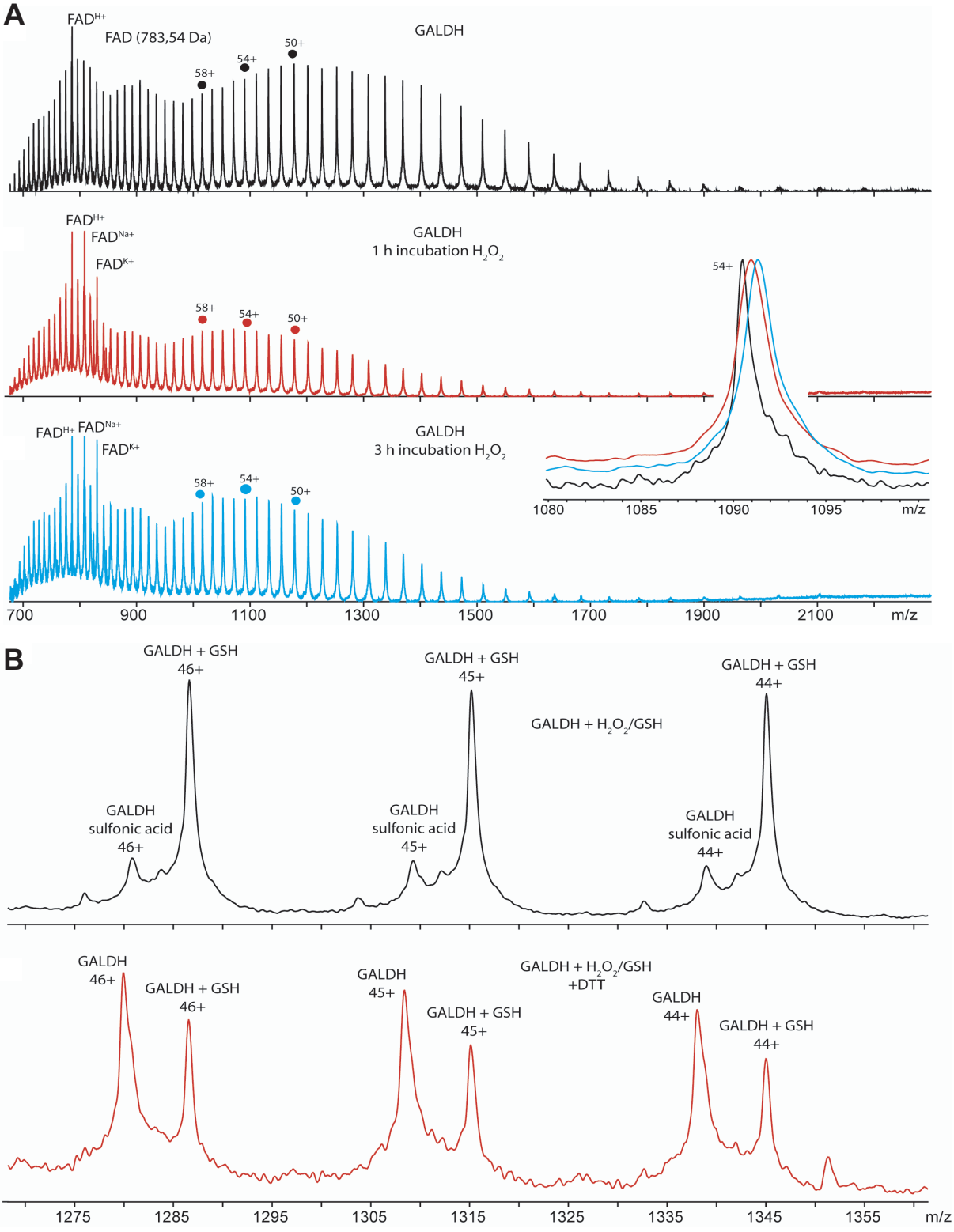

A
FAD^H+
FAD (783,54 Da)
GALDH
58+
54+
50+
FAD^H+
FAD^Na+
FAD^K+
GALDH
1 h incubation H2O2
58+
54+
50+
54+
FAD^H+ FAD^Na+
FAD^K+
GALDH
3 h incubation H2O2
58+
54+
50+
1080
1085
1090
1095
m/z
700
900
1100
1300
1500
1700
1900
2100
m/z
B
GALDH + GSH
46+
GALDH + GSH
45+
GALDH + H2O2/GSH
GALDH + GSH
44+
GALDH
sulfonic acid
46+
GALDH
sulfonic acid
45+
GALDH
sulfonic acid
44+
GALDH
46+
GALDH + GSH
46+
GALDH
45+
GALDH + GSH
45+
GALDH + H2O2/GSH
+DTT
GALDH
44+
GALDH + GSH
44+
1275
1285
1295
1305
1315
1325
1335
1345
1355
m/z

Figure 6.4. (Previous page). ESI-MS spectra of oxidized and *S*-glutathionylated GALDH. A) ESI-MS spectra of oxidized GALDH. Mass spectra of untreated wild-type GALDH (black), GALDH incubated with hydrogen peroxide for 1 h (red), and GALDH incubated with hydrogen peroxide for 3 h (blue). Spectra were recorded under denaturing conditions in 50 mM ammonium acetate solution with 5% formic acid. The inset shows a zoom in of the overlayed mass spectra at charge state 54+. Under denaturing conditions FAD dissociates from GALDH, resulting in apo-GALDH and FAD in the mass spectra. Singly charged FAD is observed via protonation, and cationization by Na^+ and K^+. B) ESI-MS spectra of *S*-glutathionylated GALDH. Mass spectrum of GALDH incubated with hydrogen peroxide and GSH (black) and mass spectrum of *S*-glutathionylated GALDH treated with DTT (red). Both spectra only show a zoom in on the charge states 44+ to 46+. Spectra were recorded under denaturing conditions in 50 mM ammonium acetate solution with 5% formic acid.

Table 6.3. Determination of oxidative modifications of GALDH by ESI-MS.

Treatment	Rel. activity (%)	Average mass (Da)	Δ mass (Da)	Intensity (%)	Modification
WT GALDH					
control[a]	100	58832.64 ± 8.69	-	100	-
H_2O_2 (1 h)	24	58863.29 ± 10.48	+30.65	100	Sulfinic acid
H_2O_2 (1 h) + DTT	53	58866.72 ± 13.06	+34.10	100	Sulfinic acid
H_2O_2 (3 h)	1.4	58879.06 ± 10.81	+46.42	100	Sulfonic acid
H_2O_2 (3 h) + DTT	6.9	nd	nd	nd	nd
H_2O_2/GSH	0.3	59137.68 ± 6.88	+305.04	76	Glutathionylation
		58876.88 ± 9.41	+44.24	24	Sulfonic acid
H_2O_2/GSH + DTT	68	59134.11 ± 10.81	+301.95	46	Glutathionylation
		58831.60 ± 12.18	-	54	None
GALDH C340A					
control[b]	100	58800.78 ± 7.21	-	100	-
H_2O_2 (1 h)	109	58805.22 ± 6.35	-	100	None
H_2O_2 (3 h)	100	58810.82 ± 6.58	-	100	None

[a]Calculated mass apo his-tagged GALDH is 58821.95 Da

[b]Calculated mass apo his-tagged GALDH C340A is 58789.89 Da

nd, not determined

relevance of oxidative stress as a pathological factor in many (chronic) diseases (Pastore et al., 2003; Mandal et al., 2007). Mixed disulfides between protein cysteine residues and GSH can be formed upon thiol disulfide exchange with oxidized glutathione (GSSG) or by the reaction of GSH with cysteine sulfenic acid (Dalle-Donne et al., 2007). Not all protein thiols readily form mixed disulfides with GSH, the accessibility and reactivity of the cysteine residues involved are the major determinants for their susceptibility to *S*-glutathionylation (Dalle-Donne et al., 2007).

To investigate the ability of GALDH to form a mixed disulfide with GSH via Cys340, DTNB-inactivated GALDH (GALDH-TNB) was incubated with excess GSH. GALDH-TNB has near-UV spectral properties which are clearly distinct from wild-type GALDH (Fig. 6.5A). Upon incubation with GSH the spectral perturbations caused by TNB binding disappeared and another species appeared, which shows a red shift compared to the spectrum of unmodified GALDH (Fig. 6.5A). DTT readily re-activated GALDH-TNB with the concomitant release of the TNB anion. GSH, however, did slowly release the TNB anion, and could not restore the activity, suggesting that a mixed disulfide has formed between Cys340 and GSH.

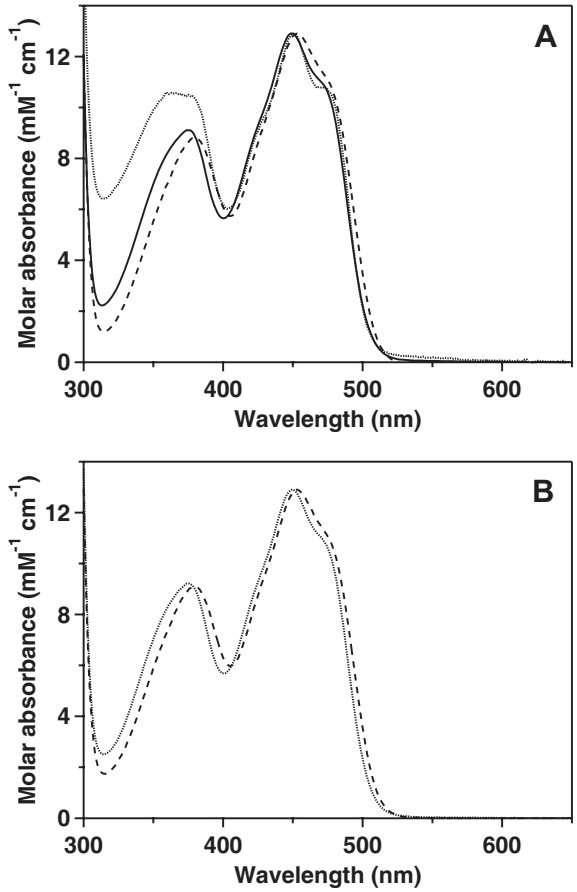


Figure 6.5. Absorption spectra of thiol-modified GALDH. A) DTNB treated GALDH (dotted-line) and DTNB inactivated GALDH treated with GSH (dashed-line) compared to untreated GALDH (solid line). B) Hydrogen peroxide inactivated GALDH (dotted-line) and hydrogen peroxide and GSH inactivated GALDH (dashed-line).

Wild-type GALDH incubated with hydrogen peroxide for 3 h is completely inactive and has spectral properties identical to the untreated enzyme. Incubation of wild-type GALDH with hydrogen peroxide in the presence of GSH yielded also inactive GALDH, but the spectral properties of this species are identical to the species obtained after incubation of GALDH-TNB with GSH (Fig. 6.5B). The activity of the hydrogen peroxide/GSH treated

GALDH could be restored upon incubation with excess DTT, indicating that the modification is reversible, in contrast to the modification with hydrogen peroxide alone. To confirm that indeed *S*-glutathionylation had occurred, the hydrogen peroxide/GSH treated GALDH sample was analyzed with ESI-MS (Table 6.3). The observed mass was 59137.68 ± 6.88 Da, an increase of 305 Da compared with unmodified GALDH, which matches with the attachment of one molecule of GSH (Fig. 3B, top panel). Upon treatment with DTT, the mass of native GALDH was obtained, showing the reversibility of the *S*-glutathionylation reaction (Fig. 6.4B, bottom panel). Incubation with GSSG did not inactivate GALDH, suggesting that *S*-glutathionylation of GALDH occurs via the sulfenic acid of Cys340.

Discussion

GALDH is the terminal enzyme of vitamin C biosynthesis in plants (Leferink et al., 2008[b]). It is already known for a long time that GALDH and related aldonolactone oxidoreductases are inactivated by thiol modifying agents (Mapson and Breslow, 1958; Nishikimi, 1979; Huh et al., 1994; Ôba et al., 1995; Østergaard et al., 1997; Imai et al., 1998; Yabuta et al., 2000; Logan et al., 2007). However, information concerning the site(s) of modification and implications for catalysis are lacking. Here we showed that GALDH from *A. thaliana* is readily inactivated by DTNB, NEM and hydrogen peroxide and that the loss of activity is retarded in the presence of the L-galactono-1,4-lactone substrate or L-ascorbate product. Cys340 was found to be the target of thiol modification. The critical involvement of this residue in substrate binding makes GALDH sensitive towards oxidative stress. Cys340 is the only conserved cysteine among the aldonolactone oxidoreductases, suggesting a similar critical function for this residue in the corresponding enzymes. From pH-dependent inactivation experiments, we estimated an apparent pK_a of 6.5 for the Cys340 side chain. This relatively low pK_a suggests that during GALDH catalysis, which is optimal at pH 8.8 (Leferink et al., 2008[b]), Cys340 participates as the thiolate anion. Interestingly, a recently discovered lactonase, that catalyzes the interconversion of L-galactonate to L-galactono-1,4-lactone in the ascorbate biosynthetic pathway of the photosynthetic algae *Euglenia gracilis*, also seems to use a cysteine for recognition of the galactonolactone (Ishikawa et al., 2008).

Due to the lack of 3D-structural information of the aldonolactone oxidoreductase family, the exact role and location of Cys340 in GALDH remains elusive. Maleimide spin-labeling of GALDH indicated that Cys340 has a buried location, and is not surface exposed. The local protein environment around Cys340 reduces the rotational mobility of the spin-label, resulting in an EPR spectrum that shows severe line-broadening and decreased amplitudes. Either Cys340 is located on the surface, and the spin-label is oriented inwards, or Cys340 is located inside the substrate entrance tunnel. The latter option seems more likely, because the

maleimide group is directly attached to the nitroxide spin label in the absence of a linker, restricting the ability of the spin-label to make large movements relative to the maleimide group.

Due to its buried location away from the flavin, Cys340 is not directly involved catalysis. This is supported by the fact that bound L-galactono-1,4-lactone protects the enzyme from fast inactivation by thiol modifying agents and that replacement of Cys340 by Ser or Ala strongly weakens substrate binding while retaining the catalytic activity. The location of Cys340 in or near the substrate entrance pocket is further supported by the crystal structure of alditol oxidase (PDB accession nr. 2VFS), a related VAO-family member. Residues lining the entrance of the catalytic pocket of alditol oxidase, including Phe279, Pro281 and Glu286 (Forneris et al., 2008), align around Cys340 of GALDH and seem to be conserved in PgGLO (Fig. 6.2). Apparently, the nature of the entrance to the catalytic pocket is different in aldonolactone oxidoreductases than in other related VAO-family members.

ESI-MS showed that the hydrogen peroxide-induced oxidative damage of GALDH is solely due to the modification of Cys340 and involves the sequential formation of sulfenic, sulfinic and sulfonic acid states. Protein sulfinic and sulfonic acids are generally stable and not reduced by low molecular weight thiols. One exception is the active site cysteine of peroxiredoxin which can be over-oxidized to the sulfinic acid, and reduced back to the sulfenic acid via the action of sulfiredoxin (Jacob et al., 2006). Protein sulfonic acids are resistant to any type of cellular repair, and their formation leads to the proteasomal degradation of the protein (Dalle-Donne et al., 2007). Oxidation of GALDH by air results in the sulfenic acid form of Cys340 causing enzyme inactivation. This mild oxidation is fully reversed by DTT.

Reactive protein thiols can be protected against over-oxidation through site-specific *S*-glutathionylation. The glutathionylated protein remains stable under oxidative stress conditions, and can be deglutathionylated once the redox balance is restored. Our results show that GSH protects GALDH from over-oxidation *in vitro* via reversible *S*-glutahionylation of Cys340. Evidence for posttranslational modification of the GALDH activity *in vivo* was found in bean nodules under oxidative stress conditions, in which the mRNA levels did not correspond to the GALDH activity and ascorbate content (Loscos et al., 2008). Furthermore, GALDH is inhibited during the early stages of programmed cell death, which is characterized by a burst of reactive oxygen species (Valenti et al., 2007). This lends support to the proposal that GALDH can be regulated *in vivo* via modification of Cys340, presumably via *S*-glutathionylation.

L-Ascorbate is one of the most abundant soluble carbohydrates in plant leaves (Noctor and Foyer, 1998). It acts, for example, as a photoprotectant during photosynthesis and confers resistance to abiotic stresses by scavenging reactive oxygen species (Noctor and Foyer, 1998). Hence, the L-ascorbate content and GALDH transcript levels are increased during high light

conditions (Tamaoki et al., 2003; Yabuta et al., 2007). The production of high levels of L-ascorbate in plants (up to 5 mM in green leaves) might be an important reason why GALDH was designed by Nature as a dehydrogenase and not, like related aldonolactone oxidoreductases (Leferink et al., 2008[a]), as an oxidase. Oxidase activity would generate high amounts of hydrogen peroxide and stimulate the irreversible inactivation of GALDH. Furthermore, high levels of hydrogen peroxide will de-regulate the expression and functioning of ascorbate peroxidases and other thiol-modulated enzymes and stimulate ageing, senescence and cell death (Giorgio et al., 2007; Navrot et al., 2007; Ishikawa and Shigeoka, 2008). Galactonolactone oxidase activity in the mitochondrial intermembrane space would also consume high amounts of oxygen, which might affect respiration. Recently we showed that *A. thaliana* GALDH can be converted into an efficient galactonolactone oxidase by a single Ala to Gly substitution. The mutation creates space in the active site allowing oxygen to access the reduced flavin (Leferink et al., 2009). This subtle gatekeeper mechanism seems to be a general strategy for regulating the oxygen reactivity of flavoenzymes and allows the easy generation of dehydrogenases that do not form reactive oxidants with a long lifetime such as hydrogen peroxide.

In conclusion, we have shown that GALDH, the ultimate vitamin C producer in plants, is susceptible to oxidative stress. The sensitivity towards oxidative damage is due to a reactive cysteine located in or near the substrate binding site. Post-translational modification of this conserved cysteine might regulate the *in vivo* activity of GALDH (Brandes et al., 2008).

Acknowledgements

We are grateful to Cor Wolfs (Laboratory of Biophysics, Wageningen University) for assistance with the EPR experiments. This research was supported by a grant from the Carbohydrate Research Centre Wageningen (CRC-W) and the Netherlands Proteomics Centre, which is part of the Netherlands Genomics Initiative.

chapter 7

Identification of a gatekeeper residue that prevents dehydrogenases from acting as oxidases

Nicole G. H. Leferink[1], Marco W. Fraaije[2], Henk-Jan Joosten[3], Peter J. Schaap[3], Andrea Mattevi[4], Willem J. H. van Berkel[1]

Journal of Biological Chemistry (2009) 284(7):4392-4397

[1] Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
[2] Laboratory of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
[3] Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands
[4] Department of Genetics and Microbiology, University of Pavia, Via Ferrata 1, 27100 Pavia, Italy

Abstract

The oxygen reactivity of flavoproteins is poorly understood. Here we show that a single Ala to Gly substitution in L-galactono-γ-lactone dehydrogenase (GALDH) turns the enzyme into a catalytically competent oxidase. GALDH is an aldonolactone oxidoreductase with a vanillyl-alcohol oxidase (VAO) fold. We found that nearly all oxidases in the VAO family contain either a Gly or Pro at a structurally conserved position near the C4a locus of the isoalloxazine moiety of the flavin, whereas dehydrogenases prefer another residue at this position. Mutation of the corresponding residue in GALDH (Ala113→Gly) resulted in a striking 400-fold increase in oxygen reactivity, while the cytochrome *c* reductase activity is retained. The activity of the A113G variant shows a linear dependence on oxygen concentration (k_{ox} = 3.4 × 10^5 M^{-1} s^{-1}), similar to most other flavoprotein oxidases. The Ala113→Gly replacement does not change the reduction potential of the flavin, but creates space for molecular oxygen to react with the reduced flavin. In the wild-type enzyme Ala113 acts as a gatekeeper, preventing oxygen to access the isoalloxazine nucleus. The presence of such an oxygen access gate seems to be a key factor for the prevention of oxidase activity within the VAO family, and is absent in members that act as oxidases.

Keywords: electron acceptor; dehydrogenase; flavoprotein; oxidase; vitamin C

Abbreviations: AldO, alditol oxidase; CO, cholesterol oxidase; GALDH, L-galactono-γ-lactone dehydrogenase; VAO, vanillyl-alcohol oxidase

Introduction

The flavoenzyme L-galactono-γ-lactone dehydrogenase (GALDH; EC 1.3.2.3) catalyzes the terminal step in the biosynthesis of vitamin C (L-ascorbate) in plants. Besides producing this essential nutrient, GALDH is required for the accumulation of plant respiratory complex I (Pineau et al., 2008). GALDH is an aldonolactone oxidoreductase that belongs to the vanillyl-alcohol oxidase (VAO) flavoprotein family (Fraaije et al., 1998). Members of this family share a two-domain folding topology with a conserved FAD-binding domain and a cap domain that defines the substrate specificity (Mattevi et al., 1997). VAO family members include enzymes involved in carbohydrate metabolism and lignin degradation, and enzymes that participate in the synthesis of antibiotics and alkaloids (Leferink et al., 2008[a]). Most VAO members contain a covalently tethered FAD and act as oxidases that use molecular oxygen to re-oxidize the flavin, resulting in the production of hydrogen peroxide. In contrast to related aldonolactone oxidoreductases like L-gulono-γ-lactone oxidase from animals (Nishikimi, 1979) and D-arabinono-γ-lactone oxidase from yeast (Huh et al., 1998), GALDH reacts poorly with molecular oxygen and contains non-covalently bound FAD (Leferink et al., 2008[b]). No crystal structure is available for the aldonolactone oxidoreductase subfamily, and little is known about the nature of the active site and the catalytic mechanism.

GALDH is localized in the mitochondrial intermembrane space where it feeds electrons into the respiratory chain. Its subcellular localization could provide a rationale why GALDH is a dehydrogenase and not, like related enzymes, an oxidase. The latter activity would result in high levels of mitochondrial hydrogen peroxide that promote GALDH inactivation (Leferink et al., 2008[c]) and induce ageing, senescence and cell death (Navrot et al., 2007; Noctor et al., 2007). Furthermore, it has been shown that the exploitation of the electron transport chain of GALDH via cytochrome *c* is essential for the proper functioning of plant mitochondria (Alhagdow et al., 2007).

GALDH from *Arabidopsis thaliana* has several properties in common with flavoprotein oxidases, including the stabilization of the red anionic flavin semiquinone and the formation of a flavin N5-sulfite adduct (Leferink et al., 2008[b]), but reacts three to four orders of magnitude slower with molecular oxygen than what is typically found for oxidases. The reaction of flavoprotein oxidases with molecular oxygen generally follows second order kinetics with rate constants in the range of 10^5 M^{-1} s^{-1} for most oxidases to 10^6 M^{-1} s^{-1} for glucose oxidase (Mattevi, 2006).

Up to now there are no clear rules that enable to predict whether or how a flavoprotein reacts with molecular oxygen. A combination of structural and dynamic features like properly positioned positive charges, solvation of the active site, and protein breathing might play a role (Mattevi, 2006). The existence of (hydrophobic) oxygen channels from the surface to the

active site has been proposed for several flavoenzymes, including the VAO-members cholesterol oxidase (CO) (Coulombe et al., 2001) and alditol oxidase (AldO) (Forneris et al., 2008).

Structure-inspired attempts to alter the oxidase activity of flavoenzymes have been mainly focused on decreasing the oxygen reactivity. Blocking putative oxygen binding sites or oxygen channels resulted in mutant proteins with poor catalytic properties and which are still active with molecular oxygen (Chen et al., 2008; Piubelli et al., 2008; Winter et al., 2008). Replacing a Lys in the active site of monomeric sarcosine oxidase decreased the oxygen reactivity by more than 1000-fold (Zhao et al., 2008). Slight improvements in the oxygen reactivity were reported for D-amino acid oxidase (Pollegioni et al., 2008), where a directed evolution approach was followed to obtain variants with increased oxygen affinity. A Tyr to Lys mutation increased the oxygen reactivity of medium-chain acyl-CoA dehydrogenase due to a better exposure of the flavin to solvent, but the reaction is far from efficient (Zeng et al., 2007).

In this study we investigated the molecular determinants for the poor oxygen reactivity of GALDH from *A. thaliana*. Because structural information for the aldonolactone oxidoreductases is lacking, we used a structure-based multiple sequence alignment strategy to find structural correlations with oxidase activity within the VAO family. Strikingly, we found that nearly all oxidase members of the VAO family contain a glycine or proline at a structurally conserved position near the C4a atom of the isoalloxazine ring of the flavin, while members that do not react with oxygen contain another residue at this position. The role of the corresponding residue in GALDH, Ala113, in the reaction with molecular oxygen was addressed by site-directed mutagenesis. We describe for the first time the characterization of a GALDH variant capable of efficiently utilizing oxygen as electron acceptor, rendering a catalytically competent L-galactono-γ-lactone oxidase.

Materials and Methods

Chemicals - Benzyl viologen, bovine heart cytochrome *c*, FAD, L-galactono-1,4-lactone and xanthine were purchased from Sigma-Aldrich. Resazurin was obtained from Acros Organics, xanthine oxidase, catalase and superoxide dismutase were from Roche Applied Science. All other chemicals were from commercial sources and of the purest grade available.

Sequence comparisons - A structure-based multiple sequence alignment of the FAD domain of the VAO family was generated with the 3DM software (Joosten et al., 2008). The 3DM alignment can be viewed at the 3DM Alignment program page (FAD-linked oxidases database). Additional Clustal W multiple sequence alignments were performed to find

corresponding residues at structurally conserved positions for VAO-members lacking in the 3DM alignment.

Cloning and site-directed mutagenesis - The cDNA encoding mature GALDH (At3g47930) has been cloned previously into the pET23a(+) vector (Novagen) to yield pET-GALDH-His_6 (Leferink et al., 2008[b]). The GALDH A113G variant was constructed using pET-GALDH-His_6 as template with the QuikChange II method (Stratagene) using the oligonucleotides A113G_fw (5'-CTC TTC AGA ACT TTG GCT CCA TTA GAG AGC AG-3') and A113G_rv (5'-CTG CTC TCT AAT GGA GCC AAA GTT CTG AAG AG-3'). Successful mutagenesis was confirmed by automated sequencing. The resulting construct pET-GALDH_A113G-His_6 was electroporated to *E. coli* BL21(DE3) cells (Novagen) for recombinant expression.

Enzyme production and purification - For enzyme production *E. coli* BL21(DE3) cells, harboring a pET-GALDH plasmid, were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin until an OD_{600} of 0.7 was reached. Expression was induced by addition of 0.4 mM isopropyl-thio-β-D-galactopyranoside and the incubation was continued for 16 h at 37°C. The recombinant His_6-tagged proteins were purified essentially as described before (Leferink et al., 2008[b]).

Protein analysis - SDS-PAGE was performed using 12.5% acrylamide slab gels essentially as described by Laemmli (Laemmli, 1970). Proteins were stained using Coomassie Brilliant Blue R-250. Total protein concentrations were estimated using the Bradford protein assay from Bio-Rad with BSA as standard. Desalting or buffer exchange of small aliquots of enzyme was performed with Bio-Gel P-6DG columns (Bio-Rad).

Spectral analysis - Absorption spectra were recorded at 25°C on a Hewlett Packard 8453 diode array spectrophotometer in 50 mM sodium phosphate buffer, pH 7.4. Spectra were collected and analyzed using the UV-Visible ChemStation software package (Hewlett Packard). The molar absorption coefficient for the mutant protein was determined by recording absorption spectra in the presence and absence of 0.1% (w/v) SDS, assuming a molar absorption coefficient for free FAD of 11.3 mM^{-1} cm^{-1} at 450 nm. Enzyme concentrations were routinely determined by measuring the absorbance at 450 nm using a molar absorption coefficient of 12.9 mM^{-1} cm^{-1} for wild-type GALDH (Leferink et al., 2008[b]).

Reduction potential determination - The reduction potentials of wild-type GALDH and the A113G variant were determined using the method of Massey (Massey, 1990). Briefly, a 1-ml suba-sealed cuvette containing 5 μM GALDH, 400 μM xanthine, 2.5 μM benzyl viologen, and 5 μM redox dye in 50 mM sodium phosphate, pH 7.4 was made anaerobic by flushing with oxygen free nitrogen. To scavenge final traces of oxygen, 1 mM vanillyl alcohol and catalytic amounts of eugenol oxidase (Jin et al., 2007) were added to the reaction mixture. To start the reaction, 2 μl xanthine oxidase (20 U/ml) was added, and spectra were collected every 2 min during the reduction using a Perkin Elmer Lambda Bio 40 spectrophotometer. The potentials at 50% reduction of GALDH (E_m) were calculated using the Nernst equation:

$$E_h = E_m + 2.303 \times \left(\frac{R \times T}{n \times F} \right) \times \log \left(\frac{[\text{ox}]}{[\text{red}]} \right) \qquad \text{(eq. 7.1)}$$

where E_h is the measured potential, E_m is the midpoint potential of the enzyme, R is the gas constant (8.31 J K^{-1} mol^{-1}), T is the temperature in K, n is the number of electrons to go from the oxidized species to the reduced species, F is the Faraday constant (96.5 kJ V^{-1} mol^{-1}), and [ox] and [red] are the concentrations of oxidized and reduced enzyme, respectively.

Activity measurements - GALDH activity was routinely assayed by following the reduction of cytochrome *c* at 550 nm (Leferink et al., 2008[b]). Because dithiothreitol interferes with the reaction, it was removed from the enzyme solution by Bio-Gel P-6DG gel filtration immediately prior to use. Initial velocities were calculated using a molar difference absorption coefficient ($\Delta\varepsilon_{550}$) of 21 mM^{-1} cm^{-1} for reduced minus oxidized cytochrome *c*. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of L-galactono-1,4-lactone per min, which is equivalent to the reduction of 2 μmol of cytochrome *c* (Ôba et al., 1995). The reaction with molecular oxygen was assayed with a polarographic oxygen uptake assay using a Clark electrode in aerated buffer (0.25 mM oxygen at 25°C) in the absence of cytochrome *c*. To identify the product of oxygen reduction, catalytic amounts of catalase (10 μg) or superoxide dismutase (10 μg) were added to the oxygen uptake assay mixture.

Stopped-flow kinetics - The re-oxidation of wild-type GALDH and A113G with molecular oxygen was studied by stopped-flow kinetics using an Applied Photophysics SX17MV instrument. All experiments were performed in 50 mM sodium phosphate, pH 7.4 at 25°C. Solutions were made anaerobic by flushing with oxygen free nitrogen. To scavenge final traces of oxygen, 1 mM vanillyl alcohol and catalytic amounts of eugenol oxidase were added to the reaction mixture. Reduced enzyme was prepared by using 1.2 eq. of substrate. The oxidative half-reaction was followed at 450 nm by mixing the substrate-reduced enzyme with buffer containing various concentrations of oxygen (0.25 – 1.25 mM). Obtained spectra were

analyzed with the Pro-K software (Applied Photophysics), and single-wavelength traces were fitted to a double exponential function:

$$A_t = A_1 \times e^{(-k1 \times t)} + A_2 \times e^{(-k2 \times t)} + C \qquad \text{(eq. 7.2)}$$

where A is absorbance difference at 450 nm, *k* are the rate constants (s^{-1}) and C the absorbance off-set at 450 nm.

Results

Structural correlations with oxidase activity in the VAO family - Most members of the VAO flavoprotein family are oxidases containing a covalently bound FAD cofactor (Fraaije et al., 1998; Leferink et al., 2008[a]). Inspection of the structure-based multiple sequence alignment of the FAD domain of many VAO-members yielded another striking correlation with oxidase activity. All known oxidases, with the exception of alditol oxidase and xylitol oxidase, contain either a glycine or a proline residue at a structurally conserved position near the C4a locus of the flavin isoalloxazine ring that is potentially reactive towards molecular oxygen. GALDH and other dehydrogenases, like cytokinin dehydrogenases and D-lactate dehydrogenase, contain an alanine, threonine or isoleucine at this position. The occurrence of amino acid residues at the structurally conserved position near flavin C4a in both oxidases and dehydrogenases of the VAO family is shown in the supplemental materials.

Inspection of known crystal structures of VAO-members revealed that the residues at the conserved position near flavin C4a are located on the *re*-side of the flavin, opposite of the substrate binding site which is on the flavin *si*-side in VAO-members. In Figure 7.1 the active sites of AldO and CO, the closest homologs of GALDH with a known 3D structure, are depicted. A high degree of conservation is observed between GALDH, AldO and CO in the substrate binding site and the area around the isoalloxazine ring of the flavin. AldO, although an oxidase, contains an alanine (Ala105), analogous to Ala113 of GALDH, near flavin C4a, CO contains a proline (Pro186) at this position. To address the potential oxidase activity of GALDH, a A113G variant was constructed by site-directed mutagenesis to create more space near flavin C4a.

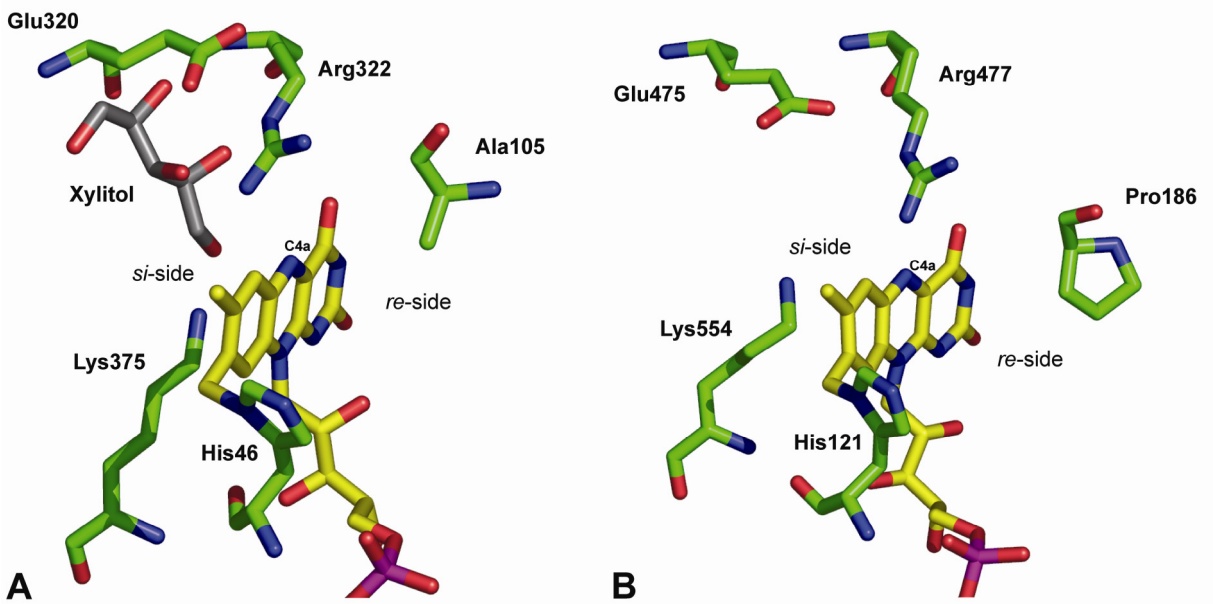


Figure 7.1. Active sites of GALDH homologs. (A) AldO in complex with xylitol (pdb, 2VFS) (Forneris et al., 2008); (B) cholesterol oxidase (pdb 1I19) (Coulombe et al., 2001). For clarity only selected amino acid residues are shown. The C4a atom of the flavin is labeled. Ligands are highlighted in black sticks, the FAD cofactors are highlighted in yellow. Oxygen atoms are in red, nitrogens are blue.

Spectral properties - Mutation of Ala113 in GALDH into Gly yielded a stable holoprotein, which could be expressed and purified in similar quantities as wild-type GALDH (Leferink et al., 2008[b]). During the purification procedure a significant fraction of the mutant protein (about one-third) lost its FAD cofactor, but the apoprotein was readily reconstituted to the holo form with commercial FAD. The Ala113 to Gly replacement gives small spectral perturbations in the flavin optical spectrum, the maxima at 376 nm and 450 nm are red and blue shifted, respectively, and the shoulder at 475 nm is less pronounced (Fig. 7.2). The molar absorption coefficient of the mutant protein is 13.0 mM^{-1} cm^{-1} at 449 nm compared to 12.9 mM^{-1} cm^{-1} at 450 nm for the wild-type protein.

Catalytic properties - The steady-state kinetic parameters of GALDH A113G for L-galactono-1,4-lactone were determined using both oxygen and cytochrome *c* as electron acceptor. The A113G mutation yields a striking increase in turnover rate with molecular oxygen, k_{cat} = 42 s^{-1} compared to 0.17 s^{-1} for wild-type, while retaining the cytochrome *c* reductase activity, k_{cat} = 116 s^{-1} versus 134 s^{-1} for the wild-type protein (Fig. 7.3A). The Michaelis constant for L-galactono-1,4-lactone is slightly higher for the mutant protein than for the wild-type enzyme (Table 7.1). The steady-state kinetic measurements with oxygen and cytochrome *c* suggest that in the A113G variant the re-oxidation with oxygen is the rate-limiting step during catalysis, and not flavin reduction or product release as is typical in flavoprotein oxidases (van Berkel et al., 1999).

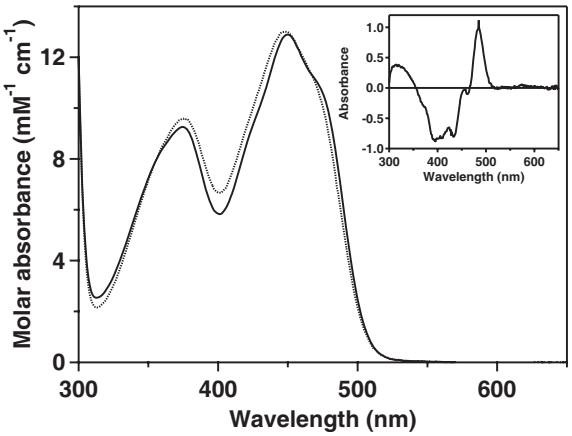


Figure 7.2. Optical spectra of oxidized wild-type GALDH (solid line) and A113G (dotted line). Spectra are taken in 50 mM sodium phosphate, pH 7.4. The inset shows the difference spectrum of wild-type GALDH and A113G.

Table 7.1. Kinetic parameters of wild-type GALDH and A113G for L-galactono-1,4-lactone using cytochrome *c* or molecular oxygen (air saturated buffer) as electron acceptor.

	Wild-type[a]	A113G
Cytochrome *c* (50 μM)		
k_{cat} (s^{-1})	134 ± 5	116 ± 5
$K_{m, GAL}$ (mM)	0.17 ± 0.01	0.45 ± 0.03
$k_{cat}/K_{m, GAL}$ (mM^{-1} s^{-1})	7.7×10^2	2.6×10^2
Oxygen (0.26 mM)		
k_{cat} (s^{-1})	0.17 ± 0.02	42 ± 6
$K_{m, GAL}$ (mM)	nd	0.46 ± 0.10
$k_{cat}/K_{m, GAL}$ (mM^{-1} s^{-1})	nd	9.1×10^1

[a] Data from ref. (Leferink et al., 2008[b])
nd, not detectable due to low oxygen consumption rates

To determine whether hydrogen peroxide or superoxide is produced as a result of oxygen reduction by GALDH A113G, the oxygen uptake assay was performed in the presence of catalase, which converts hydrogen peroxide into water and oxygen, or superoxide dismutase, which converts superoxide into hydrogen peroxide and oxygen. In the presence of catalase, the oxygen consumption rate was about half of the rate observed in the absence of catalase. Moreover, when catalase was added at the end of the reaction, about 50% of the consumed oxygen was regenerated. The addition of superoxide dismutase to the assay mixture had no effect on the oxygen consumption rate, confirming that hydrogen peroxide is indeed formed as a result of flavin re-oxidation by the A113G variant. Also for wild-type GALDH, no superoxide formation could be detected.

Oxidative half-reaction - The kinetics of re-oxidation of wild-type GALDH and the A113G variant by molecular oxygen were studied in more detail by mixing substrate-reduced enzyme with aerated buffer in the stopped-flow apparatus. Diode-array detection revealed that flavin

re-oxidation did not involve spectral intermediates in both variants. Deconvolution of the spectra taken during re-oxidation of wild-type GALDH revealed a single phase, while A113G showed two phases during the re-oxidation, a fast phase (k = ~30 s^{-1}) corresponding to flavin re-oxidation and a second slower phase (k = ~7 s^{-1}) (data shown in supplemental materials).

The effect of oxygen concentration on the re-oxidation rate of A113G was studied by single wavelength detection. Traces were best fit to a double exponential function. The first fast phase represents the re-oxidation event and shows a linear dependence on the oxygen concentration giving a bi-molecular rate constant of 3.4×10^5 M^{-1} s^{-1} for flavin re-oxidation (Fig. 7.3B), a 400-fold increase compared to wild-type GALDH which has a bi-molecular rate constant of 9.1×10^2 M^{-1} s^{-1}. Re-oxidation in the presence of excess product only had a minor effect on the rate of re-oxidation of A113G, suggesting that the data presented reflect the oxygen reactivities of the free reduced enzymes.

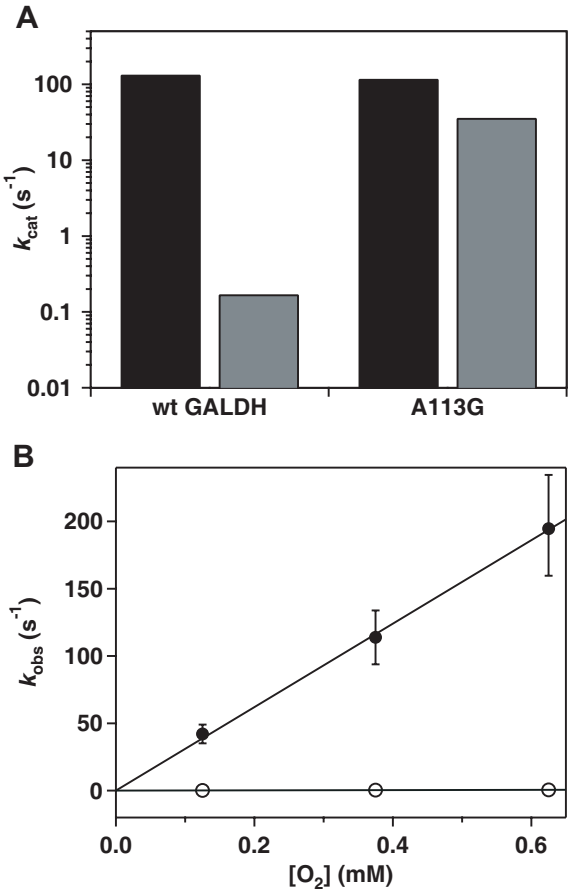


Figure 7.3. Catalytic properties and oxygen reactivity of wild-type GALDH and A113G. (A) Cytochrome *c* reductase activity (black bars) vs. oxidase activity (gray bars) of wild-type (wt) GALDH and A113G. (B) Re-oxidation of wild-type GALDH (open circles) and A113G (closed circles) by molecular oxygen. Substrate-reduced enzyme was mixed with 50 mM sodium phosphate, pH 7.4 containing various amounts of oxygen at 25°C in the stopped-flow apparatus. Kinetic traces were fit to a double exponential function. The average rates ± the S.D. of at least 7 traces are shown.

The second slower phase observed during the re-oxidation of A113G accounts for maximally 10-15% of the total absorbance change. The corresponding rates were not dependent on the oxygen concentration and were around 5-10 s^{-1} in all cases. The nature of this phase is unclear, as it is slower than the observed k_{cat}, it might represent a non-catalytic event. Alternatively, the A113G protein preparation might consist of two populations, a fast reacting species and a slow reacting one since the spectral difference in both processes is the same. A similar slow phase (around 5 s^{-1}) that is not dependent on the oxygen concentration was detected during the re-oxidation of type II CO (Piubelli et al., 2008).

Reduction potential - The protein structure around the isoalloxazine moiety of the flavin controls both the catalytic function and the redox properties of the enzyme. To find out what is the basis for the observed increase in oxidase activity in the A113G mutant, the reduction potentials of wild-type GALDH and A113G were estimated using the xanthine/xanthine oxidase method (Massey, 1990), using resazurin/resorufin (E_m resorufin = -51 mV) as reference dye. Both wild-type and A113G were reduced in a single two-electron step, without the formation of a semiquinone species (Fig. 7.4). The reduction via a single two-electron process fits well with the catalytic function of GALDH, the two-electron oxidation of L-galactono-1,4-lactone. The midpoint reduction potentials of wild-type and A113G could be estimated from the log (E_{ox}/E_{red}) versus log (dye_{ox}/dye_{red}) plots and were -44 mV for wild-type GALDH and -63 mV for A113G. These values are in the same range as has been reported for other non-covalent flavoprotein dehydrogenases and oxidases (Stankovich, 1991), while covalent flavoproteins generally have higher reduction potentials (Fraaije et al., 1999; Winkler et al., 2007). The Ala113 to Gly mutation lowers the midpoint reduction potential of GALDH by about 20 mV. Such a small decrease in reduction potential can not account for the observed increase in oxidase activity.

Discussion

GALDH from *A. thaliana* is one of the few members of the VAO flavoprotein family that poorly reacts with molecular oxygen (Leferink et al., 2008[b]). By performing a structure-based multiple sequence alignment (Joosten et al., 2008) of the FAD domain of known VAO family members we found a striking correlation between the presence of a Gly or Pro residue at a structurally conserved position near the C4a locus of the flavin and the oxidase activity of these enzymes. Replacing the corresponding residue in GALDH (Ala113) with Gly increased the oxygen reactivity of GALDH with several orders of magnitude, yielding a catalytically competent oxidase.

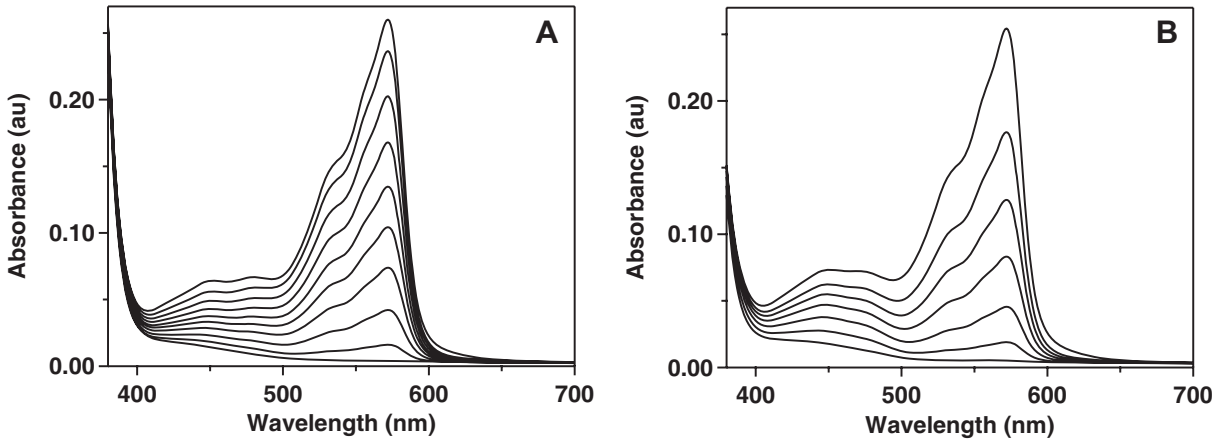


Figure 7.4. Anaerobic reduction of 5 μM wild-type GALDH (A) and A113G (B) by the xanthine/xanthine oxidase method (Massey, 1990) in the presence of 5 μM resazurin in 50 mM sodium phosphate, pH 7.4 at 25°C. Spectra were taken at regular time intervals until complete reduction was achieved after 50 min. For clarity only selected spectra are shown. au, absorbance units.

All oxidases of the VAO-family contain a covalently-bound FAD (Leferink et al., 2008[a]). The properties of the GALDH A113G variant clearly show, however, that this feature is not essential for VAO members to acquire oxidase activity. Covalent flavinylation does not enhance the reactivity of the flavin with molecular oxygen, but merely increases the flavin reduction potential, i.e. the power for substrate oxidation. The VAO H422A variant, which contains non-covalently bound FAD, has a much lower reduction potential than the wild-type enzyme, but a nearly identical reaction rate with molecular oxygen (Fraaije et al., 1999). Similarly, cytokinin dehydrogenase contains covalently linked FAD, accordingly has a relatively high reduction potential (+8 mV), but reacts poorly with molecular oxygen (Frébortová et al., 2004). In agreement with the above considerations we found that the Ala113→Gly mutation in GALDH results in a minor lowering of reduction potential of about 20 mV, but a 400-fold increase in oxygen reactivity.

Properly positioned positive charges are thought to enhance the oxygen reactivity of flavoenzymes, possibly by stabilization of the superoxide-flavin semiquinone radical pair generated after the first electron transfer from the reduced flavin to oxygen (Mattevi, 2006). For glucose oxidase it was demonstrated that a protonated His (His516) in front of the N5-C4a flavin locus is solely responsible for the observed fast reaction with molecular oxygen (k_{ox} = 1.5 × 10^6 M^{-1} s^{-1}) (Roth and Klinman, 2003). Similarly, Lys265 was found to be responsible for oxygen activation in monomeric sarcosine oxidase. Replacing this Lys with a neutral residue resulted in a 8000-fold decrease in oxygen reactivity (Zhao et al., 2008). GALDH contains two basic residues (Arg388 and Lys456) at the *si*-face of the flavin ring that are possibly important for the reaction with oxygen. These residues are conserved in AldO and CO (Fig. 7.1) where they are involved in substrate oxidation (Forneris et al., 2008). In

monomeric sarcosine oxidase, the sites for substrate oxidation and oxygen reduction are also found on opposite faces of the flavin (Trickey et al., 1999). As noted by Jorns and coworkers, such an arrangement might avoid steric crowding in the ternary complex (Zhao et al., 2008).

The chemical reaction of flavoprotein oxidases with molecular oxygen is not completely understood, but involves the transfer of one electron from the reduced flavin to oxygen resulting in the formation of a superoxide anion/flavin semiquinone radical pair. Next, the radical pair can dissociate to release the oxygen radical, it can undergo a second electron transfer to produce hydrogen peroxide, or it can form a covalent C4a-(hydro)peroxyflavin species that can further dissociate to form hydrogen peroxide. This oxygen-flavin adduct is generally unstable, but is a common feature in flavoprotein monooxygenases that use this intermediate to insert an oxygen atom into their substrates (van Berkel et al., 2006). For oxidases it is not clear whether the C4a-(hydro)peroxyflavin is formed during the catalytic cycle. Recently, for the first time such an intermediate has been experimentally detected in a flavoprotein oxidase: pyranose 2-oxidase (Sucharitakul et al., 2008). The oxygenated flavin species decayed to form oxidized flavin and hydrogen peroxide, consistent with the inability of pyranose 2-oxidase to perform oxygenation reactions.

Most flavoprotein oxidases show a linear dependence on oxygen concentration, reflecting a second order process, suggesting that the reaction is the result of a collision of oxygen and the reduced flavin cofactor and that oxygen can freely diffuse into the active site (Mattevi, 2006). For AldO, where no C4a-(hydro)peroxyflavin intermediate is detected during flavin re-oxidation (Heuts et al., 2007[a]), it was argued that the methyl side-chain of Ala105, homologous to Ala113 in GALDH, could hamper the formation of the peroxy-flavin intermediate by steric hindrance (Forneris et al., 2008). AldO and GALDH show 25% sequence identity but a high degree of conservation between these enzymes is observed in the area around the isoalloxazine ring of the flavin, including several residues in the substrate binding pocket. This supports that the A113G mutation in GALDH creates space for molecular oxygen to reach and react with the N5-C4a locus of the reduced FAD cofactor. The created space likely results in a cavity big enough to accommodate a molecule of dioxygen.

The acyl-CoA oxidases and dehydrogenases form another group of structurally similar flavoenzymes that use different electron acceptors. A distinct oxygen binding site near the C4a-N5 locus of the flavin was proposed for the acyl-CoA oxidases and assigned to the presence of a Gly (Mackenzie et al., 2006; Arent et al., 2008). The role of this residue in the oxygen reactivity was, however, never experimentally evaluated. Members of the FMN-dependent L-2-hydroxy acid oxidase family contain either a Gly or Ala at the end of a four-residue loop in close proximity of the C4a-N5 locus of the flavin (Mattevi, 2006). Changing this Gly to Ala in L-lactate monooxygenase significantly reduced the oxygen reactivity (Sun et al., 1996), whereas for mandelate dehydrogenase, a slight increase was found (Dewanti et

al., 2004). Based on these results it was argued that the oxidase activity in this family might be controlled by steric effects through the relative positions of the FMN and the four-residue peptide loop. Members with a poor oxygen reactivity show a more constrained geometry around the N5 position of the flavin (Dewanti et al., 2004).

In conclusion, we have shown for the first time that it is possible to convert a flavoprotein dehydrogenase into a catalytically competent oxidase. Our findings are of general relevance for the design of suitable biocatalysts because oxidases do not require expensive co-substrates or regeneration systems (Joosten and van Berkel, 2007). Removal of the Ala113 side-chain of GALDH creates space and allows oxygen to reach the reduced flavin. The presence of such a gatekeeper residue in dehydrogenases is a key factor for preventing oxidase activity in the VAO flavoprotein family. The question remains whether oxygen reaches the active site through defined oxygen tunnels or via multiple entrances as the result of protein breathing. What seems to be of key importance is the presence of space for molecular oxygen to reach and attack the reduced flavin

Acknowledgements

This research was supported by a grant from the Carbohydrate Research Centre Wageningen (CRC-W).

Supplementary materials

Table S7.1. Structural correlations with oxidase activity in the VAO family. Occurrence of amino acid residues at a structurally conserved position near flavin C4a in both oxidases and dehydrogenases of the VAO family. An overview of characterized VAO-members (Leferink et al., 2008[a]) with their corresponding residues is presented.

Protein	Organism	Acc. nr.	Residue near C4a	Enzyme class[a]
6-hydroxy-D-nicotine oxidase	*Arthrobacter oxidans*	P08159	Gly127	Oxidase
Aclacinomycin oxidoreductase	*Streptomyces galilaeus*	Q0PCD7	Gly171	Oxidase
Alditol oxidase	*Streptomyces coelicolor*	Q9ZBU1	Ala105	Oxidase
Arabinonolactone oxidase	*Trypanosoma brucei*	Q57ZU1	Pro112	Oxidase[b]
Berberine bridge enzyme	*Eschscholzia californica*	P30986	Gly164	Oxidase
Berberine bridge enzyme	*Papaver somniferum*	P93479	Gly168	Oxidase
Carbohydrate oxidase	*Helianthus annuus*	Q8SA59	Gly179	Oxidase
Chitooligosaccharide oxidase	*Fusarium graminearum*	XP_391174	Gly152	Oxidase
Cholesterol oxidase	*Brevibacterium sterolicum*	Q7SID9	Pro186	Oxidase
Cytokinin dehydrogenase	*Zea Mays*	Q9T0N8	**Thr168**	**Dehydrogenase**
Cytokinin dehydrogenase	*Arabidopsis thaliana*	Q9FUJ1	**Thr161**	**Dehydrogenase**
Cytokinin dehydrogenase	*Hordeum vulgare*	Q8H6F6	**Thr160**	**Dehydrognease**
D-arabinono-γ-lactone oxidase	*Saccaromyces cerevisiae*	P54783	Gly117	Oxidase
D-arabinono-γ-lactone oxidase	*Candida albicans*	O93852	Gly133	Oxidase
Dbv29	*Nonomuraea* sp. ATCC 39727	Q7WZ62	Gly149	Oxidase
D-gluconolactone oxidase	*Penicillium griseoroseium*	Q671X8	Gly131	Oxidase
D-lactate dehydrogenase	*Escherichia coli*	P06149	**Ile142**	**Dehydrogenase**
Eugenol hydroxylase	*Pseudomonas* sp. strain HR199	Q9RDU1	**Pro152**	**Dehydrogenase**
Eugenol oxidase	*Rhodococcus* sp. RHA1	Q0SBK1	Pro150	Oxidase
Galactonolactone oxidase	*Trypanosoma cruzi*	Q4DPZ5	Pro112	Oxidase[c]
Glucooligosaccharide oxidase	*Acremonium strictum*	Q6PW77	Gly153	Oxidase
Gulonolactone dehydrogenase	*Mycobacterium tuberculosis*	O06804	Gly106	Oxidase[d]
Hexose oxidase	*Chondrus crispus*	P93762	Gly136	Oxidase
Isoamyl alcohol oxidase	*Aspergillus oryzae*	Q9HGH9	Gly217	Oxidase
Isoamyl alcohol oxidase	*Fusarium graminearum*	Q2VLJ1	Gly214	Oxidase
Lactose oxidase	*Microdochium nivale*	CAI94231	Gly152	Oxidase
L-galactono-γ-lactone dehydrogenase[e]	*Arabidopsis thaliana*	Q8GY16	**Ala113[f]**	**Dehydrogenase**
L-galactono-γ-lactone dehydrogenase	*Brassica oleracea*	O47881	**Ala203**	**Dehydrogenase**
L-galactono-γ-lactone dehydrogenase	*Ipomoea batatas*	Q9ZWJ1	**Ala195**	**Dehydrogenase**
L-galactono-γ-lactone dehydrogenase	*Nicotiana tabacum*	Q9SLW6	**Ala198**	**Dehydrogenase**
L-gulono-γ-lactone oxidase	*Scyliorhinys torazame*	Q90YK3	Gly111	Oxidase
L-gulono-γ-lactone oxidase	*Rattus norvegicus*	P10867	Gly111	Oxidase
L-gulono-γ-lactone oxidase	*Mus musculus*	NP_848862	Gly111	Oxidase
Mitomycin radical oxidase	*Streptomyces lavendulae*	P43485	Gly118	Oxidase
N-acetylenolpyruvyl glucosamine reductase	*Escherichia coli*	P08373	**Leu107**	**Reductase**
N-acetylenolpyruvyl glucosamine reductase	*Staphylococcus aureus*	P61431	**Ala125**	**Reductase**
N-acetylenolpyruvyl glucosamine reductase	*Streptococcus pneumoniae*	P65466	**Ala122**	**Reductase**
Nectarin 5	*Nicotiana langsdorffii* x *N. sanderae*	Q84N20	Gly59	Oxidase
p-cresol methylhydroxylase	*Pseudomonas putida*	P09788	**Ser153**	**Dehydrogenase**
Sorbitol oxidase	*Streptomyces* sp. H-7775	P97011	Gly112	Oxidase
Tetrahydrocannabinolic acid synthase	*Cannabis sativa*	Q8GTB6	Gly174	Oxidase
Vanillyl alcohol oxidase	*Penicillium simplicissimum*	P56216	Pro169	Oxidase
Xylitol oxidase	*Streptomyces* sp. IKD472	Q9KX73	Ala105	Oxidase

[a] Dehydrogenases and reductases are indicated in bold

[b] Reported as cytochrome *c* dependent, activity with oxygen not determined (Wilkinson et al., 2005)

[c] Reported as cytochrome *c* dependent, activity with oxygen not determined (Logan et al., 2007)

[d] Reported as cytochrome *c* dependent, activity with oxygen not determined (Wolucka and Communi, 2006)

[e] Crystals of *Arabidopsis thaliana* GALDH diffract to 3.5 Å

[f] Numbering according to the mature recombinant protein (Leferink et al., 2008[b])

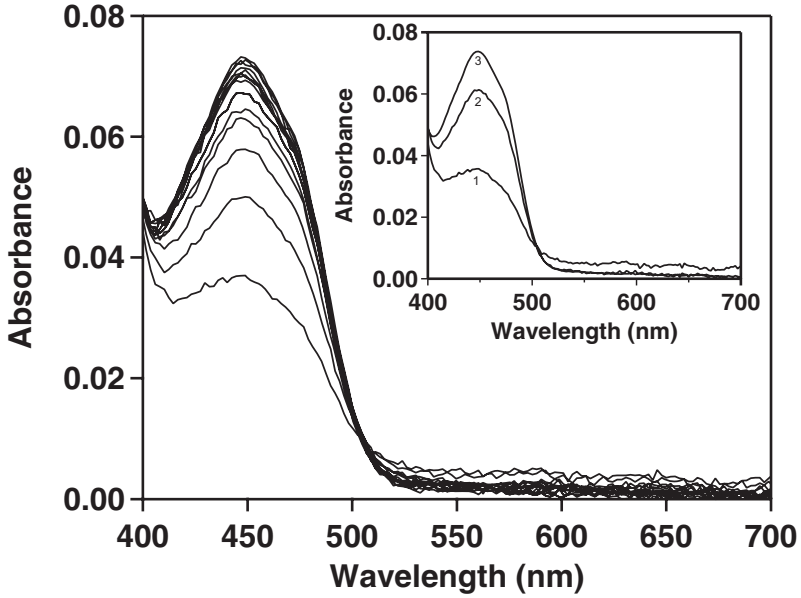


Figure S7.1. Oxidative half-reaction. Diode array detection of the re-oxidation of substrate-reduced A113G (6 μM) by molecular oxygen in the stopped-flow apparatus. Spectra were taken every 2.56 ms for 1 s, only selected spectra are shown. The inset shows the deconvoluted absorption spectra of the re-oxidation of A113G by molecular oxygen. Two-phases were distinguished, a fast phase (30 s^{-1}) leading to spectrum 2 and a second slower phase (7 s^{-1}) yielding spectrum 3.

chapter 8

Vitamin C biosynthesis in *Trypanosoma cruzi* is completed by a FAD-dependent oxidase

Nicole G. H. Leferink[1]*, Elena V. Kudryashova[1,2]*, Ilse G. M. Slot[1], Willem J. H. van Berkel[1]

[1] Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
[2] Division of Chemical Enzymology, Chemistry Department, Moscow State University, 119899 Moscow, Russia
* these authors contributed equally

Abstract

Trypanosoma cruzi, the aetiological agent of Chagas' disease, is unable to salvage vitamin C (L-ascorbate) from its environment and relies on *de novo* synthesis for its survival. Because humans lack the capacity to synthesize ascorbate, the trypanosomal enzymes involved in ascorbate biosynthesis are interesting targets for drug therapy. The terminal step in ascorbate biosynthesis is catalyzed by flavin-dependent aldonolactone oxidoreductases belonging to the vanillyl-alcohol oxidase (VAO) protein family. Here we studied the properties of recombinant *T. cruzi* galactonolactone oxidoreductase (TcGAL), refolded from inclusion bodies using a reversed micelles system. The refolded enzyme shows native-like secondary structure and is active with both L-galactono-1,4-lactone and D-arabinono-1,4-lactone. TcGAL employs a non-covalently bound FAD as redox-active cofactor, in agreement with its amino acid sequence. In addition to cytochrome *c*, TcGAL can also use molecular oxygen as electron acceptor. This is in line with the absence of a recently identified gatekeeper residue that prevents dehydrogenases of the VAO family from acting as oxidases.

Keywords: flavoprotein; galactonolactone dehydrogenase; inclusion bodies; oxidase; refolding; reversed micelles; *Trypanosoma cruzi*; vitamin C

Abbreviations: AOT, bis(2-ethylhexyl)sulfosuccinate; AtGALDH, *Arabidopsis thaliana* L-galactono-1,4-lactone dehydrogenase; CD, circular dichroism; DCPIP, 2,6-dichlorophenolindophenol; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; TcGAL, *Trypanosoma cruzi* galactonolactone oxidase

Introduction

Trypanosomatids are protozoan parasites belonging to the order of *Kinetoplastida*. They include the medically relevant human parasites *Trypanosoma brucei*, *T. cruzi* and *Leishmania* spp., the causative agents of African sleeping sickness, Chagas' disease and leishmaniasis, respectively. Together these diseases threat over 30 million people world-wide. Current treatments are unsatisfactory, since the available drugs have a limited efficacy and exhibit toxic side effects (http://www.who.int).

During their life-cycles, trypanosomatids are exposed to reactive oxygen species generated by their own aerobic metabolism and by the hosts immune response. The antioxidant response in the parasites is distinct from their mammalian hosts and includes targets that may be exploited therapeutically. Trypanosomes lack catalases and glutathione peroxidases (Irigoín et al., 2008; Krauth-Siegel and Comini, 2008), and detoxify hydrogen peroxide using a plant-like ascorbate peroxidase (Wilkinson et al., 2002; Castro and Tomás, 2008). Furthermore, they possess the unique dithiol trypanothione which is a conjugate of two glutathione molecules with one molecule of spermidine (Irigoín et al., 2008). The flavoenzyme trypanothione reductase, which keeps trypanothione in the reduced state, is an essential enzyme for the parasite as it is the only enzyme that connects hydrogen peroxide detoxification to NAD(P)H redox biology in these parasites (Krauth-Siegel and Comini, 2008).

Trypanosomes contain significant levels of ascorbate (vitamin C), which is synthesized in the glycosome, a unique single membrane organelle. Genome analysis has indicated that ascorbate biosynthesis in trypanosomes is similar to that in plants (Wilkinson et al., 2005; Logan et al., 2007). Most eukaryotic organisms can synthesize ascorbate to their own requirements. Humans and other primates, however, have lost this ability during evolution (Chatterjee, 1973), because the gene encoding the enzyme responsible for the final step is highly mutated and non-functional (Nishikimi et al., 1994). The trypanosomal enzymes involved in ascorbate biosynthesis are interesting targets for drug therapy, since the parasites lack the ability to scavenge ascorbate from the environment, and rely on *de novo* synthesis for their survival (Logan et al., 2007; Irigoín et al., 2008).

The terminal step of ascorbate biosynthesis is catalyzed by so-called aldonolactone oxidoreductases. These enzymes belong to the vanillyl-alcohol oxidase (VAO) flavoprotein family (Fraaije et al., 1998; Leferink et al., 2008[a]). Aldonolactone oxidoreductases have been isolated from various sources including animals, yeasts, fungi and plants. Most aldonolactone oxidoreductases are oxidases containing covalently bound FAD, while plants utilize a strictly cytochrome *c*-dependent galactonolactone dehydrogenase (GALDH) containing non-covalently bound FAD (Leferink et al., 2008[b]). The arabinonolactone oxidoreductase

(TbALO) from *T. brucei* and galactonolactone oxidoreductase (TcGAL) from *T. cruzi* have been isolated and characterized to some extent (Wilkinson et al., 2005; Logan et al., 2007). Both enzymes were annotated as oxidases, but the activity with molecular oxygen has never been established. Instead, cytochrome *c* was used as electron acceptor in the activity assays (Wilkinson et al., 2005; Logan et al., 2007). TcGAL and TbALO were reported to contain FMN rather than FAD as redox-active cofactor (Logan et al., 2007). Nevertheless, the PP loop for FAD binding (Fraaije et al., 1998) is in both enzymes conserved (Fig. 8.1).

In this research we have investigated the cofactor, substrate and electron acceptor specificity of recombinant TcGAL, refolded from inclusion bodies using an AOT-isooctane reversed micelles system. In contrast to an earlier report (Logan et al., 2007), we found that TcGAL employs a non-covalently bound FAD as redox active cofactor, in agreement with its amino acid sequence. Furthermore, we established that TcGAL can use molecular oxygen as efficient electron acceptor.

Materials and Methods

Chemicals - FAD, FMN, L-galactono-1,4-lactone, D-arabinono-1,4-lactone, bovine heart cytochrome *c*, 1,4-benzoquinone and bis(2-ethylhexyl)sulfosuccinate (AOT) were from Sigma-Aldrich (St Louis, MO, USA). 2,6-Dichlorophenolindophenol (DCPIP) was from Merck (Darmstadt, Germany). Mini-Complete protease inhibitor was from Roche Molecular Biochemicals (Mannheim, Germany). Restriction endonucleases, T4-DNA ligase and dNTPs were purchased from Invitrogen (Carlsbad, CA, USA). Pfu DNA polymerase was obtained from Fermentas GmbH (St. Leon-Rot, Germany). Oligonucleotides were synthesized by Eurogentec (Liege, Belgium). All other chemicals were from commercial sources and of the purest grade available.

Recombinant expression of tagged TcGAL - The pTrcHisC vector containing the TcGAL gene (from *T. cruzi* X10/6 clone) N-terminally fused to a His_6-tag, XpressTM epitope and enterokinase cleavage site (Logan et al., 2007), was kindly provided by prof. M.W. Fraaije (University of Groningen). The plasmid was electroporated into *E. coli* TOP10, BL21(DE3) and Rosetta(DE3) electrocompetent cells for recombinant expression. For enzyme production *E. coli* cells, harboring the pTrcHisCTcGAL plasmid, were grown in Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin until an OD_{600} of 0.6 was reached. Expression was induced by addition of 0.4 mM isopropyl-thio-β-D-galactopyranoside and the incubation was continued for an additional 3 h at 37°C, 6 h at 37°C, overnight at 28°C or for 3 days at 20°C.

Re-cloning of native TcGAL - To remove the large non-native N-terminal tag, the TcGAL gene was re-cloned into a modified pBAD-vector. An *Nde*I restriction site in the TcGAL gene was first removed using the QuikChange II site-directed mutagenesis method (Stratagene, La Jolla, CA, USA) using pTrcHisCTcGAL as a template. The primers used are TcGAL-qcf (5'-GCT TCG ATG TGT TCC GTC GTA CGT ACT CAC GAC GAT TGC TG- 3') and TcGAL-qcr 5'-CAG CAA TCG TCG TGA GTA CGT ACG ACG GAA CAC ATC GAA GC-3'), changed nucleotides are underlined. Successful mutagenesis was confirmed by digestion with *Nde*I. The native TcGAL gene was amplified from the mutated pTrcHisCTcGAL vector by PCR using the oligonucleotides TcGAL-f (5'-GGA ATT CCA TAT GCG TTG TGA CGT TTC CAT GCG GC-3') introducing a new *Nde*I restriction site (underlined), and TcGAL-r (5'-CCC AAG CTT ACA AAT GAC TAT TGG TGC TG-3'), introducing a *Hin*dIII restriction site (underlined). The amplified fragment was cloned between the *Nde*I and *Hin*dIII restriction sites of a modified pBAD/*Myc*-HisA expression vector, kindly provided by prof. M.W. Fraaije (University of Groningen). A stop-codon was introduced in the reverse primer to prevent fusion to the *myc* epitope and His_6-tag present in the pBAD vector. The resulting pBAD-TcGAL construct was verified by automated sequencing of both strands and electroporated into *E. coli* TOP10 electrocompetent cells for recombinant expression.

Recombinant expression and purification of native TcGAL - For enzyme production, *E. coli* BL21(DE3) cells harbouring the pBAD-TcGAL plasmid, were grown in LB medium supplemented with 100 μg/ml ampicillin until the OD_{600} reached ~0.6. Expression of TcGAL was induced by addition of 0.02% L-arabinose and the incubation was continued for 16 h at 28°C. The cells were harvested and resuspended in 50 mM Tris-HCl, 5 mM dithiothreitol (DTT), pH 7.4, supplemented with 1 tablet of mini-Complete protease inhibitor, 10 mM $MgCl_2$ and DNase I and subsequently passed twice through a pre-cooled French Pressure cell (SLM Aminco, SLM Instruments, Urbana, IL, USA) at 10 000 psi. The resulting homogenate was centrifuged at 25 000 *g* for 30 min at 4°C and the insoluble inclusion bodies were collected. The soluble extract was loaded onto a HiLoad 26/10 Q-Sepharose FF column (Pharmacia Biotech, Uppsala Sweden) equilibrated with 50 mM Tris-HCl, 5 mM DTT, pH 7.4. Proteins were eluted with a linear gradient of NaCl (0-0.5 M) in the same buffer. The TcGAL activity was collected in the flow-through, pooled and applied onto a Ceramic hydroxyapatite XK 26/11 (Pharmacia Biotech) column equilibrated with 25 mM sodium phosphate, 5 mM DTT, pH 6.5. Proteins were eluted with a gradient of sodium phosphate (25-500 mM). Again all the TcGAL activity was collected in the flow-through. The active fractions were pooled and brought to 20% ammonium sulfate saturation and applied onto a Phenyl-Sepharose CL-4B (Pharmacia Biotech) column equilibrated with 50 mM sodium

phosphate, 5 mM DTT, 20% ammonium sulphate saturation, pH 7.4. Proteins were eluted with a linear gradient of ammonium sulfate (20-0%) in the same buffer.

Isolation and refolding of TcGAL inclusion bodies - The insoluble material collected after cell lysis was washed with 6% Triton X-100 containing 60 mM EDTA and 1.5 M NaCl, pH 7.0 and dissolved in 6 M guanidinium hydrochloride as described (De Bernardez Clark et al., 1999). Subsequently, the denaturant was removed by dialysis against 10 mM sodium phosphate, 1 mM DTT, pH 8.0. A final dialysis step was performed against 10 mM sodium phosphate, pH 7.2 to remove excess DTT. After dialysis a turbid suspension of aggregated protein was obtained. This suspension was added to the reversed micelles system, 0.4 M AOT in isooctane, and shaked intensively. The hydration degree, w_0, was varied in the range 20-35 by adding different amounts of 10 mM sodium phosphate, pH 8.5. Oxidized and reduced glutathione solutions (10 mM GSSG and 30 mM GSH) in 10 mM sodium phosphate, pH 8.5 were mixed and an aliquot of 100 µl was added to 1 ml of 0.4 M AOT solution in isooctane. Refolding of the enzyme was initiated by mixing 1 volume of micellar solution containing glutathiones with 3 volumes of TcGAL micellar solution and an aliquot (10 µl per ml) of FAD or (FMN) cofactor solution in water (10-fold molar excess to enzyme concentration). The resultant solution was shaked for 10 minutes yielding a transparent TcGAL containing system. The final micellar protein concentration was 1-2 mg/ml depending on the surfactant hydration degree.

Protein analysis - SDS-PAGE was performed using 12.5% acrylamide slab gels essentially as described by Laemmli (Laemmli, 1970). Proteins were stained with Coomassie Brilliant Blue R-250. Total protein concentrations were estimated using the Bradford protein assay from BioRad with BSA as standard. Desalting or buffer exchange of small aliquots of enzyme was performed with Bio-Gel P-6DG columns (Bio-Rad).

Thin-layer chromatography (TLC) - The identity of the flavin cofactor of the soluble TcGAL preparation was determined by TLC. The flavin was extracted with a solvent mixture consisting of butanol/acetic acid/water (5:3:3). Extracted flavin and the reference compounds FAD, FMN and riboflavin were applied onto a Baker-flex silica-gel IB2 TLC plate (JT Baker, Inc., Phillipsburg, NY, USA). Butanol/acetic acid/water (5:3:3) was used as mobile phase. Fluorescent spots were visualized by UV light.

TcGAL and AtGALDH activity in aqueous solution - TcGAL and AtGALDH activity in aqueous solution was routinely assayed by following the reduction of cytochrome *c* at 550 nm (Leferink et al., 2008[b]). Initial velocities were calculated using a molar difference absorption

coefficient ($\Delta\varepsilon_{550}$) of 21 $mM^{-1}cm^{-1}$ for reduced minus oxidized cytochrome *c*. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate per min, which is equivalent to the reduction of 2 μmol of cytochrome *c* (Ôba et al., 1995). Alternatively, GALDH activity was measured with the artificial electron acceptor, 1,4-benzoquinone (ε_{290} = 2.3 $mM^{-1}cm^{-1}$) (Leferink et al., 2008[b]). For the determination of kinetic parameters, the concentration of lactone substrate was varied in the range from 5 μM to 2 mM with a constant 1,4-benzoquinone concentration of 1 mM.

TcGAL and AtGALDH activity in reversed micelles - A solution of 0.1 M AOT in isooctane was used for the preparation of the reversed micelles system. The particle size distribution of AOT reversed micelles is narrow, i.e. uniformed micelles of equal size are formed at each surfactant hydration degree (Köhling et al., 2002). The hydration degree of the micelles (w_0 = $[H_2O]/[AOT]$) was varied over the range of 12 to 40 by addition of corresponding amounts of 25 mM sodium phosphate, pH 7.2. The inner cavity radius of the micelles r_m in Å, can be calculated using the empirical equation (Levashov and Klyachko, 2001):

$$r_m = 1.5w_0 + 4 \qquad \text{(eq. 8.1)}$$

which holds for all enzyme-containing reverse micellar systems studied (Levashov and Klyachko, 2001; Köhling et al., 2002; Klyachko and Levashov, 2003).

It has been shown previously that due to strong association with the AOT interface (Pileni et al., 1985), the secondary structure of cytochrome *c* is lost in AOT-reversed micelles (Vos et al., 1987). Thus, TcGAL activity in AOT-reversed micelles was measured by following the reduction of the artificial electron acceptor 1,4-benzoquinone. The TcGAL activity in reversed micelles was measured at pH 7.2, since at higher pH values the auto-oxidation of 1,4-benzoquinone is significant (Frébortová et al., 2004). The oxidase activity of TcGAL in AOT-reversed micelles was determined by measuring the formation of ascorbic acid through its reaction with DCPIP. The absorption maximum and corresponding molar absorption coefficient of DCPIP in reversed micelles of AOT in isooctane at pH 7.2 (ε_{535} = 11.5 $mM^{-1}cm^{-1}$) are different compared to that in water (ε_{600} = 20.6 $mM^{-1}cm^{-1}$) (Klyachko et al., 2005).

The homologous AtGALDH (Leferink et al., 2008[b]) served as a control for the reaction with 1,4-benzoquinone. A113G AtGALDH, which in contrast to wild-type AtGALDH shows high oxidase activity (Leferink et al., 2009), was used as a control for the measurement of the oxygen reactivity of TcGAL in the reversed micelles system.

In a typical kinetic experiment, solutions of D-arabinono-1,4-lactone (in the case of TcGAL) or L-galactono-1,4-lactone (in the case of wild-type AtGALDH and A113G

AtGALDH) in water and 1,4-benzoquinone in DMSO or DCPIP in ethanol were solubilized in a 0.1 M AOT solution in isooctane by intensive shaking. The final concentrations were 1 mM for 1,4-benzoquinone and 100 μM for DCPIP. The reaction was initiated by addition of 20 μl TcGAL micellar system, containing 0.4 M AOT in isooctane (w_0 = 30). The background rate for non-enzymatic decomposition of 1,4-benzoquinone was subtracted from the reaction rate measured in the presence of enzyme. In case of DCPIP, the background rate for the non-enzymatic reaction was negligible. To determine kinetic parameters, the concentration of lactone substrate was varied in the range from 5 μM to 2 mM. The activity of TcGAL in reversed micelles followed Michaelis-Menten kinetics. K_m and V_{max} were calculated using nonlinear regression data analysis.

Circular dichroism - CD spectra were recorded with a Jasco-715 spectropolarimeter at 20°C in 1 mm pathlength quartz cuvettes. The CD spectra were measured in both AOT reversed micelles (0.04 M AOT, 10 mM sodium phosphate, pH 7.2, w_0 = 28; TcGAL and AtGALDH) and in aqueous solution (10 mM sodium phosphate, pH 7.2; AtGALDH). Since AOT has high absorbance in the far-UV region, for CD measurements its concentration has to be as low as possible. At 0.04 M AOT it was possible to obtain good quality spectra in the wavelength range 195-260 nm, which enabled quantitative secondary structure analysis. The protein concentrations were 0.05 to 0.1 mg/ml. The percentage of secondary structure elements was estimated using CDNN software (http://bioinformatik.biochemtech.uni-halle.de/cdnn).

Results

Sequence analysis - The mature TcGAL protein contains 505 amino acid residues and has a theoretical mass of 56 740 Da. The TcGAL used in this study, originating from *T. cruzi* clone X10/6 (Logan et al., 2007), shows 97% sequence identity to the TcGAL sequence in the database (*T. cruzi* genome reference strain CL Brener, GeneDB accession number Tc00.1047053509179.100). TcGAL shows 28%, 23% and 21% sequence identity, and 51%, 46% and 46% similarity to gulonolactone oxidase from *Rattus norvegicus*, arabinonolactone oxidase from *Saccharomyces cerevisiae* and GALDH from *Arabidopsis thaliana*, respectively. The TcGAL protein contains a relatively large number of 15 cysteine residues, compared to the 5 cysteines present in GALDH from *A. thaliana* (AtGALDH).

Multiple sequence alignment of the N- and C-terminal parts of the FAD-binding domains of various aldonolactone oxidoreductases (Fig. 8.1), shows that residues specifically interacting with the pyrophosphate and adenine parts of the FAD-cofactor are conserved in TcGAL. Furthermore, a gatekeeper residue preventing oxidase activity similar to Ala113 in

AtGALDH (Leferink et al., 2009) is absent in TcGAL, suggesting that the enzyme can use molecular oxygen as electron acceptor.

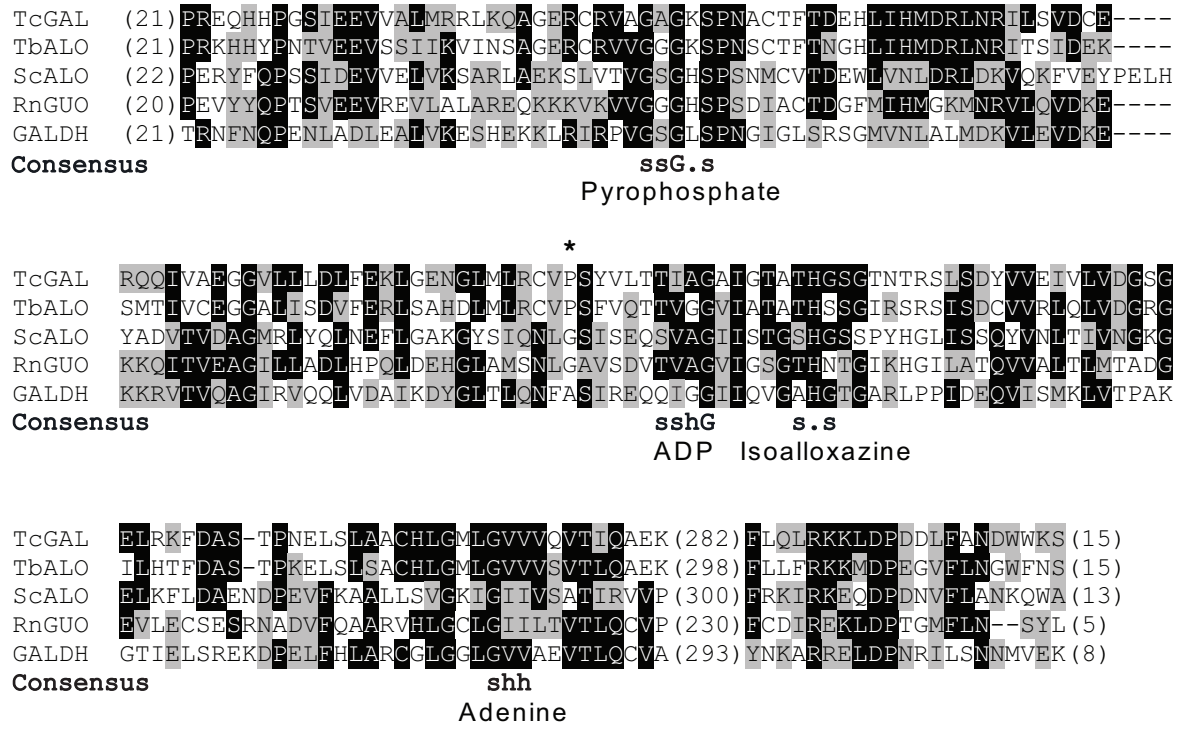


Figure 8.1. ClustalW multiple sequence alignment (Thompson et al., 1994) of the FAD-binding domains of TcGAL and related aldonolactone oxidoreductases. Identical residues are shaded in black, similar residues are shaded in gray. The consensus of conserved residues specifically interacting with parts of the FAD cofactor is shown under the alignment (s, small: G,A,S,T; h, hydrophobic: I,L,V, •, any residue) (Fraaije et al., 1998). The asterisk (*) marks the residue that determines the oxygen reactivity (Leferink et al., 2009). The number of residues present in gaps in the sequence and at the termini are indicated in parentheses. The accession numbers used are: TcGAL, Q4DPZ5; TbALO, Q57ZU1; ScALO, P54783; RnGUO, P10867; GALDH, Q8GY16.

Expression and purification of recombinant TcGAL - Expression of TcGAL from the pTrcHisCTcGAL plasmid yielded insoluble inclusion bodies, despite the use of different *E. coli* strains and induction temperatures (see Materials and Methods). Using L-galactono-1,4-lactone and cytochrome *c* as substrates, no TcGAL activity could be detected, neither in the absence or the presence of FAD or FMN. We argued that the presence of a large non-native tag of 44 amino acids fused to the N-terminus of TcGAL might hamper proper folding of the recombinant protein. Therefore, we re-cloned the TcGAL gene without any tag into the pBAD expression system. The recombinant expression of native TcGAL from the pBAD-TcGAL vector in *E. coli* yielded, at best, a mixture of soluble protein and insoluble inclusion bodies (Fig. 8.2). Using cytochrome *c* as electron acceptor, TcGAL activity was confirmed in the crude *E. coli* extract for L-galactono-1,4-lactone (0.10 U mg^{-1}) and D-arabinono-1,4-lactone (0.23 U mg^{-1}). The insoluble inclusion bodies did not contain flavin and were inactive.

Soluble TcGAL could be purified to a large extent (Fig. 8.2), but with considerable loss of enzyme activity (Table 8.1). Changing the order of purification steps did not improve the outcome. Thin-layer chromatography of the flavin released from the partially purified TcGAL preparation yielded a retention behaviour similar to that of commercial FAD, and quite different from FMN, with R_f values of 0.25, 0.32 and 0.52, for the TcGAL cofactor, FAD and FMN, respectively.

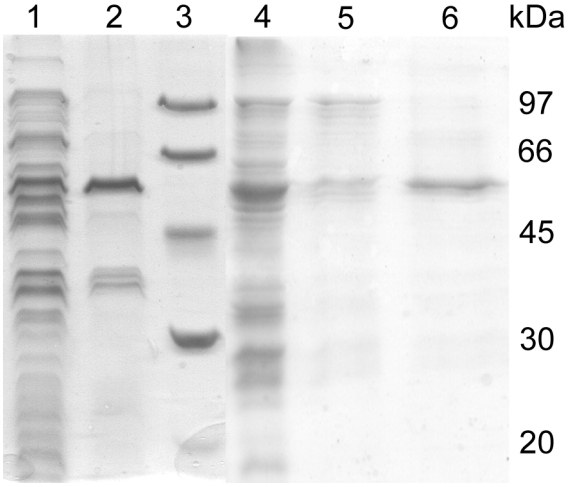


Figure 8.2. SDS-PAGE analysis of the expression and purification of soluble TcGAL in *E. coli*. TOP10 cells harbouring the pBAD-TcGAL plasmid were induced with 0.02% L-arabinose for 16 h at 28°C. Lane 1, soluble crude extract; lane 2, insoluble fraction (inclusion bodies); lane 3, LMW marker; lane 4, Q-sepharose pool; lane 5, hydroxyapatite pool; lane 6, phenyl-sepharose pool. The predicted molecular mass of native TcGAL is 56.7 kDa.

Table 8.1. (Partial) purification of soluble TcGAL expressed in *E. coli*. The activity was determined using 1 mM D-arabinono-1,4-lactone and 50 μM cytochrome *c* as substrates in potassium pyrophosphate, pH 8.8 (*I* = 25 mM).

Step	Protein (mg)	Activity (U)	Spec. activity (U mg^{-1})	Yield (%)
Cell extract	782	177	0.23	100
Q-Sepharose	469	234	0.50	132
Hydroxyapatite	57	186	3.26	105
Phenyl sepharose	16	19	1.19	11

Refolding of TcGAL inclusion bodies using reversed micelles - To obtain soluble and active TcGAL from inclusion bodies we applied a recently developed approach based on reversed micelles (Vinogradov et al., 2003). In reversed micelles each protein molecule is localized in an individual, tailor-made compartment, thus minimizing the possibility of side-reactions, such as aggregation, disulfide cross-linking, etc. A refolding reversed micelle approach has been successfully implemented with *Fusarium* galactose oxidase and *Stigmatella aurantiaca* putative galactose oxidase (Vinogradov et al., 2003).

For refolding of TcGAL, isolated inclusion bodies were first unfolded in 6 M guanidinium hydrochloride. Removal of the unfolding agent resulted in a turbid suspension. This suspension was injected into the water-AOT-isooctane micellar system containing a high concentration of AOT (0.4 M) to allow maximal protein solubilization. According to the relation: $w_0 = [H_2O]/[AOT]$, at an AOT concentration of 0.4 M, 216 µl of aqueous phase (containing the protein inclusion bodies) should be added per each ml of micellar system to form a hydration degree $w_0 = 30$ with a resultant enzyme concentration of 1-2 mg/ml.

Addition of inclusion bodies to the micellar system yielded a slightly turbid solution, indicating that the protein is still mis-folded and partially aggregated. Incubation with a mixture of reduced and oxidized glutathione (GSH/GSSG 3:1) (Sakono et al., 2004) during 10-15 minutes and subsequent saturation with FAD (or FMN) solubilized the protein completely and a fully transparent solution was obtained. This micellar enzyme system was used in subsequent experiments for kinetic and spectral characterization.

Catalytic properties of refolded TcGAL - It has been shown for a number of different enzymes that they are optimally active in reversed micelles at a w_0 where the inner radius of the micelle is equal to that of the entrapped protein: $r_{enzyme} = r_{micelle}$. It is believed that under these conditions the surfactant shell works like a molecular chaperone, re-adjusting and supporting the native protein conformation (Levashov and Klyachko, 2001). In case of enzymes existing in different oligomeric forms the activity profiles show several optima, where each of these optima reflects different oligomeric forms of the enzyme (Levashov and Klyachko, 2001).

To quantify and optimize the yield of active enzyme, the catalytic activity of solubilized and refolded TcGAL in the reversed micelles system was measured as a function of w_0 of the micelles. The activity of TcGAL in the reaction with D-arabinono-1,4-lactone and 1,4-benzo-quinone in dependence of w_0 is presented in Fig 8.3A. Two optima of TcGAL activity are observed at w_0 values of 22 and 27, which can be tentatively assigned to the monomeric and dimeric forms of TcGAL, respectively. From preliminary 3D modeling experiments (not shown) we could judge that TcGAL as well as AtGALDH have a non-spherical shape with subunit dimensions of approximately 47 × 60 × 73 Å. For proteins with a non-spherical shape the inner radius of the micelles, r_m, is equal to the maximal protein radius (half the length of the longest dimension of the protein), r_p. Thus, the r_p value for each oligomeric form can be estimated with the position of the activity optimum (w_0) using the empirical equation (Levashov and Klyachko, 2001):

$$r_p = r_m\,(\text{Å}) = 1.5w_0 + 4 \qquad \text{(eq. 8.2)}$$

From this equation and the data in Fig. 8.3A it follows that TcGAL has a r_p = 37 Å at w_0 = 22 and r_p = 46 Å at w_0 = 28. This indeed suggests the accommodation of monomeric and dimeric TcGAL in the AOT reversed micelles at hydration degrees of 22 and 28, respectively. The activity level of monomeric TcGAL is slightly higher (by 20%) compared to the dimer; the K_m values for D-arabinono-1,4-lactone are similar, 0.22 mM for w_0 = 20 and 0.25 mM for w_0 = 27, respectively.

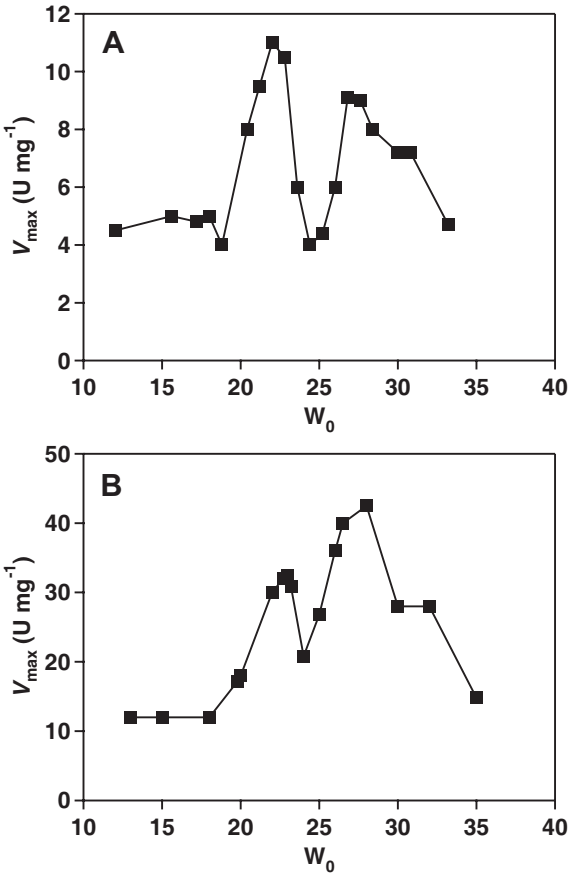


Figure 8.3. Catalytic activity of TcGAL and AtGALDH in AOT reversed micelles. A) Activity of TcGAL with D-arabinono-1,4-lactone, B) Activity of AtGALDH with L-galactono-1,4-lactone. The maximal activity was determined as a function of the hydration degree (w_0) of the reversed micelles. Experimental conditions: AOT (0.1 M), 1,4-benzoquinone (2.3 mM), 25 mM sodium phosphate, pH 7.2 and varying concentrations of lactone substrate. Kinetic parameters have maximal error values of 20%.

TcGAL is also active with molecular oxygen (aerated buffer) in the absence of alternative electron acceptors. In the reaction with molecular oxygen, a similar profile of TcGAL activity as a function of w_0 of the reverse micelles was observed. The specific activity with molecular oxygen is 2-3 times lower than that in the presence of 1,4-benzoquinone (4.4 and 3.75 U mg^{-1}, versus 11 and 9 U mg^{-1} at w_0 = 22 and w_0 = 27, respectively), while the K_m value for D-arabinono-1,4-lactone is comparable to the reaction in the presence of 1,4-benzoquinone (0.2 mM at both w_0 = 22 and w_0 = 27). No TcGAL activity could be detected (neither in the

reaction with 1,4-benzoquinone nor with molecular oxygen) when the enzyme was refolded in the presence of FMN. Thus, refolding of TcGAL inclusion bodies in reversed micelles only yields active protein in the presence of FAD and not FMN.

Interestingly, AtGALDH showed a similar activity profile in the reversed micelles system. The activity optima of AtGALDH are observed at w_0 values of 23 and 28, indicating the formation of monomeric and dimeric species as observed with TcGAL. In contrast to TcGAL, the activity of AtGALDH at w_0 = 20 is 25% lower than at w_0 = 28. The K_m values of AtGALDH for L-galactono-1,4-lactone are in the range of 0.20-0.22 mM and practically do not change in the range of w_0 from 18 to 35.

In aqueous solution, pH 7.2, AtGALDH shows the following kinetic parameters with L-galactono-1,4-lactone as the variable substrate and 1,4-benzoquinone as electron acceptor: k_{cat} = 100 ± 10 s^{-1} and K_m = 0.18 mM, in good agreement with previous results (Leferink et al., 2008[b]). The activity level of AtGALDH in reverse micelles at optimal hydration degrees (w_0 = 23 and 28) is of the same order compared to aqueous solution and the K_m value for L-galactono-1,4-lactone differs only slightly (0.20-0.22 mM). Thus, solubilization of AtGALDH in reversed micelles does not significantly change the catalytic properties of the enzyme.

Refolded TcGAL is less active than AtGALDH. Refolding TcGAL from inclusion bodies yielded approximately 15-20% of the enzymatic activity as compared to the activity of AtGALDH (Figure 8.3).

Circular dichroism - The CD spectra of TcGAL solubilized in reversed micelles are presented in Fig. 8.4, together with the CD spectra of AtGALDH in reversed micelles and in aqueous solution. The CD spectra of TcGAL in reversed micelles and AtGALDH in aqueous solution have very similar profiles. Both spectra show positive extremes around 195 nm and negative extremes of nearly equal intensity at 210 and 222 nm. The CD spectrum of AtGALDH in reverse micelles (Fig. 8.4B) has a somewhat different shape, the intensity at 210 nm is higher compared to that at 222 nm. This apparent difference in secondary structure content can be explained by FAD cofactor release upon solubilization of AtGALDH in the micellar system. Solubilization of AtGALDH in the presence of excess FAD resulted in recovery of AtGALDH structure in micelles (Table 8.2). On the other hand, apo-TcGAL solubilized in reverse micelles also shows a decreased α-helix content (by 2-3%) as compared to the holo-form of TcGAL. Quantitative analysis of the CD spectra showed that the secondary structure of both enzymes (in holo-form) is characterized by a high α-helix content (Table 8.2), consistent with the structural properties of VAO family members (Mattevi et al., 1997; Fraaije et al., 1998). Together with the activity studies this clearly demonstrates the formation of native TcGAL structure upon refolding in reverse micelles.

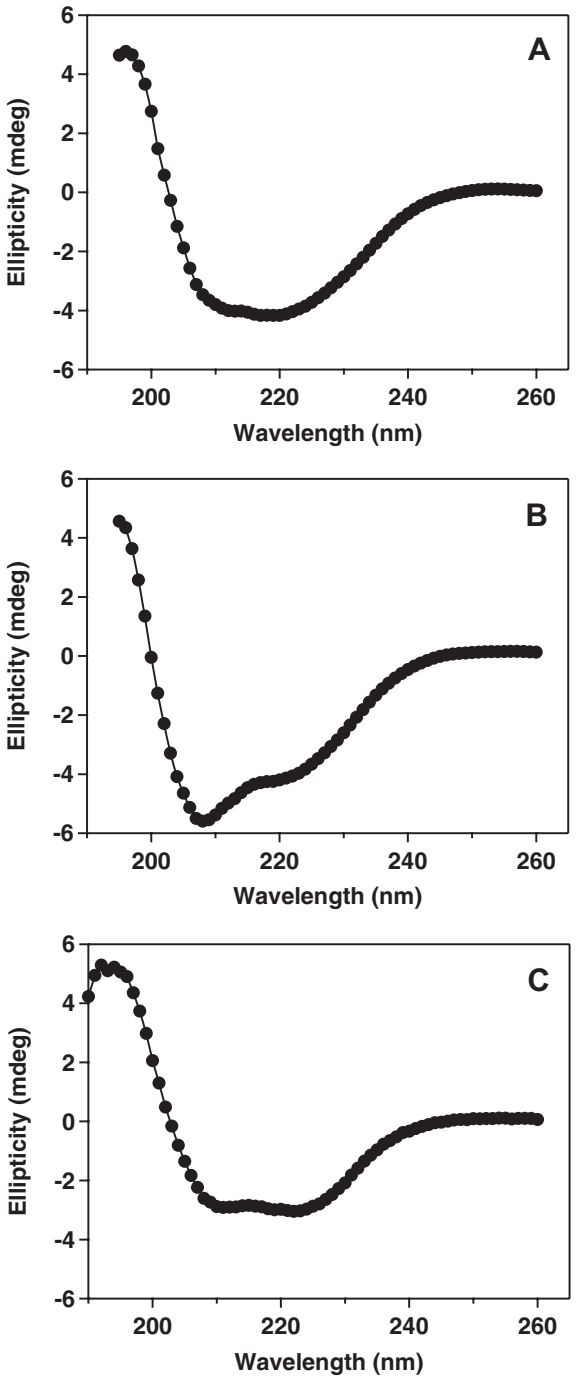


Figure 8.4. Circular dichroism spectra of TcGAL and AtGALDH. A) TcGAL in AOT reversed micelles, B) AtGALDH in AOT reversed micelles, C) AtGALDH in 25 mM sodium phosphate, pH 7.2. Experimental conditions: AOT (0.04 M), 10 mM sodium phosphate buffer, pH 7.2, 20°C. Protein concentrations were 0.05-0.1 μM.

Extraction of TcGAL from reversed micelles - After being solubilized and refolded in reversed micelles, the protein can be extracted from the micellar system to aqueous solution. Two methods were used: precipitation of the enzyme with cold dry acetone and subsequent dissolving in aqueous solution (Levashov and Klyachko, 2001), and the stratification of the micellar system with a salt solution (0.1 M KCl) (Sakono et al., 2004). With the TcGAL micellar system, the application of both these methods allowed to obtain the active enzyme in aqueous phase. The K_m for D-arabinono-1,4-lactone of extracted TcGAL in aqueous solution has the same value as determined for TcGAL in the reversed micellar system. However, enzyme extraction from the micellar system to the aqueous phase yielded a 20-30 fold lower specific activity. Thus, it is clear that further research is required to reach a better yield of the fully active enzyme in aqueous solution.

Discussion

In this work we have studied the substrate, cofactor and electron acceptor specificity of the recently discovered enzyme TcGAL (Logan et al., 2007). TcGAL completes ascorbate biosynthesis in *T. cruzi* parasites, which are unable to scavenge ascorbate from their environment.

Table 8.2. Secondary structure parameters determined from CD experiments for TcGAL inclusion bodies refolded in AOT reversed micelles. The same parameters determined for AtGALDH in aqueous solution and in reverse micelles (in the absence and presence of the excess FAD) are presented as a reference. Experimental conditions: 0.04 M AOT, 10 mM sodium phosphate buffer, pH 7.2. Protein concentrations were 0.05 to 0.1 mg/ml.

Secondary structure element	TcGAL in micelles	AtGALDH in buffer	AtGALDH in micelles	AtGALDH + FAD in micelles
	%	%	%	%
α-helix	33.1	32.4	29.1	31.3
β-sheets (antiparallel)	8.6	8.1	12.8	9.7
β-sheets (parallel)	8.9	9.1	9.2	9.3
beta-turn	16.6	16.8	17.9	17.7
loops	34.0	35.4	31.7	31.8

Recombinant expression of tagged TcGAL from the pTrcHisC vector in *E. coli* yielded inactive inclusion bodies. In contrast, expression of native TcGAL from the pBAD-TcGAL vector yielded a mixture of active soluble protein and inactive inclusion bodies. Purification of soluble TcGAL was hampered by considerable loss of activity. Possibly, the native enzyme requires disulfide bridges, which are unlikely to be formed in the reducing environment of the *E. coli* cytoplasm (Baneyx and Mujacic, 2004).

To obtain correctly folded active protein, TcGAL inclusion bodies were solubilized and refolded using an AOT-isooctane reversed micelles system. The important advantage of this approach is that upon solubilization each enzyme molecule is accommodated in an "individual compartment". The possibility to "isolate" each protein molecule minimizes side processes of protein folding, such as aggregation and intermolecular disulfide bond formation. The abovementioned suggestion that native TcGAL requires disulfide bridges is supported by the fact that refolding of TcGAL in reversed micelles requires the presence of a redox system consisting of a mixture reduced and oxidized glutathione (GSH:GSSG). The refolded TcGAL protein shows native-like secondary structure with an α-helix and β-sheet content very similar to that of AtGALDH in both aqueous solution and in the micellar system. The high α-helix content of TcGAL is consistent with the structural properties of other VAO family members (Mattevi et al., 1997; Fraaije et al., 1998).

Both TcGAL and AtGALDH show two activity optima in reversed micelles, depending on w_0, presumably corresponding to the monomeric and dimeric forms of the enzymes. It is, however, expected that both enzymes function as momomers, since for AtGALDH no dimer formation was observed in aqueous solution (Leferink et al., 2008[b]).

Previously, TcGAL was annotated as a galactonolactone oxidase, but the activity with molecular oxygen was never demonstrated. We found that, besides cytochrome *c* and 1,4-benzoquinone, TcGAL can also use molecular oxygen as electron acceptor. The reactivity with molecular oxygen is in accordance with the fact that TcGAL lacks a recently identified gatekeeper residue that prevents dehydrogenases of the VAO family from acting as oxidases

(Leferink et al., 2009). This gatekeeper residue is situated near the C4a locus on the flavin *re*-side, opposite of the substrate binding site, and likely prevents oxygen access to the reduced flavin. Replacement of the corresponding residue in AtGALDH (Ala113) into a Gly results in a catalytically competent oxidase (Leferink et al., 2009).

In line with previous reports (Wilkinson et al., 2005; Logan et al., 2007), we found that TcGAL can oxidize both L-galactono-1,4-lactone and D-arabinono-1,4-lactone, with a slight preference for the latter substrate. The preference of TcGAL and TbALO for D-arabinono-1,4-lactone discriminate the Trypanosomal enzymes from GALDH from plants, which show a clear preference for L-galactono-1,4-lactone. Despite the preference of TcGAL and TbALO for D-arabinono-1,4-lactone, genome analysis suggests that ascorbate biosynthesis in Trypanosomes proceeds via L-galactose and L-galactono-1,4-lactone (Wilkinson et al., 2005).

TcGAL has been reported to contain non-covalently bound FMN as redox-active cofactor (Logan et al., 2007). However, the trichloroacetic acid precipitation used to release the flavin cofactor promotes the acid catalyzed hydrolysis of FAD (Aliverti et al., 1999). Here we showed that soluble recombinant TcGAL employs a non-covalently bound FAD as redox active cofactor, and that the refolding of TcGAL inclusion bodies in reversed micelles only yields active protein in the presence of FAD and not FMN. Recently, also for another VAO family member, the glycopeptide hexose oxidase Dbv29, an FMN cofactor was identified (Li et al., 2007). The method used to release the cofactor is, however, not clear.

In conclusion, using an AOT-isooctane reversed micelles system we were able to obtain TcGAL in active form and investigate its secondary structure and catalytic properties. In contrast to an earlier report (Logan et al., 2007), we found that TcGAL employs a non-covalently bound FAD as redox active cofactor, in agreement with its amino acid sequence. Recombinant TcGAL oxidizes both L-galactono-1,4-lactone and D-arabinono-1,4-lactone using different electron acceptors, including molecular oxygen.

Acknowledgements

We thank prof. dr. M.W. Fraaije (University of Groningen) for kindly providing us with the plasmids pTrcHisCTcGAL and a modified pBAD/*Myc*-HisA. This work was supported by the Carbohydrate Research Centre Wageningen (CRC-W) and by a short-term EU fellowship to HK.

chapter 9

Summary and concluding remarks

Nicole G. H. Leferink[1], Willem J. H. van Berkel[1]

[1] Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

Carbohydrate oxidoreductases

Oxidoreductases are attractive biocatalysts because of their intrinsic (enantio-)selectivity and catalytic efficiency, which are often difficult to achieve by conventional chemical approaches. The discovery of new oxidoreductases together with novel insights into their catalytic mechanisms will increase the biocatalytic potential for application of these enzymes.

Chapter 1 of this thesis gives an introduction to flavoprotein carbohydrate oxidoreductases. These enzymes are valuable biocatalysts that can be used for diagnostic applications and in the food- and drinks industry (van Hellemond et al., 2006). Up to now, most carbohydrate oxidoreductases have been isolated from fungi. Extensive genome analysis revealed that also plants are a potentially rich source of carbohydrate oxidoreductases. These enzymes are, for example, involved in maintenance of the plant cell wall, active defence through the production of hydrogen peroxide, and the biosynthesis of L-ascorbic acid (vitamin C).

This thesis describes the characterization and redesign of the vitamin C producing enzyme L-galactono-γ-lactone dehydrogenase (GALDH) from the model plant *Arabidopsis thaliana*. The results presented provide a firm basis for the design of suitable biocatalysts that can be used for the biotechnological production of vitamin C or other carbohydrates with modified properties, as an alternative for the currently applied chemical methods.

The VAO flavoprotein family

GALDH and its related enzymes belong the vanillyl-alcohol oxidase (VAO) family of flavoproteins. VAO-members have a characteristic two-domain folding topology with a conserved N-terminal FAD-binding domain and a less conserved C-terminal cap-domain that determines the substrate specificity. The active site is located at the interface of the domains (Fraaije et al., 1998). **Chapter 2** gives an overview of the growing VAO flavoprotein family, the catalytic properties of some recently discovered members are described. The VAO-fold is a versatile fold, allowing a wide variety of reactions to be catalyzed. The VAO family includes members that are involved in carbohydrate metabolism, lignin degradation, and the synthesis of antibiotics and plant alkaloids. A remarkable feature of the FAD-binding module of the VAO family is that it favors the covalent attachment of the flavin cofactor. Different covalent flavin-protein linkages can be identified within this family. Among the recently discovered members are the histidyl-FAD enzymes alditol oxidase (AldO) from *Streptomyces coelicolor* and eugenol oxidase (EUGO) from *Rhodococcus* sp. RHA1, the bi-covalent FAD enzyme chitooligosaccharide oxidase (ChitO) from *Fusarium graminearum*, and the non-covalently bound FAD enzyme galactonolactone oxidase from *Trypanosoma cruzi* (TcGAL).

AldO and ChitO are carbohydrate oxidases acting on carbohydrate alcohols and chitooligosaccharides, respectively. EUGO is a bacterial VAO-homolog that is active with 4-alkylphenols. TcGAL is a trypanosomal GALDH homolog that catalyzes the oxidation of both L-galactono-1,4-lactone and D-arabinono-1,4-lactone using cytochrome *c* as electron acceptor. The VAO family is a rapidly growing family, and the increasing availability of genome sequences suggests that many more members are to be discovered in the near future.

L-Galactono-γ-lactone dehydrogenase from *Arabidopsis thaliana*

GALDH is an essential enzyme for the plant, besides producing vitamin C, it is involved in the correct assembly of respiratory complex I and the proper functioning of plant mitochondria (Alhagdow et al., 2007; Pineau et al., 2008). Although GALDH was already isolated from the mitochondria of cauliflower florets in 1958 (Mapson and Breslow, 1958), up to now the biochemical properties of this enzyme remain poorly characterized. **Chapter 3** presents the first biochemical characterization of recombinant GALDH from the model plant *Arabidopsis thaliana*. We found that GALDH oxidizes, in addition to its natural substrate L-galactono-1,4-lactone, also the animal precursor L-gulono-1,4-lactone using cytochrome *c* as electron acceptor, while it reacts poorly with molecular oxygen. In a subsequent study (see chapter 4) we demonstrated that also D-arabinono-1,4-lactone is oxidized by GALDH at relatively high rate, but the enzyme has a relatively high K_m for this substrate, indicating that the length and stereochemistry of the side-chain is important for proper binding and aligning of the substrate.

Unlike most flavoprotein dehydrogenases, GALDH forms a flavin N5 sulfite adduct, and anaerobic photoreduction involves the transient stabilization of the red anionic flavin semiquinone. Both properties are indicative for the presence of positive charges in the active site (Lederer, 1978; Fraaije and Mattevi, 2000). Most aldonolactone oxidoreductases contain a histidyl-FAD as a covalently bound prosthetic group, GALDH lacks the histidine involved in covalent FAD binding, but contains a leucine instead (Leu56). Replacing Leu56 by His did not result in covalent flavinylation but revealed the importance of this residue for both FAD-binding and catalysis. The Leu56 variants showed remarkable differences in binding capacity of L-galactono-1,4-lactone and L-gulono-1,4-lactone and released their FAD-cofactor more easily than the wild-type enzyme. Covalent attachment of the flavin commonly requires the base-assisted activation of the FAD-cofactor, this base is likely to be absent from the GALDH sequence.

No crystal structure is available for the aldonolactone oxidoreductases, hence little information is available about the nature of the active site and reaction mechanism. In **Chapter 4** two residues in the active site of GALDH from *A. thaliana* were identified by

sequence comparisons with two VAO-members of known structures, AldO (Forneris et al., 2008) and cholesterol oxidase (CO) (Coulombe et al., 2001). Both AldO and CO contain a Glu-Arg pair in the active site that is conserved among the aldonolactone oxidoreductases. The function of the corresponding residues in recombinant GALDH (Glu386 and Arg388), was investigated by site-directed mutagenesis. Glu386 appears to be crucial for the binding of L-galactono-1,4-lactone and might act as a base in substrate activation. The E386D mutant is surprisingly active with L-gulono-1,4-lactone. Arg388 is less crucial for catalysis but is important for the stabilization of negative charge generated during flavin reduction.

The interaction of GALDH with its natural electron acceptor cytochrome *c* was investigated in **Chapter 5**. Electron transfer between GALDH and cytochrome *c* presumably involves the formation of a complex between the two redox partners. A surface engineering strategy was followed to identify the role of charged residues on the surface of GALDH. In total three patches of Lys and Glu residues in close proximity were identified and mutated to Ala. Though often applied as a tool to improve crystal quality (Derewenda, 2004), the surface mutations did not improve the crystallization properties of GALDH. Using heteronuclear nuclear magnetic resonance (NMR) spectroscopy transient complex formation was demonstrated between GALDH and isotopically enriched ferric yeast iso-1-cytochrome *c*. A K_d of 50 μM was detected for the wild-type GALDH – cytochrome *c* complex, and a K_d of 44 μM was found for the 9-fold surface mutant in complex with cytochrome *c*. The transient and dynamic nature of the complex allow a fast electron transfer. The cytochrome *c* binding map for the 9-fold surface mutant is very similar to wild-type GALDH. Moreover, binding maps of cytochrome *c* in the complexes with GALDH and other physiological and non-physiological partners are strikingly similar (Worrall et al., 2001; Crowley et al., 2002; Worrall et al., 2003; Volkov et al., 2005), suggesting that cytochrome *c* employs a conserved set of surface-exposed residues for the interactions with a variety of proteins. Interestingly, the interaction with cytochrome *c* remains intact after removing 9 charged residues at the GALDH protein surface.

Oxygen reactivity of GALDH

The oxygen reactivity of GALDH was addressed in the chapters 6 and 7, and provides an explanation why GALDH was designed by Nature as a dehydrogenase.

An oxidation-sensitive cysteine required for optimal vitamin C production by GALDH was identified in **Chapter 6**. Most aldonolactone oxidoreductases, including GUO, GALDH and ALO can be inactivated by thiol reactive compounds, suggesting the involvement of cysteine residues in catalysis. We found that GALDH can be inactivated by hydrogen peroxide due to specific oxidation of Cys340, located in the C-terminal cap domain. ESI-MS revealed that the

oxidation of Cys340 involves the sequential formation of sulfenic, sulfinic and sulfonic acid states. C340A and C340S GALDH variants are insensitive towards thiol oxidation, but exhibit a poor affinity for L-galactono-1,4-lactone. Electron paramagnetic resonance (EPR) spectroscopy of spin-labeled GALDH revealed that Cys340 has a buried location, and its estimated pK_a of 6.5 suggests a critical role for the thiolate anion in substrate recognition. We also found evidence for site-specific *S*-glutathionylation of Cys340, which may be involved in protecting GALDH against irreversible oxidation under oxidative stress conditions (Dalle-Donne et al., 2007). The sensitivity towards oxidative stress and the fact that plants produce high amounts of vitamin C could provide an explanation why GALDH is a dehydrogenase and not, like related aldonolactone oxidoreductases, a hydrogen peroxide producing oxidase. High levels of hydrogen peroxide will de-regulate the expression and functioning of ascorbate peroxidases and other thiol-modulated enzymes and stimulate ageing, senescence and cell death (Giorgio et al., 2007; Navrot et al., 2007; Ishikawa and Shigeoka, 2008). In addition, mitochondrial oxygen depletion by galactonolactone oxidase activity might affect respiration.

In **Chapter 7** we investigated the molecular determinants for the poor oxidase activity of GALDH. The oxygen reactivity of flavoproteins is poorly understood and may depend on multiple factors, like solvation of the active site, charge distributions and the existence of oxygen tunnels and gating mechanisms (Mattevi, 2006). In this chapter we present for the first time the rational design of a catalytically competent galactonolactone oxidase. By performing a structure-based family alignment approach (Joosten et al., 2008) we identified a gatekeeper residue in the VAO flavoprotein family that prevents dehydrogenases from acting as oxidases. Nearly all oxidases in the VAO family contain either a Gly or Pro at a structurally conserved position near the C4a locus of the isoalloxazine moiety of the flavin, whereas dehydrogenases prefer another residue at this position (Table S7.1). Interestingly, this residue is located at the *re*-face of the flavin, in contrast to the substrate binding site, which is on the *si*-face in VAO family members. Mutation of the corresponding residue in GALDH (Ala113→Gly) resulted in a strong increase in oxygen reactivity, while the cytochrome *c* reductase activity is retained. The activity of the A113G variant is comparable to that of other flavoprotein oxidases. The Ala113→Gly mutation does not alter the redox properties of the flavin, but merely creates space for molecular oxygen to reach and react with the reduced flavin. In wild-type GALDH Ala113 acts as a gatekeeper, preventing oxygen to access the isoalloxazine nucleus. The presence of such an oxygen access gate seems to be a key factor for the prevention of oxidase activity within the VAO family, and is absent in members that act as oxidases.

Galactonolactone oxidase from *Trypanosoma cruzi*

The trypanosomal parasites *Trypanosoma brucei*, and *T. cruzi* (the causative agents of African sleeping sickness and Chagas' disease, respectively) threat millions of people world-wide. Current treatments are unsatisfactory, since the available drugs have a limited efficacy and exhibit toxic side effects. Because humans lack the capacity to synthesize ascorbate, the trypanosomal enzymes involved in ascorbate biosynthesis are interesting targets for drug therapy.

In **Chapter 8** the aldonolactone oxidoreductase of *Trypanosoma cruzi*, is characterized. Galactonolactone oxidoreductase from *T. cruzi* (TcGAL) forms an interesting drug target as *T. cruzi* is unable to scavenge ascorbate from its environment (Logan et al., 2007). Because recombinant expression of TcGAL in *E. coli* yields mostly inactive inclusion bodies, an *in vitro* refolding method was designed using an AOT-isooctane reversed micelles system. Active protein was obtained only when the refolding was performed in the presence of a redox system consisting of reduced and oxidized glutathione and FAD, but not FMN. The requirement of FAD as redox active cofactor by TcGAL is in accordance with the presence of the PP-loop in its amino acid sequence, similar to other VAO family members (Fraaije et al., 1998). Refolded TcGAL exhibits native-like structure and is active with both L-galactono-1,4-lactone and D-arabinono-1,4-lactone. In line with the predicted space near the C4a-N5 locus of the flavin, the enzyme can use molecular oxygen as electron acceptor in addition to cytochrome *c* and 1,4-benzoquinone.

Outlook

Plants are the main vitamin C producers on earth. A detailed understanding of the biosynthesis of vitamin C is essential for future efforts to find alternative methods for the commercial production of this health-promoting compound. In this research we have characterized GALDH, the enzyme that completes vitamin C biosynthesis in plants. Several crucial amino acid residues involved in cofactor and substrate binding were identified and the enzyme was redesigned into variants with altered substrate and electron acceptor specificities. Furthermore, we found out why plant GALDH was designed by Nature as a dehydrogenase, and not, like related aldonolactone oxidoreductases, as an oxidase.

The crystal structure of GALDH or a related aldonolactone oxidoreductase could shed some more light on the active site residues involved in substrate binding and catalysis. Unfortunately, all our attempts to solve the GALDH crystal structure were fruitless due to a poor diffraction of the obtained crystals. Up to now, crystals were obtained for native GALDH, His-tagged GALDH, GALDH C340A, C340S and four GALDH surface mutants.

All crystals diffract to about 3.5-4 Å resolution, which is not enough to solve the structure (NGH Leferink, E Carpanelli, A Mattevi and WJH van Berkel, unpublished results). The availability of a GALDH inhibitor could help to obtain better crystals. Lycorine, a toxic plant alkaloid, was reported as an inhibitor of ascorbate biosynthesis in plants and animals, and as a specific inhibitor of GALDH (Imai et al., 1998). We did, however, not observe a clear inhibition of GALDH activity by lycorine (NGH Leferink, unpublished results). Alternatively, an aldonolactam might be a potential competitive inhibitor of GALDH. Initial efforts to synthesize 4-deoxy-L-galactono-1,4-lactam were not successful (NGH Leferink, unpublished results). The availability of an aldonolactone oxidoreductases crystal structure will also be beneficial for the design of a specific TcGAL inhibitor, a potential drug target for Chagas' disease.

There is an increasing pressure to develop alternative methods for the Reichstein process, a mostly chemical procedure used to produce the vast majority of the world vitamin C. Innovations in recombinant DNA technology, the availability of genome sequences and recent advances in protein engineering and synthetic biology may be exploited for the biotechnological production of vitamin C. An interesting alternative pathway that deserves more investigation is the production of natural vitamin C from pectin as starting material. Pectin is a carbohydrate polymer from plant cell walls that is rich in D-galacturonic acid, a recently identified alternative intermediate in the biosynthesis of vitamin C (Agius et al., 2003). Apples and citrus fruits are particularly rich in pectin, which is a left-over product after juice making. Through the sequential action of pectinases (Kashyap et al., 2001), galacturonate reductase (Agius et al., 2003) and aldonolactonase (Ishikawa et al., 2008), pectin can be converted to L-galactono-1,4-lactone, the final precursor towards vitamin C. More research is needed to determine the feasibility of this route.

In conclusion, the results provided in this thesis provide a firm basis for further structure-function studies on VAO-type flavoenzymes in general and the aldonolactone oxidoreductases in particular. The fact that it is possible to convert a dehydrogenase into a catalytically competent oxidase is of general relevance for the design of suitable biocatalysts because oxidases do not require expensive co-substrates or regeneration systems. The knowledge acquired in this research may be applied for the biotechnological production of vitamin C or other valuable carbohydrates.

References

Agius F, González-Lamothe R, Caballero JL, Muñoz-Blanco J, Botella MA, Valpuesta V (2003) Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. *Nat Biotechnol* **21**: 177-181

Alexeev I, Sultana A, Mäntsälä P, Niemi J, Schneider G (2007) Aclacinomycin oxidoreductase (AknOx) from the biosynthetic pathway of the antibiotic aclacinomycin is an unusual flavoenzyme with a dual active site. *Proc Natl Acad Sci U S A* **104**: 6170-6175

Alhagdow M, Mounet F, Gilbert L, Nunes-Nesi A, Garcia V, Just D, Petit J, Beauvoit B, Fernie AR, Rothan C, Baldet P (2007) Silencing of the mitochondrial ascorbate synthesizing enzyme L-galactono-1,4-lactone dehydrogenase (L-GalLDH) affects plant and fruit development in tomato. *Plant Physiol* **145**: 1408-1422

Aliverti A, Curti B, Vanoni MA (1999) Identifying and quantitating FAD and FMN in simple and in iron-sulur-containing flavoproteins. *In* SK Chapman, GA Reid, eds, Flavoprotein protocols. Humana Press, Totowa, pp 9-23

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389-3402

Amako K, Fujita K, Shimohata T-a, Hasegawa E, Kishimoto R, Goda K (2006) NAD^+-specific D-arabinose dehydrogenase and its contribution to erythroascorbic acid production in *Saccharomyces cerevisiae*. *FEBS Lett* **580**: 6428-6434

Arent S, Pye VE, Henriksen A (2008) Structure and function of plant acyl-CoA oxidases. *Plant Physiol Biochem* **46**: 292-301

Bae E, Bingman CA, Bitto E, Aceti DJ, Phillips GN, Jr. (2007) Crystal structure of *Arabidopsis thaliana* cytokinin dehydrogenase. *Proteins* **70**: 303-306

Baneyx F, Mujacic M (2004) Recombinant protein folding and misfolding in *Escherichia coli*. *Nat Biotechnol* **22**: 1399-1408

Bartoli CG, Pastori GM, Foyer CH (2000) Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. *Plant Physiol* **123**: 335-344

Baud F, Karlin S (1999) Measures of residue density in protein structures. *Proc Natl Acad Sci U S A* **96**: 12494-12499

Benson TE, Filman DJ, Walsh CT, Hogle JM (1995) An enzyme-substrate complex involved in bacterial cell wall biosynthesis. *Nat Struct Biol* **2**: 644-653

Bleeg HS, Christensen F (1982) Biosynthesis of ascorbate in yeast. Purification of L-galactono-1,4-lactone oxidase with properties different from mammalian L-gulonolactone oxidase. *Eur J Biochem* **127**: 391-396

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254

Brandes N, Schmitt S, Jakob U (2008) Thiol-based redox switches in eukaryotic proteins. *Antioxid Redox Signal* in press, doi:10.1089/ars.2008.2285

Brandsch R, Bichler V (1991) Autoflavinylation of apo6-hydroxy-D-nicotine oxidase. *J Biol Chem* **266**: 19056-19062

Brandsch R, Bichler V, Nagursky H (1987) Covalent flavinylation of 6-hydroxy-D-nicotine oxidase analyzed by partial deletions of the gene. *Eur J Biochem* **165**: 559-564

Brandt K, Thewes S, Overhage J, Priefert H, Steinbüchel A (2001) Characterization of the eugenol hydroxylase genes (*ehyA/ehyB*) from the new eugenol-degrading *Pseudomonas* sp. strain OPS1. *Appl Microbiol Biotechnol* **56**: 724-730

Burns JJ, Moltz A, Peyser P (1956) Missing step in guinea pigs required for the biosynthesis of L-ascorbic acid. *Science* **124**: 1148-1149

Carter CJ, Thornburg RW (2004) Tobacco nectarin V is a flavin-containing berberine bridge enzyme-like protein with glucose oxidase activity. *Plant Physiol* **134**: 460-469

Castro H, Tomás AM (2008) Peroxidases of trypanosomatids. *Antioxid Redox Signal* **10**: 1593-1606

Cavener DR (1992) GMC oxidoreductases. A newly defined family of homologous proteins with diverse catalytic activities. *J Mol Biol* **223**: 811-814

Chatterjee IB (1973) Evolution and the biosynthesis of ascorbic acid. *Science* **182**: 1271-1272

Chen L, Lyubimov AY, Brammer L, Vrielink A, Sampson NS (2008) The binding and release of oxygen and hydrogen peroxide are directed by a hydrophobic tunnel in cholesterol oxidase. *Biochemistry* **47**: 5368-5377

Collard F, Zhang J, Nemet I, Qanungo KR, Monnier VM, Yee VC (2008) Crystal structure of the deglycating enzyme fructosamine oxidase (amadoriase II). *J Biol Chem* **283**: 27007-27016

Coulombe R, Yue KQ, Ghisla S, Vrielink A (2001) Oxygen access to the active site of cholesterol oxidase through a narrow channel is gated by an Arg-Glu pair. *J Biol Chem* **276**: 30435-30441

Crowley PB, Carrondo MA (2004) The architecture of the binding site in redox protein complexes: implications for fast dissociation. *Proteins* **55**: 603-612

Crowley PB, Rabe KS, Worrall JAR, Canters GW, Ubbink M (2002) The ternary complex of cytochrome *f* and cytochrome *c*: identification of a second binding site and competition for plastocyanin binding. *ChemBioChem* **3**: 526-533

Cunane LM, Chen ZW, McIntire WS, Mathews FS (2005) *p*-Cresol methylhydroxylase: alteration of the structure of the flavoprotein subunit upon its binding to the cytochrome subunit. *Biochemistry* **44**: 2963-2973

Custers JHHV, Harrison SJ, Sela-Buurlage MB, van Deventer E, Lageweg W, Howe PW, van der Meijs PJ, Ponstein AS, Simons BH, Melchers LS, Stuiver MH (2004) Isolation and characterisation of a class of carbohydrate oxidases from higher plants, with a role in active defence. *Plant J* **39**: 147-160

Dalle-Donne I, Rossi R, Giustarini D, Colombo R, Milzani A (2007) *S*-glutathionylation in protein redox regulation. *Free Radic Biol Med* **43**: 883-898

Davey MW, Persiau G, Bauw G, Van Montagu M (1999) Direct measurement of ascorbic acid biosynthesis in *Arabidopsis* cell suspension culture using capillary electrophoresis. *J Chromatogr A* **853**: 381-389

De Bernardez Clark E, Schwarz E, Rudolph R (1999) Inhibition of aggregation side reactions during *in vitro* protein folding. *Methods Enzymol* **309**: 217-236

de Jong E, van Berkel WJH, van der Zwan RP, de Bont JAM (1992) Purification and characterization of vanillyl-alcohol oxidase from *Penicillium simplicissimum*. A novel aromatic alcohol oxidase containing covalently bound FAD. *Eur J Biochem* **208**: 651-657

Decker K, Brandsch R (1997) Determining covalent flavinylation. *Methods Enzymol* **280**: 413-423

Derewenda ZS (2004) Rational protein crystallization by mutational surface engineering. *Structure* **12**: 529-535

Derewenda ZS, Vekilov PG (2006) Entropy and surface engineering in protein crystallization. *Acta Crystallogr D Biol Crystallogr* **62**: 116-124

Dewanti AR, Xu Y, Mitra B (2004) Role of glycine 81 in (*S*)-mandelate dehydrogenase from *Pseudomonas putida* in substrate specificity and oxidase activity. *Biochemistry* **43**: 10692-10700

Dym O, Pratt EA, Ho C, Eisenberg D (2000) The crystal structure of D-lactate dehydrogenase, a peripheral membrane respiratory enzyme. *Proc Natl Acad Sci U S A* **97**: 9413-9418

Edmondson DE, De Francesco R (1991) Structure, synthesis, and physical properties of covalently bound flavins and 6- and 8- hydroxyflavins. *In* F Müller, ed, Chemistry and Biochemistry of Flavoenzymes, Vol I. CRC Press Inc, Boca Raton, Florida, pp 73-103

Eliceiri GL, Lai EK, McCay PB (1969) Gulonolactone oxidase. Solubilization, properties, and partial purification. *J Biol Chem* **244**: 2641-2645

Ellman GL (1959) Tissue sulfhydryl groups. *Arch Biochem Biophys* **82**: 70-77

Englard S, Seifter S (1986) The biochemical functions of ascorbic acid. *Annu Rev Nutr* **6**: 365-406

Eswar N, John B, Mirkovic N, Fiser A, Ilyin VA, Pieper U, Stuart AC, Marti-Renom MA, Madhusudhan MS, Yerkovich B, Sali A (2003) Tools for comparative protein structure modeling and analysis. *Nucleic Acids Res* **31**: 3375-3380

Forneris F, Heuts DPHM, Delvecchio M, Rovida S, Fraaije MW, Mattevi A (2008) Structural analysis of the catalytic mechanism and stereoselectivity in *Streptomyces coelicolor* alditol oxidase. *Biochemistry* **47**: 978-985

Fraaije MW, Mattevi A (2000) Flavoenzymes: diverse catalysts with recurrent features. *Trends Biochem Sci* **25**: 126-132

Fraaije MW, van Berkel WJH (2006) Flavin-containing oxidative biocatalysts. *In* RN Patel, ed, Biocatalysis in Biotechnological and Pharmaceutical Industries. Marcel & Dekker, New York, pp 181-202

Fraaije MW, van Berkel WJH, Benen JAE, Visser J, Mattevi A (1998) A novel oxidoreductase family sharing a conserved FAD-binding domain. *Trends Biochem Sci* **23**: 206-207

Fraaije MW, van den Heuvel RHH, van Berkel WJH, Mattevi A (1999) Covalent flavinylation is essential for efficient redox catalysis in vanillyl-alcohol oxidase. *J Biol Chem* **274**: 35514-35520

Fraaije MW, van den Heuvel RHH, van Berkel WJH, Mattevi A (2000) Structural analysis of flavinylation in vanillyl-alcohol oxidase. *J Biol Chem* **275**: 38654-38658

Frébortová J, Fraaije MW, Galuszka P, Šebela M, Peč P, Hrbáč J, Novák O, Bilyeu KD, English JT, Frébort I (2004) Catalytic reaction of cytokinin dehydrogenase: preference for quinones as electron acceptors. *Biochem J* **380**: 121-130

Furukawa H, Wieser M, Morita H, Sugio T, Nagasawa T (1998) Purification and characterization of eugenol dehydrogenase from *Pseudomonas fluorescens* E118. *Arch Microbiol* **171**: 37-43

Gatzek S, Wheeler GL, Smirnoff N (2002) Antisense suppression of L-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate synthesis and reveals light modulated L-galactose synthesis. *Plant J* **30**: 541-553

Gibson QH, Swoboda BE, Massey V (1964) Kinetics and mechanism of action of glucose oxidase. *J Biol Chem* **239**: 3927-3934

Giffhorn F (2000) Fungal pyranose oxidases: occurrence, properties and biotechnical applications in carbohydrate chemistry. *Appl Microbiol Biotechnol* **54**: 727-740

Giorgio M, Trinei M, Migliaccio E, Pelicci PG (2007) Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nat Rev Mol Cell Biol* **8**: 722-728

Ghisla S, Massey V (1989) Mechanisms of flavoprotein-catalyzed reactions. *Eur J Biochem* **181**: 1-17

Groen BW, de Vries S, Duine JA (1997) Characterization of hexose oxidase from the red seaweed *Chondrus crispus*. *Eur J Biochem* **244**: 858-861

Habeeb AFSA (1972) Reaction of protein sulfhydryl groups with Ellman's reagent. *Methods Enzymol* **25**: 457-464

Halada P, Leitner C, Sedmera P, Haltrich D, Volc J (2003) Identification of the covalent flavin adenine dinucleotide-binding region in pyranose 2-oxidase from *Trametes multicolor*. *Anal Biochem* **314**: 235-242

Hancock RD, Viola R (2001) The use of micro-organisms for L-ascorbic acid production: current status and future perspectives. *Appl Microbiol Biotechnol* **56**: 567-576

Hancock RD, Viola R (2002) Biotechnological approaches for L-ascorbic acid production. *Trends Biotechnol* **20**: 299-305

Harada Y, Shimizu M, Murakawa S, Takahashi T (1979) Identification of FAD of D-gluconolactone dehydrogenase: D-erythorbic acid producing enzyme of *Penicillium cyaneo-fulvum*. *Agr Biol Chem* **43**: 2635-2636

Hassan-Abdallah A, Zhao G, Jorns MS (2006) Role of the covalent flavin linkage in monomeric sarcosine oxidase. *Biochemistry* **45**: 9454-9462

Heazlewood JL, Howell KA, Millar AH (2003) Mitochondrial complex I from Arabidopsis and rice: orthologs of mammalian and fungal components coupled with plant-specific subunits. *Biochim Biophys Acta* **1604**: 159-169

Hefti MH, Milder FJ, Boeren S, Vervoort J, van Berkel WJH (2003[b]) A His-tag based immobilization method for the preparation and reconstitution of apoflavoproteins. *Biochim Biophys Acta* **1619**: 139-143

Hefti MH, Vervoort J, van Berkel WJH (2003[a]) Deflavination and reconstitution of flavoproteins. *Eur J Biochem* **270**: 4227-4242

Helgstrand M, Kraulis P, Allard P, Hard T (2000) Ansig for Windows: an interactive computer program for semiautomatic assignment of protein NMR spectra. *J Biomol NMR* **18**: 329-336

Henriksson G, Johansson G, Pettersson G (2000) A critical review of cellobiose dehydrogenases. *J Biotechnol* **78**: 93-113

Heuts DPHM, Janssen DB, Fraaije MW (2007[b]) Changing the substrate specificity of a chitooligosaccharide oxidase from *Fusarium graminearum* by model-inspired site-directed mutagenesis. *FEBS Lett* **581**: 4905-4909

Heuts DPHM, van Hellemond EW, Janssen DB, Fraaije MW (2007[a]) Discovery, characterization and kinetic analysis of an alditol oxidase from *Streptomyces coelicolor*. *J Biol Chem* **282**: 20283-20291

Heuts DPHM, Winter RT, Damsma GE, Janssen DB, Fraaije MW (2008) On the role of double covalent flavin binding in chitooligosaccharide oxidase from *Fusarium graminearum*. *Biochem J* **413**: 175-183

Hiraga K, Eto T, Yoshioka I, Oda K (1998) Molecular cloning and expression of a gene encoding a novel sorbitol oxidase from *Streptomyces* sp. H-7775. *Biosci Biotechnol Biochem* **62**: 347-353

Huang CH, Lai WL, Lee MH, Chen CJ, Vasella A, Tsai YC, Liaw SH (2005) Crystal structure of glucooligosaccharide oxidase from *Acremonium strictum*: A novel flavinylation of 6-*S*-cysteinyl, 8α-*N*1-histidyl FAD. *J Biol Chem* **280**: 38831-38838

Huang CH, Winkler A, Chen CL, Lai WL, Tsai YC, Macheroux P, Liaw SH (2008) Functional roles of the 6-*S*-cysteinyl, 8α-*N*1-histidyl FAD in glucooligosaccharide oxidase from *Acremonium strictum*. *J Biol Chem* **283**: 30900-30996

Huh WK, Kim ST, Yang KS, Seok YJ, Hah YC, Kang SO (1994) Characterisation of D-arabinono-1,4-lactone oxidase from *Candida albicans* ATCC 10231. *Eur J Biochem* **225**: 1073-1079

Huh WK, Lee BH, Kim ST, Kim YR, Rhie GE, Baek YW, Hwang CS, Lee JS, Kang SO (1998) D-Erythroascorbic acid is an important antioxidant molecule in *Saccharomyces cerevisiae*. *Mol Microbiol* **30**: 895-903

Imai T, Karita S, Shiratori G, Hattori M, Nunome T, Ôba K, Hirai M (1998) L-galactono-γ-lactone dehydrogenase from sweet potato: purification and cDNA sequence analysis. *Plant Cell Physiol* **39**: 1350-1358

Irigoín F, Cibils L, Comini MA, Wilkinson SR, Flohé L, Radi R (2008) Insights into the redox biology of *Trypanosoma cruzi*: Trypanothione metabolism and oxidant detoxification. *Free Radic Biol Med* **45**: 733-742

Ishikawa T, Dowdle J, Smirnoff N (2006) Progress in manipulating ascorbic acid biosynthesis and accumulation in plants. *Physiol Plant* **126**: 343–355

Ishikawa T, Nishikawa H, Gao Y, Sawa Y, Shibata H, Yabuta Y, Maruta T, Shigeoka S (2008) The pathway via D-galacturonate/L-galactonate is significant for ascorbate biosynthesis in *Euglena gracilis*: Identification and functional characterization of aldonolactonase. *J Biol Chem* **283**: 31133-31141

Ishikawa T, Shigeoka S (2008) Recent advances in ascorbate biosynthesis and the physiological significance of ascorbate peroxidase in photosynthesizing organisms. *Biosci Biotechnol Biochem* **72**: 1143-1154

Jacob C, Knight I, Winyard PG (2006) Aspects of the biological redox chemistry of cysteine: from simple redox responses to sophisticated signalling pathways. *Biol Chem* **387**: 1385-1397

Jin J, Mazon H, van den Heuvel RHH, Janssen DB, Fraaije MW (2007) Discovery of a eugenol oxidase from *Rhodococcus* sp. strain RHA1. *FEBS J* **274**: 2311-2321

Joosten H-J, Han Y, Niu W, Vervoort J, Dunaway-Mariano D, Schaap PJ (2008) Identification of fungal oxaloacetate hydrolyase within the isocitrate lyase/PEP mutase enzyme superfamily using a sequence marker-based method. *Proteins* **70**: 157-166

Joosten V, van Berkel WJH (2007) Flavoenzymes. *Curr Opin Chem Biol* **11**: 195-202

Kashyap DR, Vohra PK, Chopra S, Tewari R (2001) Applications of pectinases in the commercial sector: a review. *Bioresour Technol* **77**: 215-227

Kenney WC, Edmondson DE, Singer TP (1976) Identification of the covalently bound flavin of L-gulono-γ-lactone oxidase. *Biochem Biophys Res Commun* **71**: 1194-1200

Kenney WC, Edmondson DE, Singer TP, Nishikimi M, Noguchi E, Yagi K (1979) Identification of the covalently-bound flavin of L-galactonolactone oxidase from yeast. *FEBS Lett* **97**: 40-42

Kim J, Fuller JH, Kuusk V, Cunane L, Chen ZW, Mathews FS, McIntire WS (1995) The cytochrome subunit is necessary for covalent FAD attachment to the flavoprotein subunit of *p*-cresol methylhydroxylase. *J Biol Chem* **270**: 31202-31209

Kiuchi K, Nishikimi M, Yagi K (1982) Purification and characterization of L-gulonolactone oxidase from chicken kidney microsomes. *Biochemistry* **21**: 5076-5082

Klyachko NL, Levashov AV (2003) Bioorganic synthesis in reverse micelles and related systems. *Curr Opin Colloid Interface Sci* **8**: 179-186

Klyachko NL, Shchedrina VA, Efimov AV, Kazakov SV, Gazaryan IG, Kristal BS, Brown AM (2005) pH-dependent substrate preference of pig heart lipoamide dehydrogenase varies with oligomeric state: response to mitochondrial matrix acidification. *J Biol Chem* **280**: 16106-16114

Koetter JW, Schulz GE (2005) Crystal structure of 6-hydroxy-D-nicotine oxidase from *Arthrobacter nicotinovorans*. *J Mol Biol* **352**: 418-428

Köhling R, Woenckhaus J, Klyachko NL, Winter R (2002) Small-angle neutron scattering study of the effect of pressure on AOT - *n*-octane - water mesophases and the effect of α-chymotrypsin incorporation. *Langmuir* **18**: 8626-8632

Krauth-Siegel RL, Comini MA (2008) Redox control in trypanosomatids, parasitic protozoa with trypanothione-based thiol metabolism. *Biochim Biophys Acta* **1780**: 1236-1248

Kutchan TM, Dittrich H (1995) Characterization and mechanism of the berberine bridge enzyme, a covalently flavinylated oxidase of benzophenanthridine alkaloid biosynthesis in plants. *J Biol Chem* **270**: 24475-24481

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685

Lederer F (1978) Sulfite binding to a flavodehydrogenase, cytochrome b_2 from baker's yeast. *Eur J Biochem* **88**: 425-431

Leferink NGH, Fraaije MW, Joosten H-J, Schaap PJ, Mattevi A, van Berkel WJH (2009) Identification of a gatekeeper residue that prevents dehydrogenases from acting as oxidases. *J Biol Chem* **284**: 4392-4397

Leferink NGH, Heuts DPHM, Fraaije MW, van Berkel WJH (2008[a]) The growing VAO flavoprotein family. *Arch Biochem Biophys* **474**: 292-301

Leferink NGH, van den Berg WAM, van Berkel WJH (2008[b]) L-Galactono-γ-lactone dehydrogenase from *Arabidopsis thaliana*, a flavoprotein involved in vitamin C biosynthesis *FEBS J* **275**: 713-726

Leferink NGH, van den Berg WAM, van Berkel WJH (2008[c]) Oxygen reactivity of L-galactono-1,4-lactone dehydrogenase. *In* S Frago, C Gómez-Moreno, M Medina, eds, Flavins and Flavoproteins 2008. Prensas Universitarias de Zaragoza, Zaragoza, pp 75-80

Levashov AV, Klyachko NL (2001) Reverse micellar systems. *In* EN Vulfson, PJ Halling, HL Holland, eds, Methods in Biotechnology, Vol 15: Enzymes in Nonaqueous Solvents: Methods and Protocols. Humana Press, Totowa, NJ, pp 575-586

Li YS, Ho JY, Huang CC, Lyu SY, Lee CY, Huang YT, Wu CJ, Chan HC, Huang CJ, Hsu NS, Tsai MD, Li TL (2007) A unique flavin mononucleotide-linked primary alcohol oxidase for glycopeptide A40926 maturation. *J Am Chem Soc* **129**: 13384-13385

Lin SF, Yang TY, Inukai T, Yamasaki M, Tsai YC (1991) Purification and characterization of a novel glucooligosaccharide oxidase from *Acremonium strictum* T1. *Biochim Biophys Acta* **1118**: 41-47

Linster CL, Gomez TA, Christensen KC, Adler LN, Young BD, Brenner C, Clarke SG (2007) Arabidopsis VTC2 encodes a GDP-L-galactose phosphorylase, the last unknown enzyme in the Smirnoff-Wheeler pathway to ascorbic acid in plants. *J Biol Chem* **282**: 18879–18885

Linster CL, van Schaftingen E (2007) Vitamin C. Biosynthesis, recycling and degradation in mammals. *FEBS J* **274**: 1-22

Logan FJ, Taylor MC, Wilkinson SR, Kaur H, Kelly JM (2007) The terminal step in vitamin C biosynthesis in *Trypanosoma cruzi* is mediated by a FMN-dependent galactonolactone oxidase. *Biochem J* **407**: 419-426

Lorence A, Chevone BI, Mendes P, Nessler CL (2004) *myo*-Inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. *Plant Physiol* **134**: 1200-1205

Loscos J, Matamoros MA, Becana M (2008) Ascorbate and homoglutathione metabolism in common bean nodules under stress conditions and during natural senescence. *Plant Physiol* **146**: 1282-1292

Macheroux P (1999) UV-visible spectroscopy as a tool to study flavoproteins. *In* SK Chapman, GA Reid, eds, Flavoprotein protocols. Humana Press, Totowa, pp 1-7

Mackenzie J, Pedersen L, Arent S, Henriksen A (2006) Controlling electron transfer in acyl-CoA oxidases and dehydrogenases - a structural view. *J Biol Chem* **284**: 31012-31020

Malito E, Coda A, Bilyeu KD, Fraaije MW, Mattevi A (2004) Structures of michaelis and product complexes of plant cytokinin dehydrogenase: implications for flavoenzyme catalysis. *J Mol Biol* **341**: 1237-1249

Mandal AK, Woodi M, Sood V, Krishnaswamy PR, Rao A, Ballal S, Balaram P (2007) Quantitation and characterization of glutathionyl haemoglobin as an oxidative stress marker in chronic renal failure by mass spectrometry. *Clin Biochem* **40**: 986-994

Mapson LW, Breslow E (1958) Biological synthesis of ascorbic acid: L-galactono-γ-lactone dehydrogenase. *Biochem J* **68**: 395-406

Mapson LW, Isherwood FA, Chen YT (1954) Biological synthesis of L-ascorbic acid: the conversion of L-galactono-γ-lactone into L-ascorbic acid by plant mitochondria. *Biochem J* **56**: 21-28

Margoliash E, Frohwirt N (1959) Spectrum of horse-heart cytochrome *c*. *Biochem J* **71**: 570-572

Massey V (1990) A simple method for the determination of redox potentials. *In* B Curti, S Ronchi, G Zanetti, eds, Flavins and Flavoproteins. Walter de Gruyter & Co, Berlin and New York, pp 59-66

Massey V (1994) Activation of molecular oxygen by flavins and flavoproteins. *J Biol Chem* **269**: 22459-22462

Massey V, Müller F, Feldberg R, Schuman M, Sullivan PA, Howell LG, Mayhew SG, Matthews RG, Foust GP (1969) The reactivity of flavoproteins with sulfite. Possible relevance to the problem of oxygen reactivity. *J Biol Chem* **244**: 3999-4006

Mathews FS, Chen ZW, Bellamy HD, McIntire WS (1991) Three-dimensional structure of *p*-cresol methylhydroxylase (flavocytochrome *c*) from *Pseudomonas putida* at 3.0-A resolution. *Biochemistry* **30**: 238-247

Mattevi A (2006) To be or not to be an oxidase: challenging the oxygen reactivity of flavoenzymes. *Trends Biochem Sci* **31**: 276-283

Mattevi A, Fraaije MW, Mozzarelli A, Olivi L, Coda A, van Berkel WJH (1997) Crystal structures and inhibitor binding in the octameric flavoenzyme vanillyl-alcohol oxidase: the shape of the active-site cavity controls substrate specificity. *Structure* **5**: 907-920

Mauch L, Bichler V, Brandsch R (1989) Site-directed mutagenesis of the FAD-binding histidine of 6-hydroxy-D-nicotine oxidase. Consequences on flavinylation and enzyme activity. *FEBS Lett.* **257**: 86-88

McIntire W, Edmondson DE, Hopper DJ, Singer TP (1981) 8α-(*O*-Tyrosyl)flavin adenine dinucleotide, the prosthetic group of bacterial *p*-cresol methylhydroxylase. *Biochemistry* **20**: 3068-3075

McIntire W, Hopper DJ, Singer TP (1985) *p*-Cresol methylhydroxylase. Assay and general properties. *Biochem J* **228**: 325-335

Mewies M, McIntire WS, Scrutton NS (1998) Covalent attachment of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to enzymes: the current state of affairs. *Protein Sci* **7**: 7-20

Millar AH, Mittova V, Kiddle G, Heazlewood JL, Bartoli CG, Theodoulou FL, Foyer CH (2003) Control of ascorbate synthesis by respiration and its implications for stress responses. *Plant Physiol* **133**: 443-447

Möhler H, Brühmüller M, Decker K (1972) Covalently bound flavin in D-6-hydroxynicotine oxidase from *Arthrobacter oxidans*. Identification of the 8α-(*N*-3-histidyl)-riboflavin-linkage between FAD and apoenzyme. *Eur J Biochem* **29**: 152-155

Morar AS, Kakouras D, Young GB, Boyd J, Pielak GJ (1999) Expression of ^{15}N-labeled eukaryotic cytochrome *c* in *Escherichia coli*. *J Biol Inorg Chem* **4**: 220-222

Mowat CG, Beaudoin I, Durley RC, Barton JD, Pike AD, Chen ZW, Reid GA, Chapman SK, Mathews FS, Lederer F (2000) Kinetic and crystallographic studies on the active site Arg289Lys mutant of flavocytochrome b_2 (yeast L-lactate dehydrogenase). *Biochemistry* **39**: 3266-3275

Müller F (1991) Nuclear magnetic resonance studies of flavoproteins. *In* F Müller, ed, Chemistry and Biochemistry of Flavoenzymes, Vol III. CRC Press, Boca Raton, FL, USA, pp 557-595

Mutsuda M, Ishikawa T, Takeda T, Shigeoka S (1995) Subcellular localization and properties of L-galactono-γ-lactone dehydrogenase in spinach leaves. *Biosci Biotechnol Biochem* **59**: 1983-1984

Nagursky H, Bichler V, Brandsch R (1988) Phospho*enol*pyruvate-dependent flavinylation of 6-hydroxy-D-nicotine oxidase. *Eur J Biochem* **177**: 319-325

Nakagawa H, Asano A (1970) Ascorbate synthesizing system in rat liver microsomes. I. Gulonolactone-reducible pigment as a prosthetic group of gulonolactone oxidase. *J Biochem (Tokyo)* **68**: 737-746

Navrot N, Rouhier N, Gelhaye E, Jacquot J-P (2007) Reactive oxygen species generation and antioxidant systems in plant mitochondria. *Physiol Plant* **129**: 185-195

Niesen FH, Koch A, Lenski U, Harttig U, Roske Y, Heinemann U, Hofmann KP (2008) An approach to quality management in structural biology: biophysical selection of proteins for successful crystallization. *J Struct Biol* **162**: 451-459

Nishikimi M (1979) L-Gulono-γ-lactone oxidase (rat and goat liver). *Methods Enzymol* **62**: 24-30

Nishikimi M, Fukuyama R, Minoshima S, Shimizu N, Yagi K (1994) Cloning and chromosomal mapping of the human nonfunctional gene for L-gulono-γ-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man. *J Biol Chem* **269**: 13685-13688

Nishikimi M, Noguchi E, Yagi K (1978) Occurrence in yeast of L-galactonolactone oxidase which is similar to a key enzyme for ascorbic acid biosynthesis in animals, L-gulonolactone oxidase. *Arch Biochem Biophys* **191**: 479-486

Nishikimi M, Tolbert BM, Udenfriend S (1976) Purification and characterization of L-gulono-γ-lactone oxidase from rat and goat liver. *Arch Biochem Biophys* **175**: 427-435

Noctor G, De Paepe R, Foyer CH (2007) Mitochondrial redox biology and homeostasis in plants. *Trends Plant Sci* **12**: 125-134

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* **49**: 249-279

Ôba K, Ishikawa S, Nishikawa M, Mizuno H, Yamamoto T (1995) Purification and properties of L-galactono-γ-lactone dehydrogenase, a key enzyme for ascorbic acid biosynthesis, from sweet potato roots. *J Biochem (Tokyo)* **117**: 120-124

Østergaard J, Persiau G, Davey MW, Bauw G, Van Montagu M (1997) Isolation of a cDNA coding for L-galactono-γ-lactone dehydrogenase, an enzyme involved in the biosynthesis of ascorbic acid in plants. Purification, characterization, cDNA cloning, and expression in yeast. *J Biol Chem* **272**: 30009-30016

Ould Boubacar AK, Pethe S, Mahy JP, Lederer F (2007) Flavocytochrome b_2: reactivity of its flavin with molecular oxygen. *Biochemistry* **46**: 13080-13088

Pastore A, Mozzi AF, Tozzi G, Gaeta LM, Federici G, Bertini E, Lo Russo A, Mannucci L, Piemonte F (2003) Determination of glutathionyl-hemoglobin in human erythrocytes by cation-exchange high-performance liquid chromatography. *Anal Biochem* **312**: 85-90

Pileni M-P, Zemb T, Petit C (1985) Solubilization by reverse micelles: solute localization and structure perturbation. *Chem Phys Lett* **118**: 414-420

Pineau B, Layoune O, Danon A, De Paepe R (2008) L-galactono-1,4-lactone dehydrogenase is required for the accumulation of plant respiratory complex I. *J Biol Chem* **283**: 32500-32505

Piubelli L, Pedotti M, Molla G, Feindler-Boeckh S, Ghisla S, Pilone MS, Pollegioni L (2008) On the oxygen reactivity of flavoprotein oxidases: An oxygen access tunnel and gate in *Brevibacterium sterolicum* cholesterol oxidase. *J Biol Chem* **283**: 24738-24747

Pollegioni L, Rosini E, Pilone MS, Orru R, Molla G (2008) Modulation of the oxygen affinity of D-amino acid oxidase: production of evolved variants for a cancer enzyme therapy. *In* S Frago, C Gómez-Moreno, M Medina, eds, Flavins and Flavoproteins 2008. Prensas Universitarias de Zaragoza, Zaragoza, pp 497-502

Pollock WB, Rosell FI, Twitchett MB, Dumont ME, Mauk AG (1998) Bacterial expression of a mitochondrial cytochrome *c*. Trimethylation of Lys72 in yeast iso-1-cytochrome *c* and the alkaline conformational transition. *Biochemistry* **37**: 6124-6131

Poole LB, Karplus PA, Claiborne A (2004) Protein sulfenic acids in redox signaling. *Annu Rev Pharmacol Toxicol* **44**: 325-347

Priefert H, Overhage J, Steinbüchel A (1999) Identification and molecular characterization of the eugenol hydroxylase genes (*ehyA/ehyB*) of *Pseudomonas* sp. strain HR199. *Arch Microbiol* **172**: 354-363

Puskas F, Braun L, Csala M, Kardon T, Marcolongo P, Benedetti A, Mandl J, Banhegyi G (1998) Gulonolactone oxidase activity-dependent intravesicular glutathione oxidation in rat liver microsomes. *FEBS Lett* **430**: 293-296

Rand T, Qvist KB, Walter CP, Poulsen CH (2006) Characterization of the flavin association in hexose oxidase from *Chondrus crispus*. *FEBS J* **273**: 2693-2703

Razeto A, Mattiroli F, Carpanelli E, Aliverti A, Pandini V, Coda A, Mattevi A (2007) The crucial step in ether phospholipid biosynthesis: structural basis of a noncanonical reaction associated with a peroxisomal disorder. *Structure* **15**: 683-692

Roth JP, Klinman JP (2003) Catalysis of electron transfer during activation of O_2 by the flavoprotein glucose oxidase. *Proc Natl Acad Sci U S A* **100**: 62-67

Sakono M, Kawashima YM, Ichinose H, Maruyama T, Kamiya N, Goto M (2004) Direct refolding of inclusion bodies using reversed micelles. *Biotechnol Prog* **20**: 1783-1787

Sali A, Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* **234**: 779-815

Salusjärvi T, Kalkkinen N, Miasnikov AN (2004) Cloning and characterization of gluconolactone oxidase of *Penicillium cyaneo-fulvum* ATCC 10431 and evaluation of its use for production of D-erythorbic acid in recombinant *Pichia pastoris*. *Appl Environ Microbiol* **70**: 5503-5510

Saraste M (1999) Oxidative phosphorylation at the fin de siècle. *Science* **283**: 1488-1493

Schiffer A, Fritz G, Kroneck PM, Ermler U (2006) Reaction mechanism of the iron-sulfur flavoenzyme adenosine-5'-phosphosulfate reductase based on the structural characterization of different enzymatic states. *Biochemistry* **45**: 2960-2967

Siendones E, González-Reyes JA, Santos-Ocaña C, Navas P, Córdoba F (1999) Biosynthesis of ascorbic acid in kidney bean. L-galactono-γ-lactone dehydrogenase is an intrinsic protein located at the mitochondrial inner membrane. *Plant Physiol* **120**: 907-912

Simpson C, Jordaan J, Gardiner NS, Whiteley C (2006) Isolation, purification and characterization of a novel glucose oxidase from *Penicillium* sp. CBS 120262 optimally active at neutral pH. *Protein Expr Purif* **51**: 260-266

Sirikantaramas S, Morimoto S, Shoyama Y, Ishikawa Y, Wada Y, Taura F (2004) The gene controlling marijuana psychoactivity: molecular cloning and heterologous expression of Δ^1-tetrahydrocannabinolic acid synthase from *Cannabis sativa* L. *J Biol Chem* **279**: 39767-39774

Smirnoff N (2001) L-ascorbic acid biosynthesis. *Vitam Horm* **61**: 241-266

Smirnoff N, Wheeler GL (2000) Ascorbic acid in plants: biosynthesis and function. *Crit Rev Biochem Mol Biol* **35**: 291-314

Smith AG, Croft MT, Moulin M, Webb ME (2007) Plants need their vitamins too. *Curr Opin Plant Biol* **10**: 266-275

Stankovich MT (1991) Redox properties of flavins and flavoproteins. *In* F Müller, ed, Chemistry and Biochemistry of Flavoenzymes, Vol I. CRC Press, Boca Raton, FL, USA, pp 401-425

Sucharitakul J, Prongjit M, Haltrich D, Chaiyen P (2008) Detection of a C4a-hydroperoxyflavin intermediate in the reaction of a flavoprotein oxidase. *Biochemistry* **47**: 8485-8490

Sugisawa T, Ojima S, Matzinger PK, Hoshino T (1995) Isolation and characterization of a new vitamin C producing enzyme (L-gulono-γ-lactone dehydrogenase) of bacterial origin. *Biosci Biotechnol Biochem* **59**: 190-195

Sun W, Williams CH, Jr., Massey V (1996) Site-directed mutagenesis of glycine 99 to alanine in L-lactate monooxygenase from *Mycobacterium smegmatis*. *J Biol Chem* **271**: 17226-17233

Tahallah N, Pinkse M, Maier CS, Heck AJR (2001) The effect of the source pressure on the abundance of ions of noncovalent protein assemblies in an electrospray ionization orthogonal time-of-flight instrument. *Rapid Commun Mass Spectrom* **15**: 596-601

Takahashi T, Yamashita H, Kato E, Mitsumoto M, Murakawa S (1976) Purification and some properties of D-glucono-γ-lactone dehydrogenase: D-erythorbic acid producing enzyme of *Penicillium cyaneo-fulvum*. *Agr Biol Chem* **40**: 121-129

Tamaoki M, Mukai F, Asai N, Nakajima N, Kubo A, Aono M, Saji H (2003) Light-controlled expression of a gene encoding L-galactono-γ-lactone dehydrogenase which affects ascorbate pool size in *Arabidopsis thaliana*. *Plant Sci* **164**: 1111-1117

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673-4680

Trickey P, Wagner MA, Jorns MS, Mathews FS (1999) Monomeric sarcosine oxidase: structure of a covalently flavinylated amine oxidizing enzyme. *Structure* **7**: 331-345

Ubbink M, Bendall DS (1997) Complex of plastocyanin and cytochrome *c* characterized by NMR chemical shift analysis. *Biochemistry* **36**: 6326-6335

Valenti D, Vacca RA, de Pinto MC, De Gara L, Marra E, Passarella S (2007) In the early phase of programmed cell death in Tobacco Bright Yellow 2 cells the mitochondrial adenine nucleotide translocator, adenylate kinase and nucleoside diphosphate kinase are impaired in a reactive oxygen species-dependent manner. *Biochim Biophys Acta* **1767**: 66-78

Valpuesta V, Botella MA (2004) Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. *Trends Plant Sci* **9**: 573-577

van Berkel WJH, Benen JAE, Eppink MHM, Fraaije MW (1999) Flavoprotein kinetics. *In* SK Chapman, GA Reid, eds, Flavoprotein protocols. Humana Press, Totowa, pp 61-85

van Berkel WJH, Kamerbeek NM, Fraaije MW (2006) Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. *J Biotechnol* **124**: 670-689

van Berkel WJH, Müller F (1987) The elucidation of the microheterogeneity of highly purified *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* by various biochemical techniques. *Eur J Biochem* **167**: 35-46

van den Heuvel RHH, Fraaije MW, Mattevi A, van Berkel WJH (2000) Asp-170 is crucial for the redox properties of vanillyl-alcohol oxidase. *J Biol Chem* **275**: 14799-14808

van Hellemond EW, Leferink NGH, Heuts DPHM, Fraaije MW, van Berkel WJH (2006) Occurrence and biocatalytic potential of carbohydrate oxidases. *Adv Appl Microbiol* **60**: 17-54

Vinogradov AA, Kudryashova EV, Levashov AV, van Dongen WMAM (2003) Solubilization and refolding of inclusion body proteins in reverse micelles. *Anal Biochem* **320**: 234-238

Volkov AN, Ferrari D, Worrall JAR, Bonvin AMJJ, Ubbink M (2005) The orientations of cytochrome *c* in the highly dynamic complex with cytochrome b_5 visualized by NMR and docking using HADDOCK. *Protein Sci* **14**: 799-811

Vos K, Laane C, Weijers SR, van Hoek A, Veeger C, Visser AJWG (1987) Time-resolved fluorescence and circular dichroism of porphyrin cytochrome *c* and Zn-porphyrin cytochrome *c* incorporated in reversed micelles. *Eur J Biochem* **169**: 259-268

Wheeler GL, Jones MA, Smirnoff N (1998) The biosynthetic pathway of vitamin C in higher plants. *Nature* **393**: 365-369

Wilkinson SR, Obado SO, Mauricio IL, Kelly JM (2002) *Trypanosoma cruzi* expresses a plant-like ascorbate-dependent hemoperoxidase localized to the endoplasmic reticulum. *Proc Natl Acad Sci U S A* **99**: 13453-13458

Wilkinson SR, Prathalingam SR, Taylor MC, Horn D, Kelly JM (2005) Vitamin C biosynthesis in trypanosomes: A role for the glycosome. *Proc Natl Acad Sci U S A* **102**: 11645-11650

Winkler A, Hartner F, Kutchan TM, Glieder A, Macheroux P (2006) Biochemical evidence that berberine bridge enzyme belongs to a novel family of flavoproteins containing a bi-covalently attached FAD cofactor. *J Biol Chem* **281**: 21276-21285

Winkler A, Kutchan TM, Macheroux P (2007) 6-*S*-cysteinylation of bi-covalently attached FAD in berberine bridge enzyme tunes the redox potential for optimal activity. *J Biol Chem* **282**: 24437-24443

Winter RT, Heuts DPHM, Popken P, van Bloois E, Fraaije MW (2008) Exploring the oxygen reactivity of alditol oxidase from *Streptomyces coelicolor*. *In* S Frago, C Gómez-Moreno, M Medina, eds, Flavins and Flavoproteins 2008. Prensas Universitarias de Zaragoza, Zaragoza, pp 237-242

Witt S, Singh M, Kalisz HM (1998) Structural and kinetic properties of nonglycosylated recombinant *Penicillium amagasakiense* glucose oxidase expressed in *Escherichia coli*. *Appl Environ Microbiol* **64**: 1405-1411

Witteveen CFB, Veenhuis M, Visser J (1992) Localization of glucose oxidase and catalase activities in *Aspergillus niger*. *Appl Environ Microbiol* **58**: 1190-1194

Wolucka BA, Communi D (2006) *Mycobacterium tuberculosis* possesses a functional enzyme for the synthesis of vitamin C, L-gulono-1,4-lactone dehydrogenase. *FEBS J* **273**: 4435-4445

Wolucka BA, van Montagu M (2003) GDP-mannose 3',5'-epimerase forms GDP-L-gulose, a putative intermediate for the *de novo* biosynthesis of vitamin C in plants. *J Biol Chem* **278**: 47483-47490

Worrall JAR, Kolczak U, Canters GW, Ubbink M (2001) Interaction of yeast iso-1-cytochrome *c* with cytochrome *c* peroxidase investigated by [^{15}N, ^{1}H] heteronuclear NMR spectroscopy. *Biochemistry* **40**: 7069-7076

Worrall JAR, Reinle W, Bernhardt R, Ubbink M (2003) Transient protein interactions studied by NMR spectroscopy: the case of cytochrome *c* and adrenodoxin. *Biochemistry* **42**: 7068-7076

Wu X, Takahashi M, Chen SG, Monnier VM (2000) Cloning of amadoriase I isoenzyme from *Aspergillus* sp.: evidence of FAD covalently linked to Cys342. *Biochemistry* **39**: 1515-1521

Xu F, Golightly EJ, Fuglsang CC, Schneider P, Duke KR, Lam L, Christensen S, Brown KM, Jorgensen CT, Brown SH (2001) A novel carbohydrate:acceptor oxidoreductase from *Microdochium nivale*. *Eur J Biochem* **268**: 1136-1142

Yabuta Y, Mieda T, Rapolu M, Nakamura A, Motoki T, Maruta T, Yoshimura K, Ishikawa T, Shigeoka S (2007) Light regulation of ascorbate biosynthesis is dependent on the photosynthetic electron transport chain but independent of sugars in *Arabidopsis*. *J Exp Bot* **58**: 2661-2671

Xu X, Reinle W, Hannemann F, Konarev PV, Svergun DI, Bernhardt R, Ubbink M (2008) Dynamics in a pure encounter complex of two proteins studied by solution scattering and paramagnetic NMR spectroscopy. **130:** 6395-6403

Yabuta Y, Yoshimura K, Takeda T, Shigeoka S (2000) Molecular characterization of tobacco mitochondrial L-galactono-γ-lactone dehydrogenase and its expression in *Escherichia coli*. *Plant Cell Physiol* **41**: 666-675

Yamashita M, Omura H, Okamoto E, Furuya Y, Yabuuchi M, Fukahi K, Murooka Y (2000) Isolation, characterization, and molecular cloning of a thermostable xylitol oxidase from *Streptomyces* sp. IKD472. *J Biosci Bioeng* **89**: 350-360

Zeng J, Liu Y, Wu L, Li D (2007) Mutation of Tyr375 to Lys375 allows medium-chain acyl-CoA dehydrogenase to acquire acyl-CoA oxidase activity. *Biochim Biophys Acta* **1774**: 1628-1634

Zhao G, Bruckner RC, Jorns MS (2008) Identification of the oxygen activation site in monomeric sarcosine oxidase: role of Lys265 in catalysis. *Biochemistry* **47**: 9124-9135

Samenvatting

Elektronenoverdrachts- of redoxreacties spelen een belangrijke rol in allerlei biochemische processen. Een redoxreactie is een chemisch proces waarbij een stof elektronen afstaat, waardoor deze wordt geoxideerd, en de afgegeven elektronen vervolgens worden opgenomen door een andere stof, die daardoor wordt gereduceerd. Dergelijke reacties worden in de natuur versneld door bepaalde biokatalysatoren, de zogenaamde redoxenzymen. Redoxenzymen bevatten een metaal ion of organische cofactor in hun reactiecentrum om de elektronenoverdrachtsreactie mogelijk te maken. Enzymen met een flavine mononucleotide (FMN) of flavine adenine dinucleotide (FAD) cofactor worden flavoenzymen genoemd. Flavoenzymen worden gekenmerkt door hun gele kleur (flavus betekent geel in het Latijn), en zijn betrokken bij een groot aantal belangrijke biochemische processen waaronder biosynthese, energie en licht productie, eiwitvouwing, ontgifting, geprogrammeerde celdood en DNA herstel. Flavoenzymen zijn ook vaak geschikt voor synthese doeleinden omdat hun katalytische efficiëntie en selectiviteit superieur zijn aan conventionele chemische methoden. Door het vinden van nieuwe flavoenzymen en een verbeterd inzicht in de werking ervan, zal het toepassen van deze enzymen in de toekomst makkelijker worden.

Suiker oxidases

Hoofdstuk 1 van dit proefschrift geeft een introductie over flavine-afhankelijke suiker oxidases. Suiker oxidases zijn waardevolle enzymen die kunnen worden gebruikt in allerlei diagnostische toepassingen en in de levensmiddelenindustrie. De meeste suiker oxidases zijn tot op heden geïsoleerd uit schimmels, maar een uitgebreide genoomanalyse liet zien dat ook planten een rijke bron zijn voor deze enzymen. Suiker oxidases zorgen o.a. voor de stevigheid van de plantencelwand, de bescherming tegen ziekteverwekkers en de aanmaak van vitamine C.

Vitamine C of ascorbaat is een belangrijk suikerderivaat dat werkzaam is als antioxidant, redox buffer en enzym cofactor. De meeste organismen kunnen zelf vitamine C aanmaken, mensen hebben deze eigenschap echter verloren en zijn afhankelijk van hun dieet voor de dagelijkse vitamine C behoefte. Een langdurig gebrek aan vitamine C kan scheurbuik veroorzaken, een ziekte die vroeger veel voorkwam bij zeelieden. Synthetisch vitamine C wordt tegenwoordig veel toegepast als conserveermiddel en antioxidant in de levensmiddelenindustrie. Daarnaast wordt dit vitamine steeds vaker toegevoegd aan diervoeding en cosmetische producten. Voor het ontwikkelen van milieuvriendelijke biotechnologische methoden om vitamine C te produceren is het noodzakelijk om

gedetailleerde kennis te hebben van de biosynthese van vitamine C en de enzymen die daarbij zijn betrokken.

In dit promotieonderzoek is het vitamine C producerende flavoenzym galactonolacton dehydrogenase (GALDH) uit de model plant *Arabidopsis thaliana* (de zandraket) bestudeerd. De kennis opgedaan in dit onderzoek kan worden gebruikt voor het ontwikkelen van biokatalysatoren die kunnen worden toegepast voor de biotechnologische productie van vitamine C of andere koolhydraten met nieuwe eigenschappen.

De VAO familie

GALDH en gerelateerde enzymen behoren tot de vanillyl-alcohol oxidase (VAO) familie van flavoenzymen. Leden van de VAO familie hebben een karakteristieke driedimensionale structuur bestaande uit twee domeinen, een sterk geconserveerd FAD-bindingsdomein en een niet geconserveerd substraat-bindingsdomein. Het reactiecentrum bevindt zich op het grensvlak van de twee domeinen. **Hoofdstuk 2** geeft een overzicht van de VAO familie en de katalytische eigenschappen van enkele recent ontdekte familieleden worden beschreven. De karakteristieke structuur van de VAO familie maakt het mogelijk veel verschillende omzettingen te katalyseren. De VAO familie bevat enzymen die betrokken zijn bij het suikermetabolisme, de afbraak van lignine en de synthese van antibiotica en alkaloïden. Een opmerkelijke eigenschap van het FAD-bindingsdomein van de VAO familie is de voorkeur voor covalente binding van de flavine cofactor. Tot de recent ontdekte familieleden behoren de mono-covalente flavoenzymen alditol oxidase uit *Streptomyces coelicolor* en eugenol oxidase uit *Rhodococcus* sp. RHA1, het bi-covalente flavoenzym chito-oligosaccharide oxidase uit *Fusarium graminearum*, en het niet-covalent gebonden flavine bevattende galactonolacton oxidase uit *Trypanosoma cruzi*. De VAO familie bestaat momenteel uit ongeveer vijftig gekarakteriseerde enzymen, en de verwachting is dat met de toenemende beschikbaarheid van genoom sequenties er in de komende jaren nog veel meer familieleden zullen worden ontdekt.

Galactonolacton dehydrogenase uit *Arabidopsis thaliana*

GALDH is een essentieel plantenenzym, naast de aanmaak van vitamine C speelt het een belangrijke rol bij de regulatie van de celgroei in de plant. GALDH is in de jaren 50 van de vorige eeuw voor het eerst geïsoleerd uit broccoli roosjes, maar over de biochemische en biofysische eigenschappen van het enzym is tot nu toe weinig bekend. **Hoofdstuk 3** beschrijft de eerste biochemische studie van recombinant GALDH uit *A. thaliana*. Naast het natuurlijk substraat L-galactono-1,4-lacton kan GALDH ook L-gulono-1,4-lacton omzetten naar

vitamine C. De oxidatie van galactonolacton verloopt echter 3000 keer efficiënter. Uit een vervolgstudie (zie hoofdstuk 4) werd duidelijk dat ook D-arabinono-1,4-lacton een substraat is voor GALDH. Dit lacton bindt echter relatief zwak, wat aangeeft dat de juiste lengte en stereochemie van de zijketen van het substraat belangrijk zijn voor de enzymspecificiteit. GALDH gebruikt cytochroom *c* als (fysiologische) elektronen acceptor, en reageert, nauwelijks met zuurstof.

GALDH vormt in tegenstelling tot de meeste flavine-afhankelijke dehydrogenases een adduct met sulfiet en een roodgekleurd flavine radicaal. Beide eigenschappen zijn een aanwijzing voor de aanwezigheid van positieve ladingen in het actief centrum. De meeste aldonolacton oxidoreductases bevatten een histidyl-FAD als covalent gebonden prosthetische groep. De histidine die betrokken is bij de covalente FAD binding ontbreekt in GALDH, in plaats daarvan bezit het enzym een leucine op dezelfde positie. Het vervangen van deze leucine door een histidine resulteerde niet in een covalente binding van de flavine, maar bracht wel het belang van dit hydrofobe aminozuur voor FAD binding en katalyse aan het licht. Voor covalente cofactor binding is een activering van de flavine of de histidine noodzakelijk. De aminozuren die deze activering bewerkstelligen zijn waarschijnlijk afwezig in GALDH.

Omdat er geen kristalstructuur van aldonolacton oxidoreductases beschikbaar is, is er maar weinig bekend over het reactiemechanisme van deze enzymen. In **Hoofdstuk 4** is een geconserveerd glutamaat-arginine paar in het reactiecentrum van GALDH geïdentificeerd door middel van sequentie vergelijkingen met alditol oxidase en cholesterol oxidase, twee VAO-homologen waarvan de ruimtelijke structuren bekend zijn. De functie van deze twee aminozuren in GALDH (Glu386 en Arg388) zijn bestudeerd door het maken van plaatsgerichte mutaties. Glu386 bleek cruciaal te zijn voor het binden van L-galactono-1,4-lacton en is mogelijk ook betrokken bij de activering van het substraat. De E386D variant vertoonde een verrassend hoge activiteit met L-gulono-1,4-lacton. Arg388 lijkt minder cruciaal te zijn voor de omzetting van het substraat, maar is erg belangrijk voor het stabiliseren van een negatieve lading die ontstaat tijdens de reductie van de flavine.

De interactie van GALDH met zijn fysiologische redox partner cytochroom *c* is bestudeerd in **Hoofdstuk 5**. Aangenomen wordt dat voor de elektronenoverdracht van GALDH naar cytochroom *c* een complex wordt gevormd tussen de beide redoxpartners. Door gebruik te maken van kernmagnetische resonantie (NMR) spectroscopie, werd complexvorming tussen GALDH en iso-1-cytochroom *c* uit gist waargenomen. De korte levensduur en het dynamische karakter van het complex maken een snelle elektronenoverdracht mogelijk. Opvallend was dat de interactie met cytochroom *c* intact blijft nadat 9 geladen residuen van het GALDH oppervlak waren verwijderd. Beide complexen hebben een vergelijkbare dissociatie constante, en de cytochroom *c* bindingskaart is in beide gevallen vergelijkbaar met

die van andere cytochroom *c* complexen. Dit impliceert dat cytochroom *c* een geconserveerde set van aminozuren gebruikt voor de interactie met verschillende redox partners.

Zuurstofreactiviteit van GALDH

De reactiviteit van GALDH met zuurstof is bestudeerd in de hoofdstukken 6 en 7, en geeft een mogelijke verklaring waarom GALDH een dehydrogenase is en geen oxidase.

Hoofdstuk 6 beschrijft de identificatie van een redox-gevoelige cysteïne in GALDH die noodzakelijk is voor de optimale productie van vitamine C. Tijdens het bewaren in de diepvries wordt GALDH langzaam door zuurstof geïnactiveerd. Studies met waterstofperoxide toonden aan dat deze inactivering komt door de specifieke oxidatie van Cys340, een aminozuur gelegen in het substraat bindingsdomein. Een massaspectrometrische analyse liet zien dat tijdens de oxidatie van Cys340 achtereenvolgens drie zuurstofatomen worden ingebouwd en dat de laatste twee oxidatiestappen irreversibel zijn. De GALDH varianten C340A en C340S zijn niet gevoelig voor cysteïne oxidatie, maar hebben een lage affiniteit voor L-galactono-1,4-lacton. pH-afhankelijke inactivatie studies suggereren dat het thiolaat anion van Cys340 een kritische rol speelt bij de substraat herkenning. Verder werd gevonden dat *S*-glutathionylering van Cys340 bescherming biedt tegen irreversibele oxidatie. De gevoeligheid voor oxidatieve stress en het feit dat planten veel vitamine C aanmaken, geven een mogelijke verklaring voor het feit dat GALDH een dehydrogenase is, en niet, een waterstofperoxide producerend oxidase zoals gerelateerde enzymen. De vorming van grote hoeveelheden waterstofperoxide in de mitochondriën zou de expressie en het functioneren van ascorbaat peroxidases en andere thiol-gemoduleerde enzymen ontregelen, en het verouderen en afsterven van plantencellen bevorderen.

In **Hoofdstuk 7** is de reden voor de geringe oxidase activiteit van GALDH nader onderzocht. Over de selectieve zuurstofreactiviteit van flavoenzymen is tot nu toe relatief weinig bekend, het is waarschijnlijk dat meerdere factoren, zoals ladingsverdelingen in het actieve centrum en het bestaan van zuurstofkanalen van invloed zijn. In dit hoofdstuk laten we voor het eerst zien dat het mogelijk is om een katalytisch competente galactonolacton oxidase te creëren. Door alle aminozuursequenties en driedimensionale structuren van VAO familieleden te vergelijken, hebben we een aminozuur geïdentificeerd dat dehydrogenases discrimineert van oxidases. Vrijwel alle oxidases van de VAO familie bezitten een glycine of proline op een geconserveerde positie nabij de C4a locus van de isoalloxazine groep van de flavine, terwijl dehydrogenases de voorkeur geven aan een ander aminozuur op deze positie. Het bewuste aminozuur bevindt zich aan de achterkant van de flavine, terwijl de substraat bindingsplaats zich aan de voorkant bevindt. De mutatie van het equivalente aminozuur in GALDH (Ala113→Gly) resulteerde in een 400-voudige toename in de reactie met zuurstof,

terwijl de cytochroom *c* reductase activiteit onaangetast bleef. De reactiesnelheid van de A113G variant met zuurstof is vergelijkbaar met andere flavoproteïne oxidases. De mutatie heeft geen invloed op de redox eigenschappen van de flavine, maar creëert ruimte voor moleculaire zuurstof om het gereduceerde flavine specifiek van één kant te benaderen en ermee te reageren. In wild-type GALDH werkt Ala113 dus als een poortwachter die voorkomt dat zuurstof toegang krijgt tot de flavine. Het afsluiten van de toegangspoort tot het actieve centrum lijkt een algemeen principe ter voorkoming van oxidase activiteit in de VAO familie, en is afwezig in leden die actief zijn met zuurstof.

Galactonolacton oxidase uit *Trypanosoma cruzi*

De eencellige parasieten *Trypanosoma brucei* en *T. cruzi*, de veroorzakers van respectievelijk de Afrikaanse slaapziekte en de ziekte van Chagas, bedreigen miljoenen mensen wereldwijd. De huidige behandeling tegen deze ziekten is ontoereikend omdat de beschikbare medicijnen maar een beperkt effect hebben en bijwerkingen kunnen veroorzaken. In **Hoofdstuk 8** is het aldonolacton oxidoreductases uit *Trypanosoma cruzi* bestudeerd. Galactonolacton oxidoreductase uit *T. cruzi* (TcGAL) is een interessant doelwit voor het ontwikkelen van een medicijn tegen de ziekte van Chagas, omdat *T. cruzi* niet in staat is om ascorbaat op te nemen uit zijn omgeving. Omdat de expressie van TcGAL in *E. coli* alleen onoplosbaar inactief eiwit, zogenaamde "inclusion bodies", opleverde, werd een *in vitro* hervouwingsmethode ontwikkeld die gebruik maakt van omgekeerde micellen. Actief eiwit werd verkregen wanneer de hervouwing werd uitgevoerd in aanwezigheid van een redox systeem bestaande uit gereduceerd en geoxideerd glutathion en FAD, en niet met FMN. Het feit dat TcGAL FAD gebruikt als redox actieve cofactor is in overeenstemming met de aminozuur sequentie, en ontkracht een recente publicatie waarin gesteld wordt dat dit enzym FMN bevat. Hergevouwen TcGAL heeft structurele eigenschappen die vergelijkbaar zijn met het oplosbare GALDH uit *A. thaliana* en is goed actief met zowel L-galactono-1,4-lacton als D-arabinono-1,4-lacton. Verder is voor het eerst aangetoond dat TcGAL actief is met zuurstof. Dit is in overeenstemming met de voorspelde toegangspoort voor zuurstof aan de achterkant van de flavine.

Vooruitzicht

Planten zijn de voornaamste producenten van vitamine C op aarde. Een gedetailleerde kennis van de biosynthese van vitamine C is essentieel voor het vinden van alternatieve productiemethoden voor deze gezondheidsbevorderende stof. In dit onderzoek is GALDH bestudeerd, het enzym dat de vitamine C biosynthese in planten voltooid. Verschillende

cruciale aminozuren die betrokken zijn bij de cofactor en substraat binding werden geïdentificeerd en varianten met een gewijzigde substraat- en elektronen acceptor specificiteit zijn ontworpen. Bovendien hebben we een verklaring gevonden voor het feit dat GALDH uit de plant een dehydrogenases is, en niet, zoals gerelateerde aldonolacton oxidoreductases, een oxidase.

De kristalstructuur van GALDH of een gerelateerd aldonolacton oxidoreductase zou meer licht kunnen werpen op de aminozuren in het reactiecentrum die betrokken zijn bij de substraat binding en katalyse. Helaas zijn alle pogingen om de kristalstructuur van GALDH op te helderen niet vruchtbaar gebleken omdat de verkregen kristallen van slechte kwaliteit waren. Tot nu toe zijn kristallen verkregen voor het natieve GALDH, GALDH met een C-terminale His-tag, GALDH C340A en C340S, en de vier GALDH oppervlakte mutanten. Alle kristallen lieten een diffractie zien van 3,5-4 Å resolutie, wat niet genoeg is om de structuur op te helderen. De beschikbaarheid van een GALDH remmer zou kunnen helpen bij het verkrijgen van kristallen met een hogere kwaliteit. Lycorine, een giftig planten alkaloïde, is in de literatuur genoemd als specifieke remmer van GALDH. Wij konden met lycorine echter geen duidelijke remming van de GALDH activiteit waarnemen. Als alternatief zou een aldonolactam een mogelijke competitieve remmer kunnen zijn voor GALDH. De eerste pogingen om 4-deoxy-L-galactono-1,4-lactam te synthetiseren waren, helaas, niet succesvol. De beschikbaarheid van een aldonolacton oxidoreductase kristalstructuur is ook belangrijk voor het ontwikkelen van een TcGAL remmer, een mogelijk medicijn voor de ziekte van Chagas.

Er is een toenemende druk om alternatieve methoden te ontwikkelen voor het Reichstein proces, een voornamelijk chemische procedure die gebruikt wordt om het leeuwendeel van de wereldwijde vitamine C voorraad te maken. Innovaties in recombinant DNA technologie, de beschikbaarheid van genoomsequenties en de recente vorderingen in eiwit “engineering” en synthetische biologie kunnen mogelijk worden geëxploiteerd voor de biotechnologische productie van vitamine C. Een interessante alternatieve route die meer onderzoek verdient is de productie van natuurlijk vitamine C uit pectine. Pectine is een koolhydraat polymeer uit de platencelwand dat rijk is aan D-galacturonzuur, een alternatief intermediair in de biosynthese van vitamine C. Appels en citrusvruchten zijn rijk aan pectine, een restproduct dat overblijft na het maken van sap. Door de werking van achtereenvolgens pectinases, galacturonaat reductase en aldonolactonase kan pectine worden omgezet naar L-galactono-1,4-lacton, de voorloper van vitamine C. Meer onderzoek is nodig om de (commerciële) haalbaarheid van deze route te bepalen.

Concluderend kunnen we stellen dat de resultaten verkregen met dit onderzoek een stevige basis vormen voor verdere structuur-functie studies aan VAO-type flavoenzymen in het algemeen en de aldonolacton oxidoreductases in het bijzonder. Het feit dat het nu mogelijk is

om een dehydrogenase om te zetten in een katalytisch competente oxidase is van algemeen belang voor het ontwikkelen van geschikte biokatalysatoren, omdat oxidases geen gebruik maken van dure co-substraten of regeneratie systemen. De kennis opgedaan met dit onderzoek kan in de toekomst worden gebruikt voor de biotechnologische productie van vitamine C of andere waardevolle koolhydraten.

Dankwoord

Het zit erop, het boekje is af! De afgelopen vier en een half jaar zijn voorbij gevlogen en nu is het tijd om terug te blikken en de mensen te bedanken die aan dit proefschrift hebben bijgedragen.

Allereerst wil ik mijn co-promotor dr. Willem van Berkel bedanken. Willem, je hebt mij geïntroduceerd in de wereld van de biochemie, jou enthousiasme voor het vakgebied werkt aanstekelijk, ik heb enorm veel van je geleerd. Je heb me de vrijheid gegeven om een eigen invulling aan het onderzoek te geven. Toen bleek dat we de beoogde suiker oxidases niet in handen konden krijgen, zochten we een homoloog enzym dat al bekend was, maar waar nog veel aan te onderzoeken viel. Ik koost toen voor het GALDH, een schot in de roos bleek achteraf! Bedankt ook voor de snelle correctie van al mijn manuscripten, ook toen er geen einde leek te komen aan de stroom aan het einde van mijn AIO periode. Ik had me geen betere begeleider kunnen wensen, bedankt voor de plezierige samenwerking! Ook wil ik mijn promotor prof. dr. Sacco de Vries bedanken voor de mogelijkheid om mijn promotie onderzoek uit te voeren bij de vakgroep Biochemie. Sacco, je stond wat verder af van mijn onderzoek, maar had altijd wel enkele nuttige tips wanneer mijn onderzoek ter sprake kwam, bedankt daarvoor.

Voor de dagelijkse gang van zaken op de vakgroep ben ik veel dank verschuldigd aan de technici Willy, Adrie, Sjef en Cathy. Willy, bedankt voor de introductie op het lab, je praktische tips, en je hulp bij het opstarten van de biochemische experimenten, die, zeker in het begin, onbekend terrein waren voor mij. Laura wil ik graag bedanken voor de snelle afhandeling van alle administratieve zaken.

I am also grateful to my students Yu, Rianne, Daan, Ilse, Mac Donald, and Justyna for their contributions to this thesis. It was a pleasure working with you, and I hope you learned as much as I did. I wish all of you the best with your future careers.

Graag wil ik ook mijn kamergenootjes Vivi Joosten en Stefania Montersino bedanken. Vief, we zijn in 2004 tegelijk begonnen in de groep van Willem, jij als postdoc ik als AIO. Als beginnend AIO heb ik veel opgestoken van jou ervaring. Ik heb ook altijd erg genoten van jou gezelschap tijdens de verschillende meetings en congressen. Bedankt dat je mijn paranimf wilt zijn! Stefi, you were my office and lab mate during the last year of my PhD period, I liked you company in the lab, the office and beyond. Having dinner with 7 Italians during the football match Netherlands-Italy (3-0) last summer in Jaca was priceless! I wish you all the best with your PhD research.

Dank ook aan alle overige (oud-)collega's van de vakgroep biochemie, José (ik, vond het erg leuk om jou paranimf te zijn), Sanne (vooral de laatste maanden gingen we gelijk op),

Helena (thanks for refolding the TcGAL inclusion bodies), Marloes (ik heb genoten van het AIO wadloop-weekend dat we samen hebben georganiseerd), Rumyana, Yee, Sofia, Ruchira, Mark, Simon en alle overige collega's die ik niet bij naam heb genoemd, bedankt voor jullie hulp en goede sfeer op het lab, de gezelligheid tijdens de koffie- en lunchpauzes, borrels, kerstdiners, labuitjes en sportdagen.

Tijdens mijn onderzoek heb ik samengewerkt met een groot aantal mensen binnen en buiten Wageningen. Henk-Jan Joosten en Peter Schaap ben ik dank verschuldigd voor het maken van de 3DM alignment van de VAO familie, Geb Visser, Carel Weijers en de PGO groep dank ik voor de hulp bij het synthetiseren van het galactonolacton substraat toen bleek dat het niet meer te koop was. Cor Wolfs bedank ik voor zijn hulp bij de EPR metingen. Marco Fraaije, Daniel Torres Pazmiño en Dominic Heuts van de Rijksuniversiteit Groningen wil ik graag bedanken voor de prettige samenwerking de afgelopen 4 jaar. Marco, ik vond het erg fijn om enkele weken in Groningen door te brengen, Danny bedankt voor je hulp bij de stopped-flow metingen. Niek, ik was aangenaam verrast toen je naast me kwam wonen, en heb erg genoten van onze etentjes waar we veel over 'onze enzymen' hebben gesproken. I thank Andrea Mattevi and Elena Carpanelli from the University of Pavia for all crystallization experiments. Marcellus Ubbink en Qamar Bashir uit Leiden wil ik graag bedanken voor de NMR metingen en Esther van Duijn, Arjan Barendregt en Albert Heck uit Utrecht bedank ik voor de ESI-MS metingen van geoxideerd GALDH.

Voor de nodige afleiding buiten het lab, wil ik graag al mijn vrienden bedanken voor de gezelligheid tijdens de vele etentjes, spellenavonden, filmavonden, sinterklaas (vr)eetfeesten, etc. Met name wil ik hier graag noemen Henriek en Ruth voor het bijkletsen tijdens onze wekelijkse zwemavonden. En Mark, we zijn bijna tegelijk begonnen als AIO, ik bij Biochemie, jij bij Microbiologie, we gingen gelijk op en hadden altijd veel te bespreken over onzer beider onderzoeken, ik wens je dan ook veel succes met het afronden van jou proefschrift.

Als laatste wil ik mijn familie bedanken, bij wie het altijd weer fijn thuiskomen is in Haaksbergen. Pap, mam, Leon & Belinda en Eline, bedankt voor jullie interesse in mijn onderzoek, ook al begrepen jullie maar weinig van wat ik op het lab allemaal uitspookte. "Ziet er mooi uit, maar ik snap er niks van", was het steevaste antwoord als ik weer trots een artikel liet zien dat was geaccepteerd. Pap en mam, jullie hebben mij altijd gestimuleerd om mijn eigen weg te gaan, en mij altijd gesteund, welke keuzes ik ook maakte, en zie waar het mij heeft gebracht! Eline, zus en vriendin, we hebben de afgelopen jaren veel met elkaar opgetrokken, ik hoop dat je me straks ook vaak komt opzoeken als ik in Engeland zit. Ik vind het erg leuk dat je mijn paranimf wilt zijn!

Nicole

Curriculum Vitae

Nicole Gerharda Henrica Leferink werd geboren op 22 november 1979 te Enschede en groeide op in het Twentse dorp Sint Isidorushoeve. In juli 1998 behaalde zij het VWO diploma aan de Scholengemeenschap Het Assink te Haaksbergen. In september van datzelfde jaar begon ze met de opleiding Biologie en Medisch Laboratorium Onderzoek aan de Saxion Hogeschool Enschede. Voor deze studie heeft zij 10 maanden stage gelopen bij NIZO food research in Ede en in januari 2002 studeerde ze af in de richting Biochemie/Biotechnologie. Vervolgens vertrok ze naar Wageningen om in september 2002 aan te vangen met de studie Biotechnologie aan de Wageningen Universiteit. Tijdens deze studie specialiseerde zij zich in de richting Cellulair/Moleculair en heeft ze afstudeeronderzoeken verricht bij de vakgroepen Virologie en Microbiologie. In september 2004 behaalde ze het Master diploma Biotechnologie met lof. Aansluitend begon ze aan haar promotieonderzoek bij het Laboratorium voor Biochemie aan de Wageningen Universiteit, waar zij onderzoek verrichtte aan het vitamine C producerende flavoenzym galactonolacton dehydrogenase uit *Arabidopsis thaliana*. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Vanaf april 2009 zal zij als postdoctoraal onderzoeker gaan werken bij het Manchester Interdisciplinary Biocentre aan de Universiteit van Manchester in Engeland.

List of publications

E.W. van Hellemond, **N.G.H. Leferink**, D.P.H.M. Heuts, M.W. Fraaije, W.J.H. van Berkel (2006). Occurrence and biocatalytic potential of carbohydrate oxidases. *Adv Appl Microbiol*, **60**:17-54

N.G.H. Leferink, W.A.M. van den Berg, W.J.H. van Berkel (2008). L-Galactono-γ-lactone dehydrogenase from *Arabidopsis thaliana*, a flavoprotein involved in vitamin C biosynthesis. *FEBS J*, **275**(4):713-726

N.G.H. Leferink, D.P.H.M. Heuts, M.W. Fraaije, W.J.H. van Berkel (2008). The growing VAO flavoprotein family. *Arch Biochem Biophys*, **474**(2):292-301

R. Machielsen, **N.G.H. Leferink**, A. Hendriks, S.J.J. Brouns, H.G. Hennemann, T. Daussmann, J. van der Oost (2008). Laboratory evolution of *Pyrococcus furiosus* alcohol dehydrogenase to improve the production of (2S,5S)-hexanediol at moderate temperatures. *Extremophiles*, **12**(4):587-594

N.G.H. Leferink, W.A.M. van den Berg, W.J.H. van Berkel (2008). Oxygen reactivity of L-galactono-1,4-lactone dehydrogenase. In: *Flavins and Flavoproteins 2008*, Prensas Universitarias de Zaragoza, pp. 75-80

N.G.H. Leferink, M.W. Fraaije, H-J. Joosten, P.J. Schaap, A. Mattevi, W.J.H. van Berkel (2009). Identification of a gatekeeper residue that prevents dehydrogenases from acting as oxidases. *J Biol Chem*, **284**(7):4392-4397

R.K.P. Kuipers, H-J. Joosten, E. Verwiel, J. Paans, J. Akerboom, J. van der Oost, **N.G.H Leferink**, W.J.H. van Berkel, G. Vriend, P.J. Schaap (2009). Correlated mutation analyses on super-family alignments reveal functionally important residues. *Proteins*, in press doi:10.1002/prot.22374

N.G.H. Leferink, E. van Duijn, A. Barendregt, A.J.R. Heck, W.J.H. van Berkel (2009). Galactonolactone dehydrogenase requires a redox-sensitive thiol for optimal production of vitamin C. *Submitted*

N.G.H. Leferink*, E.V. Kudryashova*, I.G.M. Slot, W.J.H. van Berkel (2009). Vitamin C biosynthesis in *Trypanosoma cruzi* is completed by a FAD-dependent oxidase. (*these authors contributed equally) *In preparation*

N.G.H. Leferink*, Q. Bashir*, M. Ubbink, W.J.H. van Berkel (2009). Interaction of L-galactono-γ-lactone dehydrogenase with cytochrome *c*. (*these authors contributed equally) *In preparation*

N.G.H. Leferink, M.D.F. Jose, W.A.M. van den Berg, W.J.H. van Berkel (2009). Functional assignment of Glu386 and Arg388 in the active site of L-galactono-γ-lactone dehydrogenase. *In preparation*

Overview of completed training activities

Discipline specific activities

Courses

Protein Engineering (Wageningen, 2004)

Chemistry and Biochemistry of Antioxidants (Wageningen, 2004)

Food Enzymology (Wageningen, 2005)

Bio-Nanotechnology (Wageningen, 2005)

Biomolecular Mass Spectrometry (Utrecht, 2005)

Summer School Glycosciences (Wageningen, 2006)

Meetings

CRC-Wageningen Industrial Platform Meetings (Wageningen, 2004-2007)

Annual Meetings Study Group Protein Research (Lunteren/Veldhoven 2004-2008)

European Meeting in Oxizymes (Oeiras, Portugal, 2006)

Biocatalysis in the Food and Drinks Industry (Wageningen, 2006)

Annual Meetings Study Group Biomolecular Chemistry (Lunteren, 2006-2007)

International Symposium on Flavins and Flavoproteins (Jaca, Spain, 2008)

General courses

VLAG PhD week (Bilthoven, 2004)

Organizing and supervising BSc and MSc thesis work (Wageningen, 2006)

Intermediair PhD career event (Amsterdam, 2008)

Optional activities

Preparing PhD research proposal

Foreign excursion Molecular Sciences (Hamburg, Germany/Copenhagen, Denmark, 2006)

Biochemistry biweekly seminars (Wageningen, 2004-2008)

Biochemistry biweekly journal club (Wageningen, 2004-2008)

The research described in this thesis was financially supported by the Carbohydrate Research Centre Wageningen (CRC-W).

Cover design and layout by Nicole Leferink

Printed by Gildeprint, Enschede