STUCTURE-FUNCTION OF FLAVOENZYMES OF INDUSTRIAL INTEREST

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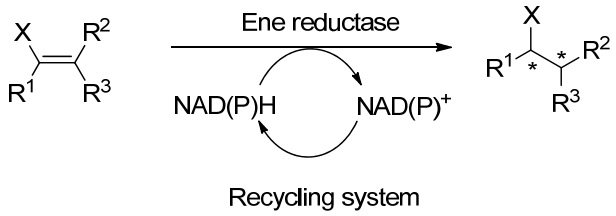
1. Introduction

Flavoenzymes are attractive biocatalysts because of the selectivity, controllability and efficiency of their reactions. Flavoenzymes typically contain a non-covalently bound FMN or FAD cofactor involved in redox chemistry (van Berkel 2008). In some flavoenzymes, the isoalloxazine moiety of the flavin is covalently linked to the polypeptide chain (Hefti et al. 2003; Heuts et al. 2009). This can be beneficial for cofactor economy, protein stability, and the enzyme oxidation power. Flavoenzymes constitute about 2% of all enzymes and can be classified according to sequence, fold, and function. Here we focus on the structure-function of flavoenzymes that are of interest for applications in the pharmaceutical, fine-chemical and food industries.

2. Flavoprotein reductases

Flavoprotein reductases primarily use NAD(P)H as electron donor and pass these electrons to a protein substrate or another electron acceptor. Flavoprotein reductases have long been ignored for biocatalytic applications, but this scenario has changed since the characterization of a number of members of the Old Yellow Enzyme (OYE) family. Ene reductases of the OYE family catalyze the asymmetric reduction of activated α,β-unsaturated alkenes to the corresponding alkanes yielding valuable products containing one or two chiral carbon centres. Combining the ene reductases with a suitable NAD(P)H recycling catalyst enables highly stereoselective alkene reductions on a preparative scale that are difficult to perform by conventional means (Stuermer et al. 2007):



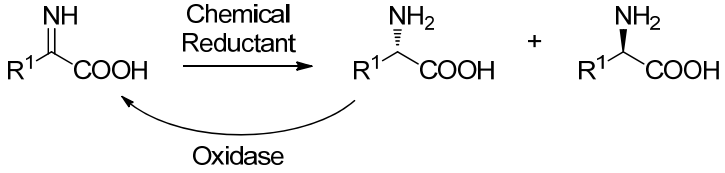
Well-studied members of the OYE family include OYE1 (brewers yeast), morphinone reductase (*Pseudomonas putida*), pentaerythritol tetranitrate reductase (*Enterobacter cloacea*), 12-oxophytodienoate reductase (tomato) and YqjM (*Bacillus subtillis*) (Adalbjçrnsson et al. 2010). These FMN-containing enzymes have structures based on a TIM barrel fold and can exist in different functional oligomeric forms. Ene reductases display a broad substrate specificity and are active with α,β-unsaturated ketones, aldehydes, nitroalkenes, carboxylic acids and derivatives.

TOYE, a NAD(P)H-dependent oxidoreductase from *Thermoanaerobacter pseudethanolicus*, is the first hyperthermophilic OYE homolog with known structure. The enzyme exists in solution in multiple oligomeric states and is most active with maleimide substrates (Adalbjçrnsson et al. 2010). Reduction of the *N*-phenyl substituted maleimide by TOYE yields the (*R*)-enantiomer with characteristically high yields and enantiopurity (Fryszkowska et al. 2009).

3. Flavoprotein oxidases

Flavoprotein oxidases catalyze the conversion of a substrate single bond to a double bond. The reduced flavin generated during this reaction is reoxidized by molecular oxygen to form hydrogen peroxide. Flavoprotein oxidases can have different folds and topologies (Fraaije and Mattevi 2000) and are active with many different substrates, including (amino) acids, mono- and polyamines, nitroalkanes, aliphatic and aromatic alcohols, mono- and oligosaccharides, thiols and thioesters.

D-amino acid oxidase (DAAO) is one of the most extensively studied flavoprotein oxidases. The homodimeric enzyme catalyzes the strictly stereospecific oxidative deamination of neutral and hydrophobic D-amino acids to give α-keto acids and ammonia. Mammalian and yeast DAAO share the same catalytic mechanism, but differ in kinetic mechanism, catalytic efficiency, substrate specificity, protein stability and mode of subunit interaction (Pollegioni et al. 2007). DAAO is well suited for chiral organic synthesis including the deracemization of unnatural amino acids (Fotheringham et al. 2006):



The above depicted chemoenzymatic procedure results in the production of chiral amines that are important for pharmaceutical development and a number of other applications. Other flavoprotein oxidases such as L-amino acid oxidase and monoamine oxidase can also be used for the deracemization process while laboratory evolution is applied to broaden the scope of substrates thar are not favoured for the native enzymes.

Vanillyl-alcohol oxidase (VAO; Figure 1) is the prototype of a multifunctional family of flavoenzymes that favors the covalent binding of FAD (Leferink et al. 2008). VAO catalyzes the oxidation, demethylation, deamination, or hydroxylation of phenolic substrates via the formation of a quinone-methide product intermediate. By doing so, the enzyme produces different flavors and fragrances, including vanillin and 4-hydroxycinnamyl alcohol. Studies with site-directed mutants demonstrated that the enantioselectivity of VAO can be inversed by transferring the catalytic base involved in water attack of the intermediate quinone methide product to the other site of the substrate-binding pocket (van den Heuvel et al. 2000). Furthermore, by using a directed evolution approach, it was possible to turn the suicide inhibitor creosol into a readily processed substrate (van den Heuvel 2004).

Aryl-alcohol oxidase (AAO) is a fungal enzyme that belongs to the GMC (glucose-methanol-choline oxidase) family of flavoenzymes. AAO catalyzes the dehydrogenation of various primary polyunsaturated alcohols, yielding the corresponding aldehydes. The crystal structure of AAO was recently solved (Fernandez et al. 2009). Two conserved histidines play a role in alcohol activation. Three aromatic side chains are proposed to form an aromatic gate that may regulate the access of the enzyme substrates to the active site.

Members of the GMC family bind the FAD cofactor through a specific structure known as the Rossmann fold. This domain is often found in combination with other domains of different folding

types. For example, flavoprotein disulfide reductases have two domains of the Rossmann-fold type, one FAD-binding domain that is interrupted by an NAD(P)-binding domain (Argyrou and Blanchard 2004). Kallberg and Persson developed a tool to identify Rossmann folds and predict their coenzyme specificity (NAD, NADP or FAD) using only the amino acid sequence as input (http://www.ifm.liu.se/bioinfo). The method was applied on a set of 68 genomes, giving a prediction sensitivity of 79% and selectivity close to 100% (Karllberg and Persson 2006).

Several industrial relevant FAD-dependent carbohydrate oxidases have recently become available (van Hellemond et al. 2006). Pyranose oxidase converts (hemi)cellulose-derived aldopyranoses at C2 to the corresponding ketoaldoses. Lactose oxidase, hexose oxidase, glucooligosaccharide oxidase and chitooligosaccharide oxidase (ChitO) are VAO family members with a bi-covalent flavin cofactor. Lactose oxidase catalyzes the production of lactobionic acid, which can be used as a food additive and in a range of other applications. ChitO catalyzes the regioselective oxidation of N-acetylated oligosaccharides. Besides tuning the redox properties, the bi-covalent binding of the FAD cofactor in ChitO is essential for a catalytically competent conformation of the active site (Heuts et al. 2008).

Figure 1. Crystal structure of VAO (pdb code 1VAO). The covalently bound FAD cofactor is depicted in yellow, the substrate binding domain in red and the FAD binding domain in green.

Alditol oxidase displays a high reactivity towards xylitol and sorbitol. As with VAO, substrate binding occurs through a lock-and-key mechanism. Protein structural analysis and molecular dynamics simulations revealed possible pathways for diffusion of molecular oxygen and a small cavity on the re-side of the flavin that host oxygen during FAD reoxidation (Baron et al. 2009). Creating space for oxygen to access this cavity in galactonolactone dehydrogenase turned this vitamin C producing flavoenzyme into a competent oxidase (Leferink et al. 2009).

Sulfhydryl oxidases (SOX) participate in the net generation of disulfide bonds during oxidative protein folding. SOX enzymes contain a redox-active disulfide (CXXC) that communicates with the non-covalently bound FAD cofactor. SOX enzymes might be applied for the cross-linking of proteins or peptides in food product preparations (Joosten and van Berkel 2007). QSOX, isolated from chicken egg white, introduces disulfide bridges directly into a wide range of unfolded proteins and peptides. *Trypanosoma brucei* QSOX lacks an entire domain, but shows catalytic activity and substrate specificity similar to the avian QSOX (Kodali and Thorpe 2010).

4. Flavoprotein monooxygenases

Flavoprotein monooxygenases catalyze the insertion of a single atom of molecular oxygen into the substrate, while the other oxygen atom is reduced to water. Activation of molecular oxygen in these enzymes is achieved by the generation of a flavin(hydro)peroxide. Based on fold and function, flavoprotein monooxygenases can be divided into six subfamilies (van Berkel et al. 2006). Single-component flavoprotein hydroxylases (class A) typically react with aromatic substrates. They are very regioselective and display a subtle mechanism of substrate, coenzyme and oxygen recognition (Joosten and van Berkel 2007).

Single-component Baeyer-Villiger monooxygenases (BVMOs) belong to class B. They contain two Rossmann fold domains and keep the coenzyme bound during catalysis. BVMOs convert ketones (or aldehydes) into esters or lactones and are widely used for the preparation of enantiopure compounds (Rehdorf et al. 2010). Several new (thermostable) BVMOs have been described (Torres Pazmino et al. 2010) and the stereopreference and substrate acceptance of selected BVMOs have been improved by directed evolution (Reetz and Wu 2009; Wu et al. 2010). Recently, a new generation of self-sufficient BVMOs was reported (Torres Pazmino et al. 2009). In these systems the BVMO is fused to a thermostable phosphite dehydrogenase for cofactor regeneration.

Two-component flavoprotein monooxygenases (class C-F) are composed of a NAD(P)H-dependent flavin reductase and a flavin-specific monooxygenase. Class C flavoprotein monooxygenases display a TIM-barrel fold, while Class D flavoprotein hydroxylases have an acyl-CoA dehydrogenase fold (Joosten and van Berkel 2007). Class E flavoprotein monooxygenases are relatively rare. These enzymes oxidize styrene derivatives to the corresponding epoxides and provide a highly enantioselective alternative to chemical epoxidation catalysts (Schmid et al. 2001). The monooxygenase component of the styrene converting system has many structural properties in common with class A aromatic hydroxylases (Ukaegbu et al. 2010). Recently, the first self-sufficient single-component styrene monooxygenase/reductase system was discovered in a *Rhodococcus opacus* strain (Tischler et al. 2009). This system is highly enantioselective but less efficient in substrate oxygenation.

Class F flavoprotein monooxygenases catalyze the regioselective chlorination and bromination of activated organic molecules. These enzymes are of interest for the production of antibiotics, antitumor agents and other natural products. The 3D structure of tryptophan 7-halogenase suggests a catalytic mechanism involving the formation of hypohalous acid, which is guided to the substrate binding site for the regioselective halogenation of the tryptophan (Dong et al. 2005). More recent work suggests that the chloride addition reaction requires the critical involvement of a Lys and Glu residue (Flecks et al. 2008) and provides interesting information about the structural determinants for regioselectivity control (Zu et al.2009).

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