Gluconate formation and polyol metabolism in *Aspergillus niger*

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aan mijn ouders

1 Vele artikelen zijn verschenen over de rol die polyolen spelen bij osmoregulatie in schimmels. Daarin wordt ten onrechte geen aandacht besteed aan de plaats van accumulatie van polyolen in de cel.

2 Bij de door Henrissat voorgestelde classificatie van glycosyl hydrolases, gebaseerd op overeenkomsten in aminozuurvolgordes, hadden de voor de verschillende families karakteristieke kenmerken van aminozuursamenstelling aangegeven moeten worden.
(Henrissat (1991), Biochem J **280**:309-316; Henrissat and Bairoch (1993), Biochem J **293**:781-788)

3 Door Yoder *et al.* (1993) wordt een verband gelegd tussen de ß-helix structuur van pectaat lyase C (PelC) en de eigenschap van het eiwit om door een dialyse membraan te lekken. Dezelfde eigenschap zou het voor PelC mogelijk maken om in de plantecelwand verborgen polygalacturonaat te bereiken. De flexibiliteit en openheid van structuur die hiervoor nodig is lijkt strijdig met de hoge stabiliteit van het pectaat lyase.
(Yoder *et al.* (1993), Science **260**:1503-1507)

4 De conclusie van Kelley en Reddy dat glucose oxidase het belangrijkste H_2O_2 genererende enzym is tijdens de afbraak van lignine door *Phanerochaete chrysosporium*, wordt onvoldoende door experimentele data ondersteund.
(Kelley en Reddy (1986), J Bacteriol **166**:269-74)

5 Er is nooit tijd om een experiment goed te doen maar er is altijd tijd om er een over te doen.

6 Menig dierenliefhebber houdt een dier in gevangenschap.

7 De mogelijkheden om aansluiting te verkrijgen op internet zouden even groot moeten zijn als de mogelijkheden voor aansluiting op kabeltelevisie.

8 Kostenbeheersing in de gezondheidszorg is slechts mogelijk als veel nieuwe technieken en medicijnen niet worden toegepast.

Wageningen, 27 september 1993 Cor Witteveen

Contents

Chapter 1

Introduction[1]

Among the filamentous fungi overproduction of primary metabolites is quite common. The carbon source available is thus used for the synthesis of biomass, production of energy and metabolites which can accumulate in the cell or are excreted. The function of this overflow metabolism often is not clear. In many fungi it can be stimulated and in some cases conditions have been found where a carbon source is converted almost quantitatively in one or more overflow metabolites. In *Aspergilli* two groups of compounds are overproduced under certain conditions, namely: organic acids and polyols. Synthesis of these compounds is part of carbon metabolism and common mechanisms might be involved in the production of both organic acids and polyols.

1 ORGANIC ACID PRODUCTION BY *ASPERGILLI*

The *Aspergilli* are well-known for their ability to produce organic acids of which the most important ones are listed in Fig. 1. Apparently these fungi have an intrinsic tendency to overproduce such metabolites. The organic acids can be divided in two groups: the TCA cycle related acids (citric-, itaconic-, malic-, fumaric-, succinic- and oxalic acid) and those directly derived from glucose (gluconic and kojic acid). The conditions necessary to achieve appreciable formation of the acids vary. The main factors involved are a high concentration of a carbon-source which is readily metabolised, the external pH and adequate aeration of the cultures. Besides these factors other nutritional parameters have been described to influence acid formation, amongst which trace metals, and nitrate and phosphate levels (reviewed e.g. by Miall (1978) and Röhr *et al.* (1992)).

1.1 TCA cycle related organic acids

Considering the biochemical problems for the fungus that must be overcome during the extracellular production of the TCA related acids four aspects are of importance:

1. A high flux through the Embden-Meyerhof pathway (EMP), must be maintained.

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[1]Part of this chapter will be published: J Visser, HJ Bussink and C Witteveen. Gene expression in filamentous fungi: the expression of pectinases and glucose oxidase in *Aspergillus niger*. In: Recombinant microorganisms and gene expression. A Smith (ed). Marcel Dekker, New York. In press.

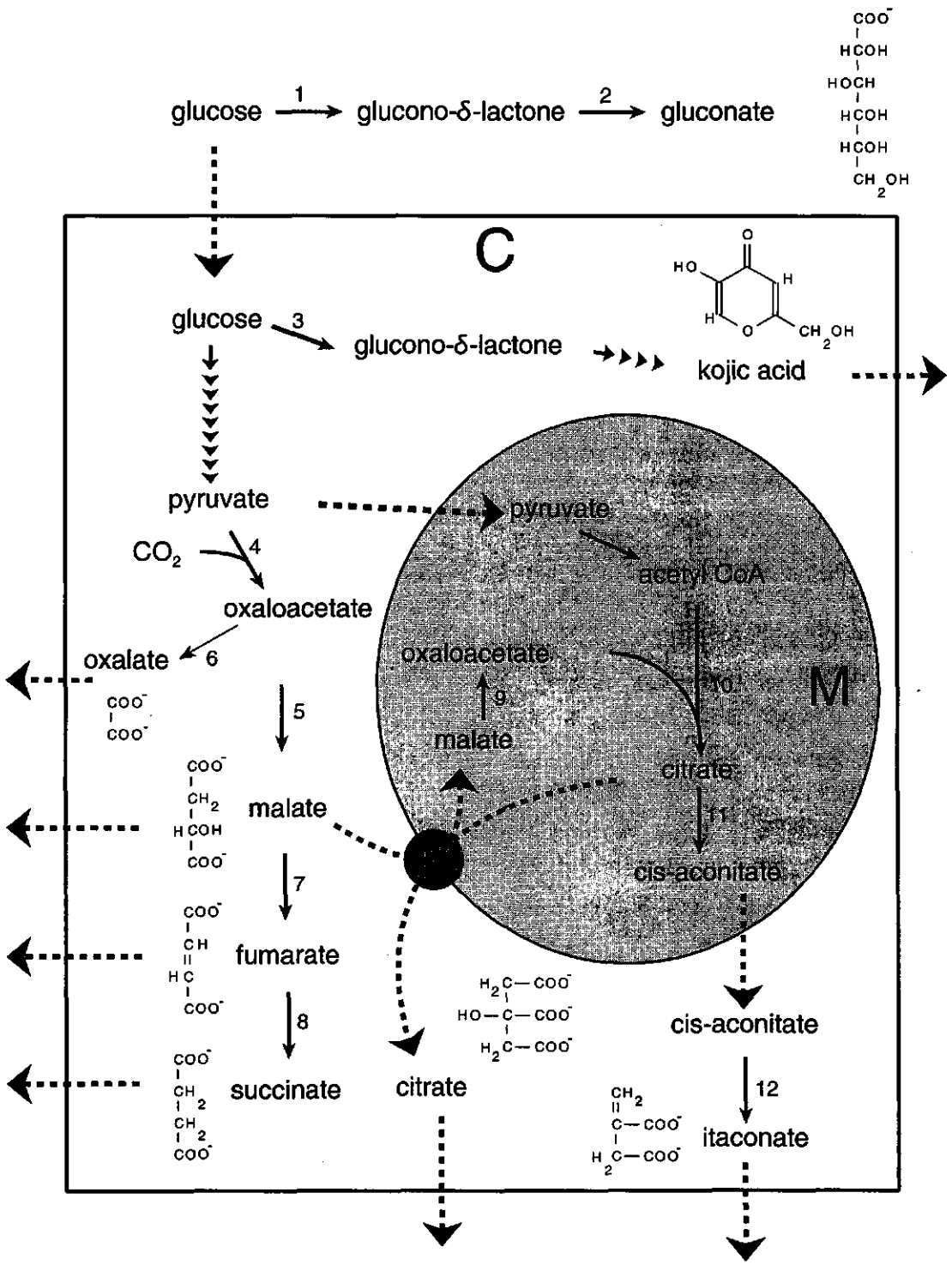
glucose
1
glucono-δ-lactone
2
gluconate
C
glucose
3
glucono-δ-lactone
kojic acid
pyruvate
CO2
4
oxaloacetate
oxalate
6
5
malate
7
fumarate
8
succinate
citrate
pyruvate
acetyl CoA
oxaloacetate
9
malate
10
citrate
11
cis-aconitate
M
cis-aconitate
12
itaconate

Fig 1 Schematic representation of biosynthetic pathways of organic acids in *Aspergilli* and their localization in the cell. C = cytosol, M = mitochondrion. Hyphenated arrows represent transport over a membrane. 1: glucose oxidase, 2: lactonase, 3: glucose dehydrogenase, 4: pyruvate carboxylase, 5: malate dehydrogenase, 6: oxaloacetate hydrolase, 7: fumarase, 8: succinate dehydrogenase, 9: malate dehydrogenase, 10: citrate synthase, 11: aconitate hydratase, 12: cis-aconitate decarboxylase.

2. Transport of the metabolites in and out of the mitochondria must take place.
3. Cofactor and ATP balances must be maintained.
4. Excretion of the acids into the medium requires transport across the plasmic membrane.

In fungi the biochemical details of these processes are still surprisingly unknown. Good aeration and a high level of an easily metabolisable substrate are supposed to be of importance for a high flux through the EMP. Xu *et al.* (1989) showed that an increase of the sugar level caused induction of citric acid formation. This occurred with a concomitant increase of the fructose-2,6-bisphosphate level, an activator of phosphofructokinase.

Some of the reactions in the biosynthetic pathway for the synthesis of the organic acids are catalyzed exclusively in the mitochondrion. In relation to the transport of metabolites into and out of the mitochondria it is presumably essential that pyruvate carboxylase is a cytosolic enzyme (Bercovitz *et al.*, 1990). The involvement of this enzyme in organic acid formation has been demonstrated using labeled substrates (Cleland and Johnson, 1954; Bentley and Thiessen, 1957; Winskill, 1983; Kubicek, 1988a; Peleg *et al.*, 1988). For citrate formation it has been suggested that malate is made in the cytosol (Fig. 1) and then exchanged for citrate (formed in the mitochondria) via the tricarboxylic acid carrier, thus ensuring an efficient transport of citric acid out of the mitochondria (Kubicek, 1988b).

Cis-aconitate decarboxylase, which is induced specifically under conditions of itaconic acid production, catalyzes the decarboxylation of cis-aconitate to itaconate and is localized in the cytosol (Jaklitsch *et al.*, 1991). Since aconitate hydratase is localized exclusively in the mitochondria it can be concluded that cis-aconitate in this case and not citrate is exported from the mitochondria.

For optimal citric and itaconic acid formation the external pH has to be low (Kubicek and Röhr, 1986; Miall, 1978). The internal pH is effected only to a small extent. The reason for the low pH requirement is not clear. However two possibilities can be indicated: (1) it is important for the excretion of the organic acids over the plasma membrane and (2) it is important for maintaining the energy balance in the cell. The low external pH may cost the cell a lot of energy because of leakage of H^+ over the membrane (Sigler and Hofer, 1991). Since citric and itaconic acid formation from glucose are processes that result in a net synthesis of 1 ATP and 3 NADH, it is possible that the energy required for the pH

correction contributes to maintaining the energy balance in the cell (Röhr *et al.*, 1992). Another factor of importance in relation to this is that high oxygen tensions induce an alternative respiratory pathway (the salicylhydroxamic acid (SHAM) sensitive respiratory pathway) which is important for the reoxidation of NADH (Röhr *et al.*, 1992).

Oxalic acid, which is mainly studied because of its toxicity which makes it an unwanted byproduct which arises for example in gluconic acid and citric acid fermentations, is formed from oxaloacetic acid by an oxaloacetate hydrolase which is localized in the cytosol (Kubicek *et al.*, 1988a). This enzyme has been purified from *A. niger* (Lenz *et al.*, 1976). The optimal pH for oxallic acid formation is in the range of 4-6.

L-Malic acid is not produced commercially by fermentation, although good yields of malic acid formation by *Aspergillus flavus* starting from glucose have been described (Battat *et al.*, 1991). A molar yield of 128% from glucose was reported. Succinic and fumaric acid are produced as well in this process. The production conditions for these acids are different from those reported for citric and itaconic acid. They have in common that adequate aeration is required but in this case the pH of the medium has to be around 6. This is achieved by adding $CaCO_3$ to the medium. Low nitrogen and phosphate levels are required for optimal production conditions. The biochemical pathway of the C4 acid formation is through the reductive branch of the TCA cycle. L-malic acid is formed from pyruvate mainly via oxaloacetate (Peleg *et al.*, 1988, 1989). This was demonstrated by showing that [1-^{13}C] glucose resulted in C3 labeled L-malic acid (Peleg, 1989). The cytosolic localization of pyruvate carboxylase and of an isoenzyme of NAD^+- dependent malate dehydrogenase suggests that malic acid formation is completely a cytosolic process. The molar yields from glucose above 100% confirm the cytosolic route via pyruvate carboxylase, since biosynthesis via the oxidative reactions of the TCA cycle leads to a maximal molar yield of 100%. The highest efficiencies are obtained when $CaCO_3$ is present as a neutralizing agent. Possibly HCO_3^- is taken up by the hyphae as substrate for pyruvate carboxylase, thus stimulating the efficiency of acid formation.

1.2 Glucose derived organic acids

Kojic acid (2-hydroxymethyl-5-hydroxy-γ-pyrone) has been produced commercially on a small scale using *Aspergillus oryzae* or *Aspergillus flavus* (Miall, 1978). The conditions for production are similar to those for citric and itaconic acid production: a low pH of around 2 favours kojic acid formation. The main pathway for the formation of kojic acid is directly from glucose without splitting of the sugar molecule. However, kojic acid can also be synthesized from smaller molecules (Arnstein and Bentley, 1953). From an analysis of the enzymes induced during kojic acid fermentation Bajpai *et al.* (1981) concluded that glucose dehydrogenase and gluconic acid dehydrogenase are involved in the first two

steps. Glucose oxidase, catalyzing the oxidation of glucose to glucono-δ-lactone, is not involved in the first step (Bajpai *et al.*, 1981). No information is available about the metabolic steps after the oxidation of glucono-δ-lactone, but a hypothesis has been formulated by Bajpai *et al.*(1981).

The formation of gluconic acid is different from the patway of synthesis of the other acids. The process is extracellular (Chapter 2 of this thesis) and is in fact a one step conversion of glucose. The result of this is that production of gluconic acid is much faster than the production of the other acids (1 day for the conversion of 30% glucose to gluconic acid versus 7 to 10 days for conversion of 20% or less sugar into the other organic acids). Gluconic acid formation is one of the main topics of this thesis and aspects of gluconic acid formation and the enzymes involved will be described in more detail in the next paragraph.

2 THE GLUCOSE OXIDATION SYSTEM OF *ASPERGILLUS NIGER*

2.1 Glucose oxidase: a brief introduction

The reaction catalyzed by glucose oxidase (ß-D-glucose:oxygen 1-oxidoreductase; EC 1.1.3.4.) is the oxidation of glucose to glucono-δ-lactone with concomitant reduction of oxygen to hydrogen peroxide. In *Aspergillus niger* the glucono-δ-lactone can be hydrolyzed by a lactonase (EC 3.1.1.17) or is hydrolyzed spontaneously while the hydrogen peroxide is decomposed by catalases (EC 1.11.1.6) (Fig. 2).

Glucose oxidase in *A. niger* was first described by Müller in 1928. Franke and Lorenz (1937) reported the formation of H_2O_2 in the reaction and demonstrated that other hydrogen acceptors can replace oxygen. They partially purified glucose oxidase and obtained evidence that glucose oxidase is a flavoprotein (Franke and Deffner, 1939). In *Penicillium notatum* glucose oxidase was initially described as an antibiotic compound and named Penicillin A, but was soon recognized as glucose oxidase and renamed notatin, an enzyme similar to the one already described for *Aspergillus niger* (Coulthard *et al.*, 1942 and 1945). Keilin and Hartree (1948a) characterized a highly purified glucose oxidase from *P. notatum*.

In addition to *A. niger* and several *Penicillium* species (Nakamatsu *et al.*, 1975) glucose oxidase has been found in a number of other filamentous fungi including *Phanerochaete chrysosporium* where it might have a function in the degradation of lignin by lignin peroxidases (Ramasamy *et al.*, 1985; Kelley and Reddy, 1986). The hydrogen peroxide formed by glucose oxidase of *Talaromyces flavus* is thought to be involved in the biocontrol of another fungus, *Verticillium dahliae* (Kim *et al.*, 1988). The physiological function of glucose oxidase is not always clear, since the organism cannot utilize the energy released in the oxidation reaction. The most likely function is that it contributes to the competitiveness of the organism, by the removal of the easily degradable glucose, by the concomitant acidification of the immediate environment or by the

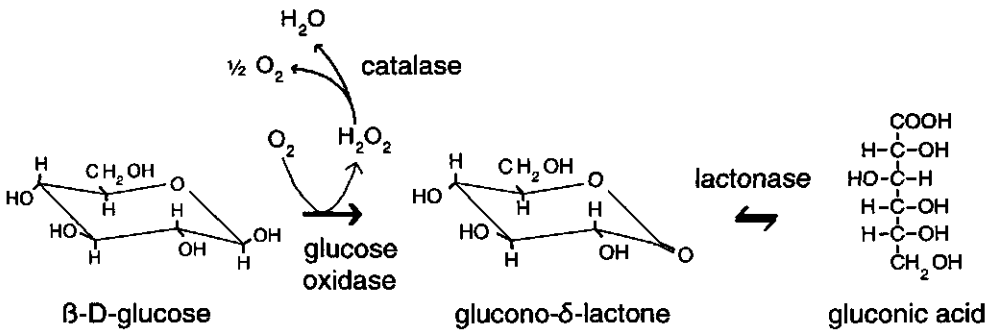

Fig. 2. Enzymatic reactions of the oxidation of ß-D-glucose to gluconic acid by *A. niger*

formation of the toxic H_2O_2, to which *A. niger* is highly resistant. In some organisms the provision of H_2O_2 as a substrate for a peroxidase might be an important function as is suggested for *P. chrysosporium*.

2.2 Applications of glucose oxidase

Glucose oxidase has several commercially interesting applications, which can be roughly divided into the production of gluconic acid, in which case usually the intact fungus containing the enzyme is used, and into applications for which the isolated enzyme is required. Different approaches for strain improvement are required for these two applications. Strains used for the production of gluconic acid have to be more resistant to the conditions used in the fermentation process including high oxygen levels, mechanical shear, high sugar and product concentrations and high levels of hydrogen peroxide, whereas this is not of less importance for strains used for production of the enzyme. The availability of the structural gene for glucose oxidase allows for separate production of the enzyme in other microorganisms (Frederick *et al.*, 1990).

Commercial preparations of glucose oxidase usually contain other enzymes, especially catalase. For some applications of glucose oxidase the presence of catalase is necessary to counteract the toxic H_2O_2. In other cases, e.g. analytical purposes, the catalase has to be removed (Frost and Moss, 1987). *A. niger* catalase is also commercially available.

Applications of glucose oxidase are all based on the capacity of the enzyme to form gluconate and hydrogen peroxide and to remove glucose and oxygen. All these four functions have found commercial applications (Scott, 1975). The use of *A. niger* for the formation of gluconate is discussed later in paragraph 2.3. Glucose removal by glucose oxidase was used for "desugaring eggs" in order to prevent the Maillard reaction (the condensation of an aldehyde and an amino group) (Scott, 1975). Oxygen removal by glucose oxidase is used to prevent oxidation and thus prevent colour and flavour changes. For this application it is

used sometimes in soft drinks, beer, wine, dried foods and foods containing oil emulsions in water like mayonnaise (Scott, 1975; Richter, 1983). A relatively new application is the use in toothpaste together with amyloglucosidase. The glucose oxidase forms H_2O_2 which is used by an antibacterial system in the saliva based on lactoperoxidase. The high specificity for glucose in combination with the formation of the easily detectable H_2O_2 and the high stability of the enzyme render it a very attractive system for the determination of glucose in biological samples (Keilin and Hartree, 1948b).

Finally, glucose oxidase is probably the most frequently used enzyme in biosensors. The stability, the high specificity and several possibilities to detect the activity of the enzyme make it an ideal enzyme for application in biosensors. Determination of glucose with high specificity has many, including biomedical applications. For reviews on the application of glucose oxidase in biosensors we refer to Schmid and Karube (1988) and Janata (1992) and references therein.

2.3 Production of gluconic acid

A. niger has been used for the production of gluconate since the 1930's. In that period efficient processes for the production of gluconic acid using submerged *A. niger* cultures were developed. Elevated air pressure and neutralization with calcium carbonate were applied in these processes. In 1952 Blom *et al.* developed a process for the production of sodium gluconate, using sodium hydroxide for the neutralization of the gluconic acid formed. For a more detailed description of the history of the gluconate fermentation process we refer to Miall (1978) and Röhr *et al.* (1983).

The high Michaelis Menten constant of glucose oxidase with respect to oxygen (K_m for oxygen = 0.48 mM at 27°C, Gibson *et al.*, 1964) makes the dissolved oxygen level a key variable in the process kinetics of gluconate formation. Increasing the oxygen concentration under production conditions by elevation of the air pressure is the easiest and cheapest method to increase the formation rate of gluconate (May *et al.*, 1934). A mathematical model describing the kinetics of growth and production coupled with gas/liquid oxygen transfer rates has been developed by Reuss *et al.* (1986). Their model takes into account the effects of pH on growth and production rate and the influence of sugar and gluconate concentrations on the solubility of oxygen and the k_La (the mass transfer coefficient) for oxygen. The main parameters and their effects included in the model are:

- The product formation rate is proportional to the dissolved oxygen concentration under normal production conditions because of the high K_m for oxygen.
- The optimal pH for growth is lower than the optimal pH for enzyme activity.
- An increase in the glucose and/or gluconate concentration results in a lower solubility and k_La of oxygen.

From experimental data for product formation they calculated an apparent K_m for

oxygen. This value (0.36 mM) is comparable to the value of the isolated enzyme. The model predicts an increased product formation rate when glucose is added continuously to the culture compared to a culture where the glucose is added batchwise. This is because of the better oxygen solubility and higher k_La at lower sugar concentrations. Adapting the pH at the beginning of the fermentation to values resulting in higher biomass formation does not give a relevant increase in the product formation under normal conditions (using 1 bar air for aeration). This is because the increase in biomass and thus in glucose oxidase is counterbalanced by a decrease in the oxygen level due to the higher amount of enzyme, resulting in a lower turnover per unit of enzyme. However, when the air pressure is increased to 4 bar more biomass does result in an increased product formation rate.

Glucose oxidase overproducing strains become of course interesting as well under these high pressure conditions. They have the advantage that lower amounts of biomass are necessary to obtain the same or even higher activities. This means that a lower amount of glucose is used for the formation and maintenance of biomass and also that the amount of other organic acids like oxalic acid, that are formed under these fermentation conditions will decrease. Another advantage is the lower viscosity of the culture fluid, which will lead to higher k_La values. Two approaches are available for constructing strains with higher glucose oxidase levels: mutagenesis and subsequent selection of overproducers or the use of molecular biological techniques. The advantage of a mutant selection strategy is that mutations with a broader effect can be isolated. For instance the *gox*B mutant (see Chapter 4) has, besides an increased level of glucose oxidase, also a higher level of two catalases which might be important for the protection of the cell during the gluconic acid fermentation process.

2.4 Enzyme properties

2.4.1. Glucose oxidase

Glucose oxidase from *A. niger* is a glycoprotein with a molecular weight of approximately 150 kDa (Nakamura and Hayashi, 1974,Pazur *et al.*, 1963; Pazur and Kleppe, 1964). Estimations of the carbohydrate content vary from 10 to 25% (Nakamura and Hayashi, 1974; Pazur *et al.*, 1965, Swoboda and Massey, 1965, Kalisz *et al.*, 1991). The native enzyme consists of two identical subunits (O'Malley and Weaver, 1972; Pazur *et al.*, 1984; Frederick *et al.*, 1990) and two tightly but non-covalently bound FAD's (Pazur and Kleppe, 1964; Swoboda, 1969) The holoenzyme contains two free SH groups and two disulfide groups (Swoboda and Massey, 1965, O'Malley and Weaver, 1972, Tsuge *et al.*, 1974). O'Malley and Weaver (1972) reported an intersubunit disulfide bridge whereas Tsuge *et al.* (1975), Jones *et al.* (1982) and Ye and Combes (1989) found that the subunits were bound non-covalently.

Both O- and N-linked carbohydrates were reported (Pazur *et al.*, 1963; Takegawa *et al.*, 1989, 1991a and b). The exact composition of the carbohydrates bound varies and depends on the production lots of manufacturers (Hayashi and Nakamura, 1976). When separated on the basis of their isoelectric point, usually several bands of glucose oxidase are found differing only in carbohydrate composition (Hayashi and Nakamura, 1981). The carbohydrates are mainly mannose and glucosamine (6:1) and a small amount of galactose. Several studies in which the function of the carbohydrates of glucose oxidase was investigated have been published (Pazur *et al.*, 1965; Nakamura *et al.*, 1974, 1976; Pazur *et al.*, 1984; Takegawa *et al.*, 1989; Kalisz *et al.*, 1990, 1991). Summarizing it can be said that at pH 5 - 6, where glucose oxidase is most stable, the carbohydrates do not contribute to the stability, but under less favourable pH conditions they do contribute to the stability of the protein. Using deglycosylated protein Kalisz *et al.* (1990) were able to obtain crystals of *A. niger* glucose oxidase. Kusai *et al.* (1960) described the crystallization of native *Penicillium amagasakiense* glucose oxidase. According to Hendle *et al.* (1992) these crystals were not suitable for structure determination so they used deglycosylated glucose oxidase from this organism for crystallization. The availability of the crystal structure (as recently published by Hecht *et al.* (1993)) and the structural gene for glucose oxidase opens new possibilities for research, because modified glucose oxidases can be studied.

The specificity of glucose oxidase was the subject of several reports (Keilin and Hartree, 1952; Sols and De La Fuente, 1957; Adams *et al.*, 1960; Pazur and Kleppe, 1964; Feather, 1970). Both the *Aspergillus* and the *Penicillium* enzymes are highly specific for the ß-form of D-glucose. D-galactose, D-mannose and D-xylose are oxidised at less than 1% of the rate of oxidation of glucose. Both 2-deoxyglucose and 6-deoxyglucose are oxidized by glucose oxidase, although at lower rates than glucose.

Kleppe (1966) and Greenfield *et al.* (1975) showed that glucose oxidase of *A. niger* is inactivated by H_2O_2 and the enzyme is much more sensitive when the flavin groups are in the reduced state. The mechanism of inactivation is not clear but the most likely mechanism seems to be the oxidation of methionine residues to methionine sulfoxide.

The kinetic mechanism of the reaction catalyzed by glucose oxidase has been studied in detail by steady state and stopped flow methods for the *Aspergillus* and *Penicillium* enzymes (Gibson *et al.*, 1964; Nakamura and Ogura, 1968; Bright and Appleby, 1969; Nakamura and Ogura, 1962). The experimental data can be explained by the following mechanism:

$$E_o + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_o\text{-}S \xrightarrow{k_2} E_r + P_1$$

$$E_r + O_2 \xrightarrow{k_3} E_o\text{-}P_2 \xrightarrow{k_4} E_o + P_2$$

Where S = glucose, P_1 = gluconic acid and P_2 = hydrogen peroxide.

Only a few compounds are known to inhibit glucose oxidase. Both D-glucal, a structural analog of glucose, and halide ions are competitive inhibitors of glucose oxidase with respect to glucose and uncompetitive with respect to oxygen (Rogers and Brandt, 1971a and b). These two compounds bind at different loci in the active site of the enzyme (Rogers and Brandt, 1971c).

2.4.2. Catalases

Catalase activity is necessary for the detoxification of the hydrogen peroxide during the production of gluconic acid. This is essential for the viability of the cells and the stability of the glucose oxidase. Witteveen *et al.* (1992) (Chapter 2) showed that *A. niger* forms at least 4 different catalases, two of which are induced in parallel with glucose oxidase (CAT III and CAT IV). Several publications described the purification and characterization of an *A. niger* catalase. Since these catalases are glycosylated and are purified from commercial preparations, which very likely originate from glucose oxidase fermentations, they probably correspond with CAT IV, the cell wall bound catalase which is induced in parallel with glucose oxidase. This catalase is a tetramer with a reported molecular weight which varies strongly: 323 kDa (Gruft *et al.*, 1978), 338 kDa (Mosavi-Movahedi, 1987), 385 kDa (Kikuchi-Torii, 1982) and 460 kDa (Mosavi-Movahedi, 1987). This is probably caused by differences in the carbohydrate content (10-33%). The amino acid composition has been determined by Gruft *et al.* (1978) and Mosavi-Movahedi (1987). The enzyme is much more stable than beef liver catalase, especially with respect to H_2O_2 resistance, proteolytic degradation and temperature and pH, especially at low pH (Scott and Hammer, 1960; Wasserman and Hultin, 1981) and is relatively insensitive to inhibition by HCN, HN_3 and HF (Gruft *et al.*, 1978; Kikuchi-Torii *et al.*, 1982). However, the *A. niger* enzyme has a specific activity which is approximately 10-fold lower than that of bovine liver catalase.

2.4.3. Lactonase

The physiological importance of this enzyme is not clear since the hydrolysis of glucono-δ-lactone also occurs spontaneously (half time approximately 0.5 h, strongly dependent on the pH). Glucono-δ-lactone is known to be a strong inhibitor of ß-glucosidase, therefore lactonase is supposed to contribute to cellulose degradation by the hydrolysis of the lactones (Moeller, 1973; Bruchmann *et al.*, 1987). The induction of the enzyme is coordinately regulated with glucose oxidase (see Chapter 4). Lactonase was partially purified from a commercial *A. niger* enzyme preparation by Bruchmann *et al.* (1987). They concluded that there are several lactonase isoenzymes. The molecular weight is approximately 70 kDa.

2.5 Localization of the glucose oxidase system

For a long time it was assumed that glucose oxidase of *A. niger* was located

intracellularly (Pazur, 1966). This was further supported by ultrastructural studies by van Dijken and Veenhuis who concluded that the enzyme was located in peroxisomes (Van Dijken and Veenhuis, 1980). Mischak *et al.*(1985) showed that under manganese deficient growth conditions glucose oxidase is found almost quantitatively in the culture fluid. They explained this by a cell wall localized glucose oxidase which enters the culture fluid because of an altered cell wall composition resulting from manganese deficiency. In Chapter 2 of this thesis evidence is provided for a cell wall localized glucose oxidase system. Thus the oxidation of glucose, which is a highly toxic process because of the hydrogen peroxide generation, takes place extracellularly where it is less harmful.

The cell wall is not a separate compartment so there must be a mechanism by which the proteins are retained. The proteins might be bound to cell wall components or they might be retained because the pore size is too small to let them diffuse out of the cell wall. In Chapter 2 it is shown that not all the activity of glucose oxidase, catalase and lactonase is retained in the cell wall. A certain amount is found in the medium. Aproximately 20% of glucose oxidase, less than 5% of catalase and 40-50% of lactonase is found in the medium. This might be explained by the difference in size of the proteins. Catalase, having a very high molecular weight (at least 350 kDa) is retained almost completely, whereas lactonase (70 kDa) passes the cell wall more easily. Glucose oxidase (150 kDa) takes an intermediate position. In yeast it was shown that the passage of proteins through the cell wall is not just determined by the molecular weight of the proteins but is influenced by several factors (De Nobel and Barnett, 1991). However, the distribution of catalase, glucose oxidase and lactonase does suggest that the molecular size is an important factor. Expression of glucose oxidase in *S. cerevisiae* resulted in a strongly overglycosylated protein which apparently could easily pass the cell wall (Frederick *et al.*, 1990). A comparable result was obtained when the cellobiohydrolase from *Trichoderma reesei* was synthesized by *S.cerevisiae* (Pentillä *et al.*, 1988).

2.6 Induction of the glucose oxidase system

Factors important for optimal induction of glucose oxidase are:

1. high glucose concentrations (Zetelaki and Vas, 1968).
2. a pH around 5.5. (Röhr *et al.*, 1983)
3. a high level of dissolved oxygen (Zetelaki, 1970).

Control of the external pH can be achieved either by titration with alkali or by adding $CaCO_3$ to the medium. The presence of manganese has been claimed to be important for glucose oxidase induction (Lockwood, 1975) but Mischak *et al.* (1985) showed that this is not true. Nitrogen limitation is also not a prerequisite in obtaining efficient induction of glucose oxidase (Chapter 3), although this was previously reported to stimulate the formation of glucose oxidase in both *Penicillium* (Linton *et al.*, 1984) and *Aspergillus* (Müller, 1986). The latter

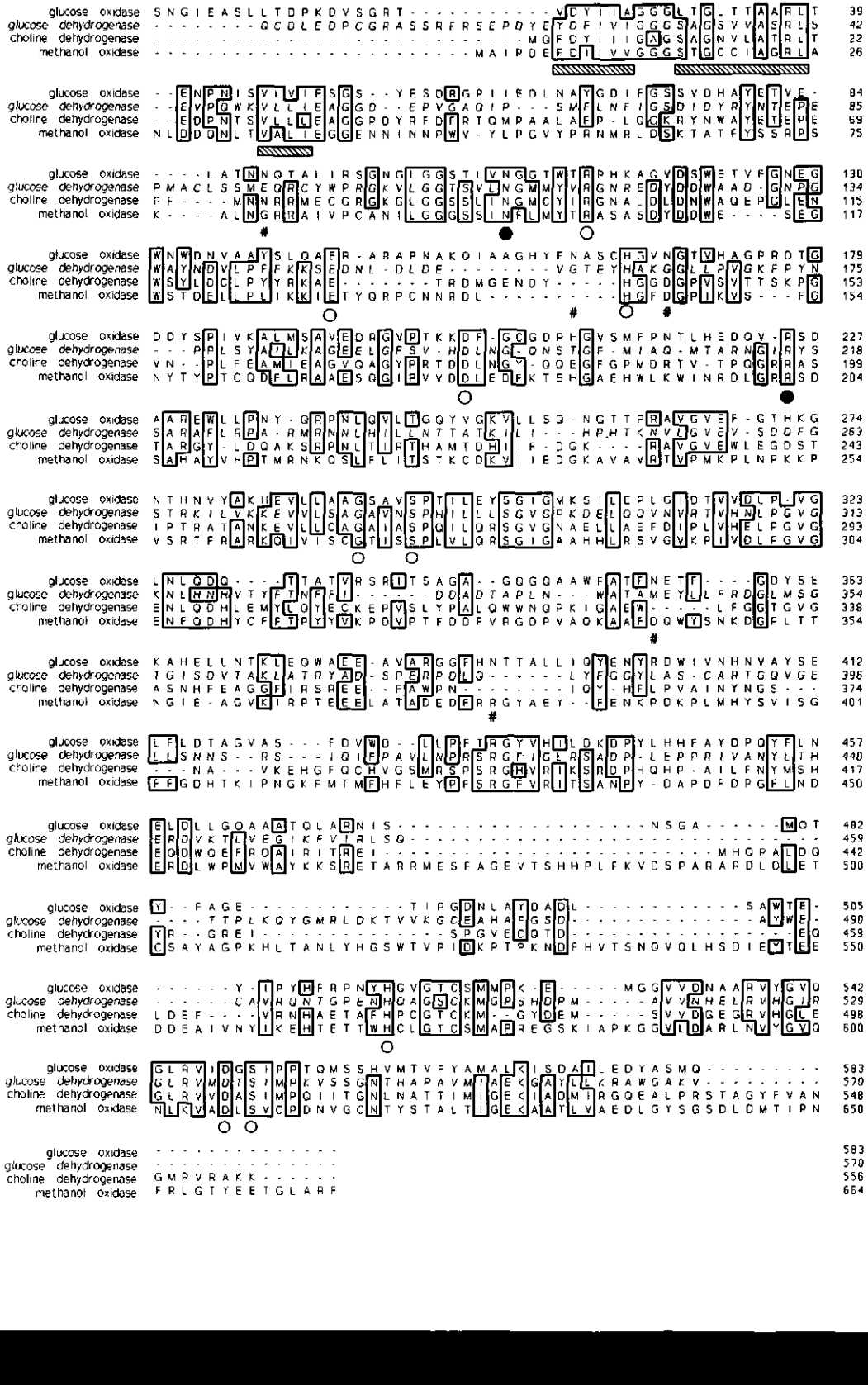
glucose oxidase
glucose dehydrogenase
choline dehydrogenase
methanol oxidase

Fig 3 Alignment of amino acid sequences of glucose oxidase (*A. niger*), glucose dehydrogenase (*Drosophila melanogaster*), choline dehydrogenase (*Escherichia coli*) and methanol oxidase (*Hansenula polymorpha*). The boxes at the N-terminus indicate the ß-α-ß motif involved in binding of the ADP part of FAD. Glycosylation sites are indicated by #. The filled circles indicate the asn and arg involved in the bending of the flavin and the open circles indicate conserved amino acids which are part of strong hydrogen bond interactions or buried salt bridges.

publication relates to surface cultures of *Aspergillus niger* and is therefore not directly comparable. Induction of glucose oxidase requires the presence of glucose or of 2-deoxyglucose (Mischak *et al.*, 1985; Witteveen *et al.*, 1990a (chapter 3 of this thesis)). The effect of the pH is more complicated. There is no doubt that the optimal pH for induction is around 5.5, but at pH's as low as 2.5 biosynthesis of glucose oxidase could still be demonstrated (Heinrich and Rehm, 1982; Witteveen *et al.*, 1990a). At a pH lower than 2.5 glucose oxidase is no longer stable. When raising the pH from 2 to 5.5 Mischak *et al.* (1985) could demonstrate *de novo* synthesis of glucose oxidase and Wirsel *et al.* (1990) demonstrated the induction of glucose oxidase mRNA.

All these data do not contain any information on the molecular mechanisms of glucose oxidase induction. In Chapter 4 of this thesis data are presented which result in a hypothesis for the mechanism of induction of glucose oxidase, lactonase and catalase.

2.7 The glucose oxidase gene

Four recent publications described the isolation of the glucose oxidase gene (Kriechbaum *et al.*, 1989, Frederick *et al.*, 1990, Whittington *et al.*, 1990, Witteveen *et al.*, 1993 ;see also Chapter 4 of this thesis). The methods used involved the use of protein sequence derived oligonucleotide probes (Kriechbaum *et al.*, 1989, Frederick *et al.*, 1990, Whittington *et al.*, 1990) or the screening of a cDNA expression library using antibodies (Chapter 4). Kriechbaum *et al.* (1989) and Frederick *et al.* (1990) used a cDNA library constructed from the *A. niger* ATCC 9029 strain. Whittington *et al.* (1990) isolated the glucose oxidase gene directly from a genomic library of the *A. niger* B60 strain (Kubicek and Röhr, 1978). The *gox* gene described in Chapter 4 is isolated from the CBS 120.49 strain. The sequence of Whittington *et al.* (1990) shows some minor differences in the promoter region compared to the sequence of the ATCC 9029 strain (at positions -476, -464 and -395). Comparison of the cDNA sequence and the genomic sequence revealed that no introns are present in the glucose oxidase gene. Nuclease S1 mapping of the 5' end of the *gox* transcript indicated the initiation of transcription to be at position -38, 43 bp downstream of the TATAA sequence (Whittington *et al.*, 1990). Protein sequence data showed that the mature enzyme is preceeded by a 22 amino acid preprosequence which has a

monobasic processing site (Frederick *et al.*,1990). The mature enzyme consists of 583 amino acids, contains 8 potential sites for N-linked glycosylation and 3 cysteine residues.

Comparison of the glucose oxidase amino acid sequence with a protein sequence database (Swisspir) resulted in 5 proteins with more extended homology: *E.coli* choline dehydrogenase, two *Drosophila* glucose dehydrogenases, and two yeast methanol oxidases (Lamark *et al.*, 1991, Whetten *et al.*, 1988, Krasney *et al.*, 1990, Ledeboer *et al.*, 1985, Sakai and Tani, 1992) . All 5 proteins are flavoproteins. Fig. 3 shows alignments of these sequences with glucose oxidase. Of the two glucose dehydrogenases and the two methanol oxidases only one in each case was used in the alignment. The stretches with highest homology are involved in FAD binding (Hecht *et al.*, 1993). At the N-terminal end the ß-α-ß motif is found which is involved in binding of the ADP moiety of the FAD (Wierenga *et al.*, 1986). A second (AA 243-289) and a third stretch (near the C-terminus) contain conserved blocks of amino acids that are also involved in the FAD binding. Besides these stretches a number of amino acids are conserved in all 4 sequences which are described by Hecht *et al.* (1993) to be important for the tertiary structure. These amino acids form salt bridges buried in the protein or strong H-bridges. They are indicated in the figure. For the activity of the protein it is essential that the FAD group is non-planar. Asn 107 is involved in preventing the planarity of the FAD group. Arg 226 enforces this effect by forming a strong bond to the Asn 107. Both residues are conserved in all 4 sequences. The region which is involved in substrate binding is less homologous than the FAD binding parts. However, in these areas there are two stretches (around AA 325 and 434) that show a series of conserved amino acids. The function of these parts is not known.

5 of the 8 potential N-glycosylation sites in glucose oxidase are indeed glycosylated. All these sites are in regions with lower homology to the other proteins. This is in agreement with previously published results that enzyme activity and stability are not effected by deglycosylation (see paragraph 2.4.1).

The quite extensive overall homology between the 6 proteins, even though they are from evolutionary distant organisms, is remarkable. They probably form a distinct class of structurally related flavoproteins.

2.8 Overexpression of glucose oxidase and expression of the glucose oxidase gene in other organisms

In filamentous fungi it is often possible to achieve overproduction of an enzyme just by increasing the copy number of the encoding structural gene, which can be achieved by (co)transformation (Visser *et al.*, 1993). Chapter 4 describes that an increase in the copy number of the glucose oxidase gene results only in a limited increase of the glucose oxidase level. High levels of glucose oxidase seem to have a negative effect on growth.

Vectors containing the glucose oxidase gene have been used to transform

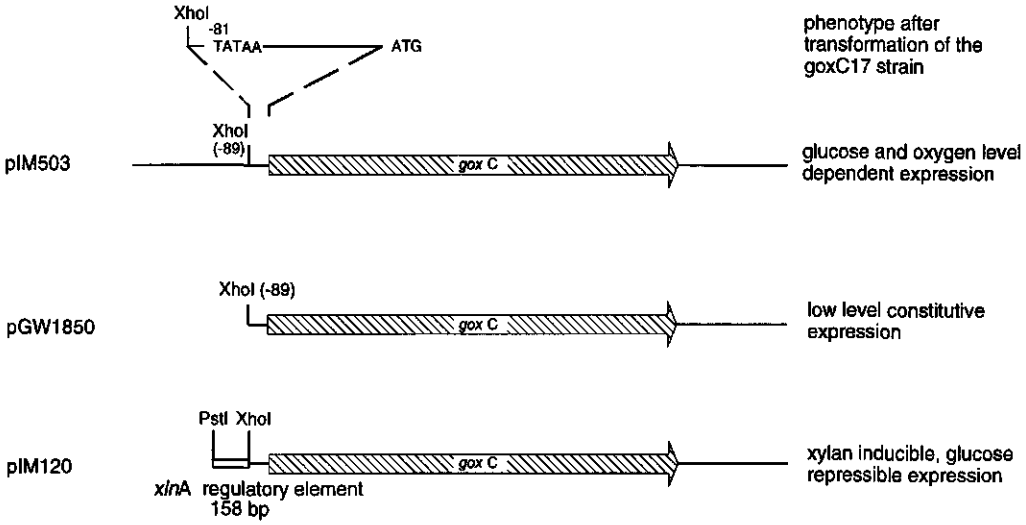

Fig 4 The glucose oxidase reporter system for analysis of upstream regulatory sequences. The upstream regulatory region of the *gox*C gene (pIM503) is deleted leaving the TATA box unaltered resuting in pGW1850. In front of the *gox*C structural gene and the remaining basic transcription unit fragments containing potential upstream regulatory sequences are cloned such as a 158 regulatory element of the *A.tubigensis xln*A gene. (Adapted after de Graaff *et al.*(1992)).

Saccharomyces cerevisiae and *Aspergillus nidulans*. *S.cerevisiae* (Frederick *et al.*, 1990, De Baetselier *et al.*, 1991) was transformed with the plasmid pSGO2 in which the glucose oxidase gene (including the preprosequence) is under the control of the regulated ADH2-GAPDH hybrid promoter. This resulted in a production of glucose oxidase of 3 g per liter. The glucose oxidase produced this way was strongly overglycosylated, and had a molecular weight of 350-400 kDa as estimated by gel permeation chromatography. This enzyme had a normal activity but showed an increased heat resistance and pH stability.

2.9 The glucose oxidase gene used in a reporter system

The relatively easy and sensitive detection of the enzyme activity in intact mycelium and in solution , as well as the stability of the enzyme, allow the glucose oxidase gene to be used as an *Aspergillus* derived reporter system for gene expression and regulation studies. De Graaff *et al.* (1992) made a construct in which a promoter element derived from the *xln*A gene of *A.tubigensis* which encodes an endo-xylanase was ligated to a fragment containing the *gox*C structural gene and the core promoter (TATA box and transcription initiation site) but without regulatory sequences of the glucose oxidase gene (Fig. 4). This construct was used to transform a strain with the *gox*C mutation (Chapter 3). In this way they were able to show that a 158 bp element contains the information

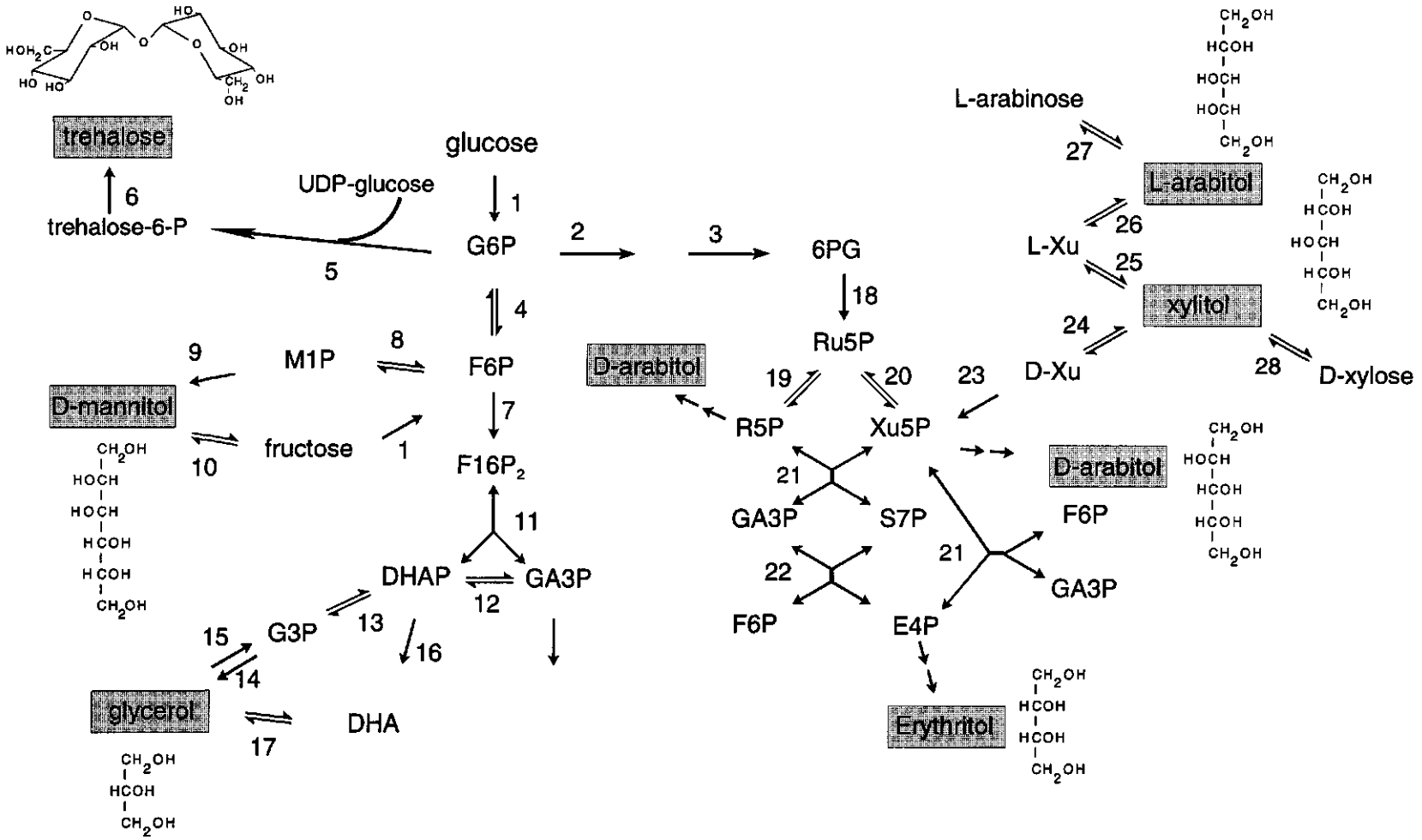
trehalose
6
trehalose-6-P
UDP-glucose
5
glucose
1
G6P
2
3
6PG
4
18
Ru5P
9
M1P
8
F6P
D-arabitol
19
20
23
R5P
Xu5P
D-mannitol
10
fructose
1
7
F16P2
21
GA3P
S7P
D-arabitol
F6P
11
22
21
DHAP
12
GA3P
F6P
GA3P
15
G3P
13
16
E4P
14
glycerol
17
DHA
Erythritol
L-arabinose
27
L-arabitol
26
L-Xu
25
xylitol
24
D-Xu
28
D-xylose

Fig 5 Schematic representation of part of the hexose monophosphate pathway, the pentose phosphate pathway and metabolic routes involved in polyol synthesis and degradation. 1: hexokinase, 2: glucose-6-phosphate dehydrogenase, 3: 6-phosphogluconolactonase, 4: hexosephosphate isomerase, 5: , 6: trehalose-6-phosphate phosphatase, 7: phosphofructokinase, 8: NAD^+-dependent mannitol-1-phosphate dehydrogenase, 9: mannitol-1-phosphate phosphatase, 10: mannitol dehydrogenase, 11: aldolase, 12: triosephosphate isomerase, 13: glycerol-3-phosphate dehydrogenase, 14: glycerol-3-phosphate phosphatase, 15: glycerol kinase, 16: dihydroxyacetonephosphate phosphatase, 17: glycerol dehydrogenase, 18: 6-phosphogluconate dehydrogenase, 19: ribosephosphate isomerase, 20: ribulose-phosphate 3-epimerase, 21: transketolase, 22: transaldolase, 23: D-xylulose kinase, 24: NAD^+-dependent xylitol dehydrogenase, 25: $NADP^+$-dependent xylitol dehydrogenase, 26: NAD^+-dependent L-arabitol dehydrogenase, 27: L-arabinose reductase, 28: D-xylose reductase

for xylan-dependent induction and glucose repression. Also the effect of other carbon sources and other potential inducers could be investigated very efficiently using this system. A *gox* reporter system which includes the *gox* core promoter, is particularly useful to monitor transcription activation elements.

3 POLYOL METABOLISM IN *ASPERGILLUS*

Another group of compounds which can be considered in some ways as products of overflow metabolism are the polyols. Fig. 5 gives a schematic view of the polyols that are synthesized in *A. niger* and of their (assumed) biosynthetic pathways. Mannitol, glycerol and erythritol are the main polyols in *A. niger*. Low levels of arabitol are usually found whereas the other pentitols are only present under specific conditions. Trehalose can be considered as a cyclic polyol and is indicated as well in the scheme. Sorbitol, ribitol and galactitol have not been observed in *A. niger* except under specific conditions as for example growth on ribose in the case of ribitol. When these compounds are studied it is important to be aware of the possible functions of the polyols. The following functions have been postulated:

1 Balancing the osmotic pressure
2 Carbon source reserve compounds
3 Regulation of cofactor balance
4 Transport of carbon substrates through the hyphae.

The information about the role of the various polyols in *Aspergilli* is fragmentary and our knowledge about the function of each specific polyol is scarce. The main polyols which occur in *A. niger* are discussed below.

3.1 Mannitol

Mannitol accumulation has been reported for many *Aspergilli*. It is an abundant compound in conidiospores of *A. oryzae* (Horikoshi *et al.*, 1965) and it is a common polyol in the hyphae of most fungi studied (see for example Blumenthal, 1976).

There are several possibilities for the biosynthesis and degradation routes of mannitol. These are summarized by Blumenthal (1976). It is assumed that it is formed from and also degraded to fructose-6-phosphate. This implies both a reduction/oxidation and a dephosphorylation/phosphorylation step. The variation in the order in which these reactions occur and the cofactors used suggest a series of possibilities for the routes used. There is evidence that the biosynthetic route is by reduction of fructose-6-phosphate (F6P) to mannitol-1-phosphate (M1P) by an NAD^+-dependent M1P dehydrogenase and subsequent dephosphorylation by a specific M1P phosphatase (Horikoshi *et al.*, 1965; Lee, 1967; Corina and Munday, 1971, Boonsaeng *et al.*, 1976). The M1P dehydrogenase has been purified from *A. niger* (Kiser and Niehaus, 1981). The degradation of mannitol is assumed to involve oxidation by an $NAD(P)^+$-dependent mannitol dehydrogenase and subsequent phosphorylation of the fructose formed by hexokinase (Horikoshi *et al.*, 1965). However, no conclusive evidence has been presented in the literature for this route. Niehaus and Dilts (1982) purified an $NADP^+$-dependent mannitol dehydrogenase form *A. parasiticus*.

Hult and Gatenbeck (1978) postulated the presence of a mannitol cycle in the fungus *Alternaria alternata*. Synthesis of mannitol from F6P by NAD^+-dependent M1P dehydrogenase and M1P phosphatase and remetabolizing the mannitol by oxidizing it to fructose by $NADP^+$-dependent mannitol dehydrogenase and phosphorylation of the fructose to F6P would result in the net formation of NADPH at the cost of an NADH and an ATP. Hult *et al.* (1980) concluded from the presence of the enzyme activities that the mannitol cyle is an important pathway in imperfect fungi. However, the presence of the enzyme activities can not be taken as sole evidence for an active mannitol cycle. Singh *et al.* (1988) could not find a correlation between the activity of the mannitol cycle enzymes and the need of the cell for NADPH, whereas they found an increase of glucose-6-phosphate dehydrogenase, gluconate-6-phosphate dehydrogenase and $NADP^+$-dependent isocitrate dehydrogenase under conditions of increased requirement for NADPH. Therefore, the involvement of the mannitol cycle in generating NADPH is doubtful, although it can not be excluded that it plays a role under certain specific conditions.

Although mannitol is accumulated in *Aspergilli* under most circumstances, the levels show strong variations. Dijkema *et al.* (1985, 1986) found mannitol accumulation in *A. nidulans* associated with low oxygen levels, low pH and ammonium as a nitrogen source. *A. clavatus* grown on glucose converted a considerable part of the sugar (30%) into mannitol which was reconsumed after exhaustion of the glucose (Corina and Munday, 1971). In conidiospores of

A.oryzae mannitol is present in high amounts and is metabolized during the very early stages of germination (Horikoshi *et al.*, 1965). Several reports have been published on the production of mannitol by fungi (Smiley *et al.*, 1967, Hendriksen *et al.*, 1988). The fermentation process is quite slow: about 80 g mannitol per liter is produced from glucose in 12 days by *Aspergillus candididus* with yields that approached 50% of the glucose consumed (Smiley *et al.*, 1967). The process was strongly dependent on the initial pH of the medium and showed an optimum in the aeration level required. In an earlier publication it was observed that very low aeration gave optimal results (Birkenshaw *et al.*, 1931). No dissolved oxygen tensions are given for these experiments so it is difficult to make a comparison. However, in contrast to the organic acid fermentations a limited aeration seems to be favourable. Mannitol accumulates in the medium as long as there is external glucose available, but it is remetabolized as soon as the glucose has been consumed.

These observations leave us with numerous questions on the function of mannitol for the fungal cell and about how mannitol pool formation and degradation is regulated. Except for the apparent function as a storage compound in conidia of *A. oryzae* no clear answers can be given. The role of mannitol in *A. niger* is discussed in Chapter 8 of this thesis.

3.2 Glycerol

Glycerol is a common polyol in fungi. Like in many other fungi it is reported to play a major role in the osmotic adjustment of *A. nidulans* (Beever and Laracy, 1986). In *A. nidulans* glycerol formation is stimulated by strong aeration. Upon starvation it is the first polyol which is metabolized by the fungus (Dijkema *et al.*, 1985). In *Phycomyces blakesleeanus* large amounts of glycerol were synthesized during the early stages of germination (Van Laere and Hulsmans, 1987).

The degradation route of glycerol has been extensively studied especially in *A. nidulans.* Several mutants have been isolated in the degradation pathway of glycerol. A glycerol uptake mutant (*glc*C), unable to grow on glycerol, was isolated (Visser *et al.*, 1988). Mutants in glycerol kinase (*glc*A) and in an FAD-dependent glycerol-3-phosphate dehydrogenase (*glc*B, *glc*D and *glc*G) demonstrated that the route of degradation is via glycerol-3-phosphate and subsequent oxidation to dihydroxyacetonephosphate (Arst *et al.*, 1990, Hondmann *et al.*, 1991). Mutants in the regulation of glycerol metabolism have been isolated as well (Hondmann and Visser, 1992). Witteveen *et al.* (1990b) (Chapter 7 of this thesis) demonstrated that the same route of glycerol degradation is followed in *A. niger.*

The biosynthetic route of glycerol is supposed to involve reduction of dihydroxyacetonephosphate by an NAD^+-dependent glycerol-3-phosphate dehydrogenase. In *Saccharomyces cerevisiae* the level of this enzyme was increased during osmotic stress situations (Blomberg and Adler, 1989). The glycerol-3-phosphate is dephosphorylated by a phosphatase. The glycerol-3-

phosphate phosphatase from the green alga *Dunaliella tertiolecta* was characterized (Belmans and Van Laere, 1988). An important factor determining the *in vivo* activity of the enzyme was found to be the glycerol-3-phosphate level in the cell. This means that the main control point in this organism is at the level of the glycerol-3-phosphate dehydrogenase. An $NADP^+$-dependent glycerol dehydrogenase has been isolated from *A. nidulans* and *A. niger* (Schuurink *et al.*, 1990). The enzyme is constitutive in both fungi and catalyzes the $NADP^+$-dependent oxidation of glycerol to dihydroxyacetone. The K_m for glycerol is very high (around 1 M), and the enzyme shows a strong inhibition by $NADP^+$. Therefore it was assumed by the authors that the enzyme catalyzes *in vivo* the reduction of dihydroxyacetone. It is not clear whether this enzyme plays a role in glycerol formation since an alternative route via reduction of dihydroxyacetonephosphate to glycerol-3-phosphate is assumed to be the predominant biosynthetic route of glycerol.

Glycerol is also involved in the catabolic pathway of dihydroxyacetone and D-galacturonic acid of *A. nidulans* and *A. niger* (Hondmann *et al.*, 1991, Witteveen *et al.*, 1990 or Chapter 7 of this thesis).

Glycerol accumulates in an early stage of citric acid producing mycelium. It was suggested to play a role in the initiation of this process (Legiša and Mattey, 1986). In Chapter 8 the role of glycerol in *A. niger* discussed.

3.3 Erythritol

Although erythritol is a common polyol in many fungi it has received little attention. According to Dijkema *et al.* (1985, 1986) its biosynthesis is related to the flux through the pentose phosphate pathway. Beever and Laracy (1986) showed that this polyol plays also a role in osmotic adjustment of *A. nidulans*. In line with the biosynthetic routes of glycerol and mannitol it is presumably formed from erythrose-4-phosphate by subsequent reduction and dephosphorylation. The role of erythritol in *A. niger* is discussed in Chapter 8 of this thesis.

Erythritol catabolism has been recently investigated in *A. nidulans* in our laboratory. It involves an NAD^+-dependent erythritol dehydrogenase which is highly specific for erythritol in the oxidative reaction and is most active with L-erythrulose in the reductive reaction (Hondmann *et al.*, unpublished results).

3.4 Pentitols

Arabitol has been reported to accumulate under certain conditions in some filamentous fungi like *A. nidulans* (Dijkema *et al.* 1985). Ribitol is found to accumulate less often but plays a prominent role in some fungi (see for example Corina and Munday, 1971).

Chiang and coworkers established the catabolic pathway for D-xylose and L-arabinose (Chiang *et al.*, 1958; Chiang and Knight 1959, 1960a and b, 1961). Two polyols play a role in these pathways, namely: L-arabitol and xylitol. Information on pentitol metabolism in filamentous fungi is scarce and has only

received attention in relation to pentose catabolism.

Pentose metabolism is of importance in relation to the degradation of hemicellulose. *A. niger* is able to produce a whole spectrum of enzymes for the degradation of these heteropolysaccharides (Johnson *et al.*, 1989). The involvement of the pentitols in the induction of these enzymes has already been shown (v.d. Veen *et al.*, 1993). Witteveen *et al.* (1989) (Chapter 6 of this thesis) elucidated the catabolic pathway of L-arabinose and D-xylose. They isolated a D-xylulose kinase mutant of *A. niger*. Two xylitol dehydrogenases which are part of the catabolic pathway of L-arabinose have been purified and characterized (Chapter 7).

4. AIM AND OUTLINE OF THE THESIS

The organism of study in this thesis is *A. niger*. Although it has been used already for many years in several industrial processes for organic acid production, there is still little information available about the details of the regulation of overflow metabolism. This thesis is a contribution to this field. It can be divided in two main topics: (1) regulation of gluconic acid formation and (2) polyol metabolism. Both the polyols and the organic acids are products of overflow metabolism and although there are many differences in the conditions under which they are produced and their function in the cell, there are also similarities. The aim of this thesis is to try to reach some understanding about the basic regulatory mechanisms of these processes. The work presented here was done as part of a project to study the possibilities for strain improvement for gluconic acid formation. The purely applied aspect of that work has been left out of this thesis but has played an important role in the choices that have been made during this study.

The gluconic acid biosynthesis is the simplest of all the organic acids produced by *Aspergillus*. This has the advantage that it is easier to get insight in the regulation of the process. The actual formation of gluconic acid requires only one enzyme: glucose oxidase. Furthermore catalases and a lactonase are involved. There were contradictory reports on the localization of these enzymes in the hyphae. In Chapter 2 evidence is provided for a cell wall localized glucose oxidation system. For studying the regulation of induction of especially glucose oxidase, a useful approach is the isolation of regulatory mutants. The extracellular localization of the enzyme and the fact that H_2O_2 is formed by glucose oxidase enabled easy detection of glucose oxidase in intact mycelium grown on agar plates. This detection system was used to isolate mutants. A first description of the mutants and their genetic characterization is given in Chapter 3. A thorough genetic analysis is important for the construction of strains in a strain improvement program. Mutations can be combined in a single strain by standard genetic techniques (Debets, 1990) when detailed information is available on the

localization and type of the mutations. An accurate analysis of the induction parameters combined with an analysis of some of the mutants described in Chapter 3 resulted in a model for the induction of glucose oxidase, catalase and lactonase. The isolation of the glucose oxidase gene allowed further analysis of the regulatory mechanism at the molecular level. In Chapter 4 the data leading to a model for the regulation of induction of the enzymes are presented.

As for the studies on polyol metabolism there was a general lack of biochemical information about the pathways involved in *A. niger*. Therefore it was decided to analyze some of the metabolic pathways involved in polyol metabolism first. In Chapter 5 the catabolic pathway of glycerol is studied. Glycerol is an important polyol in *A. niger* and is also one of the most mobile pools, so degradation of glycerol might also play a role during growth on other carbon sources than glycerol. In Chapter 6 the D-xylose and L-arabinose catabolism is described which involves the corresponding polyols as intermediates in the first steps of the degradation of these compounds. There were several reasons for studying pentose metabolism. There first was the indication that polyols were important intermediates in the first steps of pentose catabolic pathways (Chiang and Knight, 1961), therefore studying this pathway would contribute to the understanding of the kinetics of polyol synthesis and degradation. Another reason was the relation with hemicellulolytic enzymes. One of the options to improve gluconic acid formation was to investigate the possibilities of using relatively raw substrates and not just pure glucose. Hemicelluloses form an important impurity in raw starch. Pentoses are the major components of the hemicelluloses. Understanding the degradation pathway of D-xylose and L-arabinose is therefore important for the analysis of the regulation of hemicellulolytic enzymes. *A. niger* is an important producer of these enzymes. In Chapter 7 the purification and characterization of two xylitol dehydrogenases is described. These enzymes are part of the L-arabinose catabolic pathway. They catalyze similar reactions, the oxidation of xylitol to either L- or D-xylulose, but *in vivo* in exactly the opposite direction. Special attention was given to their kinetic properties with respect to their common product/substrate xylitol. This analysis contributes to our understanding of the type of mechanisms that the cell uses to control the levels of its polyol pools. Chapter 8 contains the description of a series of experiments carried out in an attempt to obtain more information on the role which the various polyols play in the physiology of *A. niger*. This chapter also describes that *A. niger* can accumulate large amounts of polyols in the medium in a way that bears similarities with the accumulation of TCA cycle related organic acids.

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Chapter 2

Localization of glucose oxidase and catalase activities in *Aspergillus niger*[1]

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SUMMARY

The subcellular localization of glucose oxidase (EC 1.1.3.4) in *Aspergillus niger* N400 (CBS 120.49) was investigated by (immuno)cytochemical methods. By these methods, the bulk of the enzyme was found to be localized in the cell wall. In addition, four different catalases (EC 1.11.1.6) were demonstrated by nondenaturing polyacrylamide gel electrophoresis of crude extracts of induced and noninduced cells. Comparison of both protoplast and mycelial extracts indicated that, of two constitutive catalases, one is located outside the cell membrane whereas the other is intracellular. Parallel with the induction of glucose oxidase, two other catalases are also induced, one located intracellularly and one located extracellularly. Furthermore, lactonase (EC 3.1.1.17) activity, catalyzing the hydrolysis of glucono-δ-lactone to gluconic acid, was found to be exclusively located outside the cell membrane, indicating that gluconate formation in *A. niger* occurs extracellularly.

INTRODUCTION

The subcellular localization of glucose oxidase in *Aspergillus niger* has been a point of discussion. For a long time the enzyme was assumed to be located intracellularly (Pazur, 1966). This was further supported by ultrastructural studies by van Dijken and Veenhuis (1980) who concluded that the enzyme was located in peroxisomes. Mischak *et al.* (1985) showed that under manganese deficient growth conditions glucose oxidase is found almost quantitatively in the culture fluid. Their explanation for this was that a cell wall localized glucose oxidase entered the culture fluid because of an altered cell wall composition resulting from manganese deficiency. Two other arguments are in favor of an extracellular localization of glucose oxidase: (1) glucose oxidase is strongly glycosylated (Pazur *et al.*, 1965), a phenomenon which has never been observed for any peroxisomal protein; and (2) the amino acid sequence derived from the glucose oxidase DNA sequence shows a typical secretion signal peptide (Frederick *et al.*,

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1990). In this study we reexamined the localization of glucose oxidase in *A. niger*.

An extracellular localization of glucose oxidase activity implies an extracellular production of hydrogen peroxide. The sequestration of this toxic process will prevent damage to the cell. However, there remains the necessity for an efficient removal of H_2O_2 since this compound is known to inactivate glucose oxidase (Kleppe, 1966) and can easily diffuse over the cell membrane. Mycelium containing high levels of glucose oxidase can produce 50-100 mmol hydrogen peroxide per h per g of mycelium (dry weight) without large scale cell lysis or inactivation of glucose oxidase; therefore, an effective protective system must exist. Both catalases and peroxidases might be involved in this process. The induction and localization of catalases under glucose oxidase-inducing conditions were therefore also investigated.

MATERIALS AND METHODS

Strains, Media and Growth. All experiments were performed with *A. niger* N400 (CBS 120.49). Mycelium was grown in a 3-liter fermenter using the same medium as described previously (Witteveen *et al.*, 1990a). To obtain mycelium which was induced for glucose oxidase, the oxygen level was kept above 30% air saturation (i.e. the dissolved oxygen concentration is 30% of the oxygen level in a solution saturated with air) during the whole growth period, or glucose oxidase was induced by increasing the oxygen level to 50 % air saturation 4 - 6 h before harvesting the mycelium. The latter method gave somewhat lower activities of glucose oxidase. The noninduced mycelia for the catalase induction and localization experiments were grown at low oxygen levels (less than 7 % air saturation) while the other conditions were identical to those under inducing conditions. Mycelium grown this way contains no detectable glucose oxidase activity (Witteveen *et al.*, 1990a). In all cases the mycelium was grown at 30°C and harvested at approximately 24 h after inoculation.

Preparation of the protoplasts. Protoplasts were released from the mycelium using 1-3 mg of Novozyme 234 ml^{-1} in protoplast stabilizing buffer (PSB) containing 10 mM Tris HCl (pH 7.5), 50 mM $CaCl_2$, and 1.33 M sorbitol. The mycelium concentration was approximately 50 mg (wet weight) ml^{-1}. Protoplasts were separated from the mycelium by filtration over glass wool, washed twice with PSB and subsequently lysed in 20 mM sodium phosphate buffer (pH 6.0). To complete cell lysis, the suspension was sonicated. The supernatant, obtained after 5 min centrifugation at 10,000 x g, was used for enzyme measurements and analysis by polyacrylamide gel electrophoresis. Cell wall-bound catalase was released by partial degradation of the cell wall. This was achieved by incubation of the mycelium for 30 min in PSB with 0.1 mg Novozyme 234 ml^{-1}. These levels of Novozyme did not cause detectable degradation of the catalase, and no catalase activity bands were detectable on gel when only the Novozyme solution was applied on gel.

Biochemical analyses. Crude cell-free extracts were prepared as described previously (Witteveen *et al.*, 1989) except that 20 mM sodium phosphate (pH 6.0) was used as the extraction buffer.

Separation and visualization of catalase was performed by using a modification of the procedure described by Gregory and Fridovich (1974). Crude extracts were separated on a 1.5 mm thick, 7.5 % polyacrylamide gel containing 20 % glycerol. For visualizing catalase activities the gel was incubated for 30 min in 50 ml of 50 mM sodium phosphate (pH 7.0) containing 0.2 mg of horseradish peroxidase ml^{-1}. Twelve microliters 30% H_2O_2 was then added, and the mixture was incubated for 10 min. Subsequently, the liquid was washed away quickly and an o-dianisidine solution (0.5 mg ml^{-1}) was added to visualize the presence of H_2O_2. At positions where catalase was present, no staining occurred.

Glucose oxidase was measured spectrophotometrically as described previously (Witteveen *et al.*, 1990a). Catalase activity was measured by monitoring the decrease in A_{240} caused by H_2O_2 degradation (Aebi, 1974). Glucose-6-phosphate dehydrogenase and $NADP^+$-dependent glutamate dehydrogenase were assayed by the method of Bruinenberg *et al* (1983). Dihydroxyacetone (DHA) reductase was assayed by the method of Witteveen *et al.* (Witteveen *et al.*, 1990b). Citrate synthase was assayed as described by Stitt (1984). Lactonase activity was determined by titration with 50 mM sodium hydroxide of a 50 ml 10 mM sodium phosphate buffer (pH 6.0) with an appropriate amount of extract or culture filtrate to which 0.45 g glucono-δ-lactone was added at the start of the assay. The acidification was followed for 5 min. Corrections were made for spontaneous hydrolysis.

SDS polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970) using the Pharmacia-LKB midget electrophoresis system. For detection of glucose oxidase on Western blot (immuno blot) an alkaline phosphatase assay (Biorad) was used.

Protein concentrations were estimated, after denaturation and precipitation of protein with sodium deoxycholate and trichloroacetic acid (Bensadoun and Weinstein, 1976), by a microbiuret method (Itzhaki and Gill, 1964) using bovine serum albumin as standard.

Generation of antisera. Rabbit polyclonal antibodies against glucose oxidase were raised by standard procedures by using purchased glucose oxidase (Boehringer, grade I) which, prior to injection, was further purified as follows. The enzyme was dissolved in 20 mM Tris HCl (pH 7.4) containing 0.5 M NaCl, 0.25 mM $MnCl_2$ and 0.25 mM $CaCl_2$ and loaded to a concanavaline-Sepharose 4B column (Pharmacia). The enzyme was eluted with 20 mM sodium acetate buffer (pH 4.5) containing 0.5 M NaCl and 1 M α-methyl mannoside. The eluted protein was dialyzed extensively against 0.1 M sodium phosphate buffer (pH 7.5). The glucose oxidase solution thus obtained was denatured and deglycosylated using N-glycanase (Genzyme) according to the procedure described by Tarentino *et al.* (1985) and subsequently used for immunization. The protein prepared this way showed a single, sharp band on an SDS polyacrylamide gel. The antisera obtained were further purified by incubation with D-xylose- and acetate-grown *A. niger* mycelia, which lack glucose oxidase, to remove non-specific antibodies, directed against antigens other than glucose oxidase. Finally, the IgG fraction was purified on a Biorad Affi-Gel Blue column according to the manufacturer's procedure.

The antibodies thus obtained were used in the experiments described below.

Immunocytochemical and cytochemical experiments. For immunocytochemical experiments, the mycelium was fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 90 min at 0°C, dehydrated in a graded ethanol series and embedded in Lowicryl K4M (Zagers *et al.*, 1987). Immunolabeling was performed on ultrathin sections by the protein A-gold method described by Slot and Geuze (1984).

Cytochemical staining experiments were performed on glutaraldehyde-fixed cells. Catalase activity was demonstrated by the 3-3' diaminobenzidine (DAB) based method, glucose oxidase activity was demonstrated by using $CeCl_3$ as the H_2O_2-capturing agent (Veenhuis *et al.*, 1976).

RESULTS

The overall cell morphology of *A. niger*, grown on glucose, is shown in Fig.1. The cells contain the usual cell organelles such as nucleus, mitochondrial and vacuolar profiles and strands of endoplasmic reticulum. Remarkably, the proliferation of microbodies (both in size and number) is low in these cells. Cytochemically, catalase activity could be demonstrated inside these microbodies. In addition, the outer layer of the cell wall was densely stained (Fig. 2). In cytochemical experiments using $CeCl_3$ for the detection of glucose oxidase activity, the bulk of the reaction products were confined to extracellular regions. Apart from the periplasmic space, staining was also observed in the cell wall and associated with the outer cell wall layer (Fig. 3). It is, however, unclear to what extent the staining of this outer layer is specific. During incubation of cells with $CeCl_3$ and glucose, the incubation mixture assumed a yellow/brownish color, most probably due to precipitation of cerium perhydroxyde formed from H_2O_2 that had leaked from the site of its generation. Therefore, staining of the outer cell wall layer may, at least partly, be due to adsorption of cerium precipitate formed in the incubation mixture.

The procedure given to prepare antigen and antibody turned out to be essential to obtain reproducible results. Polyclonal antibodies against nondeglycosylated glucose oxidase turned out to be nonspecific when tested by Western blot after SDS-PAGE of mycelial extracts. However, antibodies raised against deglycosylated and denatured glucose oxidase revealed only the broad protein band characteristic for this enzyme. When used in immunocytochemical experiments these antisera showed a minor reactivity against cell walls of cells of *A. niger* which lacked glucose oxidase. This non-specific labeling could be removed by incubation of the antisera with intact D-xylose- and acetate-grown mycelia and subsequent purification of the IgG fraction by using an Affi-Gel Blue column. When applied to glucose oxidase-containing mycelium the purified antibodies resulted in an intense labeling of the cell wall (Fig. 4).

The possibility of an exclusive localization of glucose oxidase in the cell wall of *A. niger* was further studied biochemically. We measured the specific activities

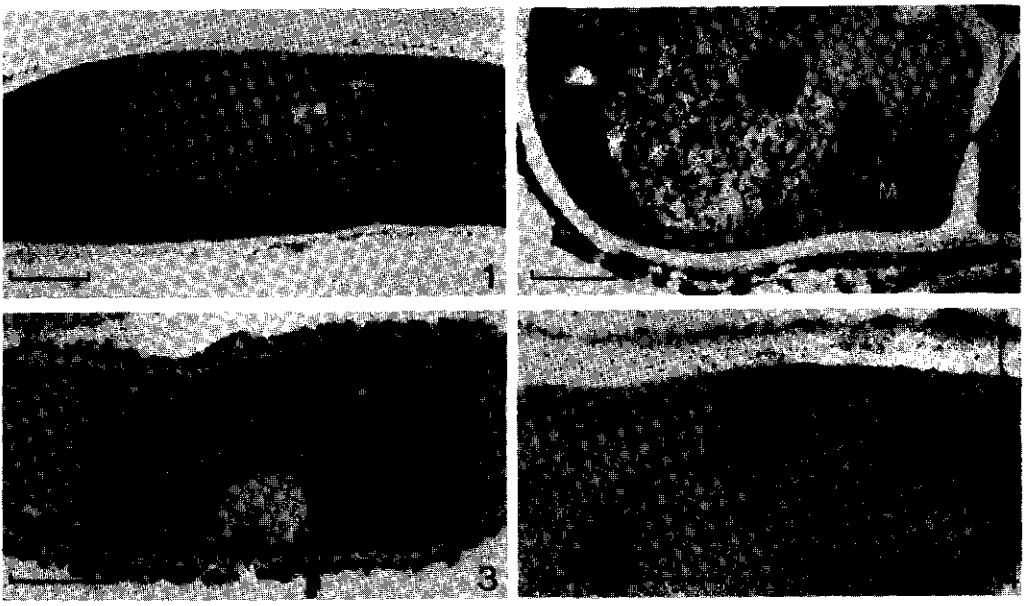

Fig. 1 Thin section of a $KMnO_4$-fixedcell of glucose-grown *A. niger* to show overall cell morphology. The small microbody is indicated by an arrow. N, nucleus. Bar, 0.5 μm.

Fig. 2. Cytochemical staining of catalase activity (3,3'-diaminobenzidine-H_2O_2) in a glucose-induced cell of *A. niger*. Inaddition to the peroxisomes (arrow), the outer cell wall layer isdensely stained.Mitochondrial staining is due to cytochrome c oxidase or peroxidase activity.V, vacuole; M, mitochondria. Bar, 0.5 μm.

Fig. 3. Cytochemical staining of glucose oxidase activity after aerobic invubations of cells with $CeCl_3$ and glucose. Specific reaction products are confined to the periplasmic space and the cell wall. Bar, 0.5 μm.

Fig. 4. Immunochemical staining using specific antibodies against glucose oxidase and protein A-gold. Labeling is confined to the cell wall. Peroxisomes are not labeled. N, nucleus; P, peroxisome. Bar, 0.5 μm.

in lysates from protoplasts of glucose oxidase-induced mycelium and compared these with the specific activities in extracts of intact mycelium. This method also gave information on the distribution of catalase activities. Besides catalase and glucose oxidase three cytoplasmic enzymes were measured as a control, together with citrate synthase which is known to be localized in mitochondria (Table 1). The results convincingly indicate a localization outside the cell membrane of a catalase activity and of glucose oxidase activity.

The intermediary value for the protoplast/mycelium specific activity ratio of catalase as compared to that of glucose oxidase and the intracellularly located enzymes can be explained by the presence of more than one catalase. To clarify this, we separated mycelial and protoplast extracts on a nondenaturing polyacrylamide gel and visualised the catalases by activity staining. These experiments revealed the presence of at least four different catalases, two of which were induced parallel with glucose oxidase. Under noninducing conditions, a weak

Table 1 Enzyme activities in extracts of mycelium and protoplasts of glucose oxidase-induced and non-induced cells.

	INDUCED						NON-INDUCED	
	Gdh	DHAred	G6Pdh	CS	CAT	GOX	G6Pdh	CAT
protoplast	0.68	0.62	0.80	0.06	21	0.11	0.46	3.9
mycelium	0.42	0.62	0.89	0.04	178	5.4	0.6	38.6

GDH = $NADP^+$-dependent glutamate dehydrogenase, DHAred = NADPH dependent dihydroxyacetone reductase, G6Pdh = glucose-6-phosphate dehydrogenase, CS = citrate synthase, CAT = catalase, GOX = glucose oxidase. The activities are in $\mu mol\ min^{-1}$ (mg protein)$^{-1}$

band and a stronger catalase activity band were visible (Fig. 5, lane A). Protoplast extracts of this mycelium showed only the weaker upper band (Fig. 5, lane B). From this, we conclude that this particular catalase is intracellularly located (catalase I), whereas the more rapidly migrating form is apparently localized in the cell wall (catalase II). The presence of a catalase localized outside the cell membrane under noninducing conditions was also indicated by the difference in relative specific activities in protoplast and mycelial extracts (Table 1). Under conditions where glucose oxidase was induced, an additional catalase band of high mobility was found (catalase III) whereas at the level of catalase I a strong band became visible (Fig. 5, lane C and E). In protoplasts of an induced mycelium only the high mobility band was visible, whereas at the position of catalase I only a weak activity was found (Fig. 5, lane D). From this we conclude that the high mobility band, which becomes visible under inducing conditions (catalase III), is intracellular and that the low mobility band appearing under inducing conditions is not the intracellular catalase I but a different extracellular catalase (catalase IV). To obtain further confirmation on the cell wall localization, we treated induced mycelium with low concentrations of Novozyme 234 to see whether eventual cell wall localized catalases could be released from the mycelium. Analysis of catalase activity on gel showed that at the position of the two catalases which we assumed to be cell wall localized, activity bands were visible (Fig. 5, lane F). This was not the case at the position of catalase III.

Besides glucose oxidase and catalase we also measured lactonase. Activities of this enzyme were found both in mycelial extracts and in the culture fluid (approximately 50 %). We were not able to measure this enzyme in the protoplast extracts. These data indicate that lactonase is also transported across the cell membrane and, therefore, that all enzyme activities involved in gluconate biosynthesis reside extracellularly.

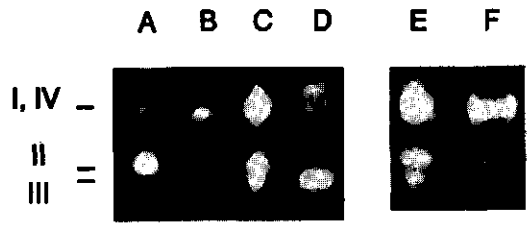

Fig 5. Catalases in mycelial and protoplast extracts of induced and noninduced mycelia visualized on nondenaturing polyacrylamide gels. lanes: A and B, extracts of mycelium and protoplasts of noninduced mycelium; C and D, extracts of mycelium and protoplasts of induced mycelium; E, same as that described for lane C; F, material released from induced mycelium when low concentrations of Novozyme 234 were used.

DISCUSSION

The results described in this paper confirm the conclusion of Mischak *et al.* (1985) that, in *A. niger* glucose oxidase is located in the cell wall. The (immuno)-cytochemical data (Figs 3 and 4) show this very clearly and the localization outside the cell membrane is confirmed by the ratio of the specific glucose oxidase activities in protoplast and mycelial extracts (Table 1). The results of van Dijken and Veenhuis (1980), who described a peroxisomal localization of glucose oxidase, are in contradiction with these results. Their cytochemical data may be explained as an artifact of the method used. Within this view, the observed positive staining of microbodies, caused by catalase, may result from H_2O_2 generated by glucose oxidase located in the cell wall after the addition of glucose to the incubation sample, which subsequently diffuses inside the cell. The high glucose oxidase activities in the cell wall might produce sufficient H_2O_2 for the peroxisomal staining observed. Peroxisomal staining has been observed by us after adding H_2O_2 to the cell suspension. The observation of van Dijken and Veenhuis that the size and number of peroxisomes increased with glucose oxidase synthesis is more difficult to explain and might be an indication of strain differences. The extracellular localization of glucose oxidase is in line with the finding that glucose oxidase is glycosylated (Pazur, 1966) and that the amino acid sequence derived from the DNA sequence (Frederick *et al.*, 1990) clearly shows a characteristic secretion signal peptide. Similarly, in Penicillium strains glucose oxidase is also excreted in the culture fluid (Kusai *et al.*, 1960). The peroxisomal localization of glucose oxidase in other *A. niger* strains cannot be ruled out but is considered unlikely by us.

The differences in localization and induction patterns of catalase permits the conclusion that at least four different catalase proteins are formed by *A. niger* and that the bands visible on the gels are not degradation products of a single

catalase. The catalases I and II represent more or less constitutive catalases, whereas the catalases III and IV are induced under conditions when glucose oxidase is formed. Chary and Natvig (1989) showed the presence of three catalase genes in *Neurospora crassa*. However, the information available is insufficient for a comparison of the individual enzymes to be made.

The extracellular localization of a catalase as concluded from the cytochemical experiments (Fig. 2), the patterns on native gel of mycelial and protoplast extracts stained for catalase activity (Fig.5), and the relative specific activities of catalase in mycelial and protoplast extracts, is further supported by the observation that a catalase isolated from *A. niger* is glycosylated (Kikuchi-Torii *et al.*, 1982; Wasserman and Hultin, 1981).

The protection of glucose oxidase in the cell wall against inactivation by H_2O_2 might very well be achieved by catalase, despite its relative low affinity for H_2O_2, because inactivation as observed by Kleppe (1966) plays only a role at H_2O_2 concentrations up to at least a few millimolar. The very strong induction of a catalase which is localized at the site of H_2O_2 formation, in the cell wall, makes it very likely that this enzyme plays a major role in the breakdown of H_2O_2 formed by glucose oxidase. The role of catalase in the protection of the cell interior is less clear. Studies in the methylotrophic yeast *Hansenula polymorpha* showed that cytochrome c peroxidase may play an essential role in the detoxification of H_2O_2. In this organism catalase could only effectively compete with cytochrome c peroxidase when it was located at the site of H_2O_2 generation in the peroxisomes (Verduyn *et al.*, 1988). The induction of an intracellular catalase is an indication that, in *A. niger* catalase plays a role in the intracellular detoxification. However, it is very well possible that other enzymes are involved.

Lactonase activity has been demonstrated before in *A. niger.* It has been purified from Novozyme 188, a commercial enzyme preparation from *A. niger* (Bruchmann *et al.*, 1987). The presence of a lactonase is not obligatory since the hydrolysis of the lactone also occurs spontaneously, although at a lower rate. The extracellular localization of lactonase implies that the complete glucose oxidation system is localized outside the cell membrane.

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Chapter 3

Glucose oxidase overproducing and negative mutants of *Aspergillus niger*: a phenotypic and genetic analysis.[1]

Cor F.B. Witteveen, Peter JI van de Vondervoort, Klaas Swart, Jaap Visser

SUMMARY

A colony staining method was used to isolate mutants in *Aspergillus niger* which showed altered glucose oxidase (GOX) induction. The mutants were isolated under weakly or non-inducing conditions. A stable glucose oxidase negative, a low producing and a series of overproducing mutants were found. Among the overproducing mutants, different phenotypes were found with respect to glucose oxidase induction. Genetic analysis showed that these overproducing mutants could be classified into seven complementation groups and that the two glucose oxidase negative mutants originate from different complementation groups. These nine *gox* loci were assigned to linkage groups using master strains with marked chromosomes. Three *gox* loci are in linkage group II, one is in III, two are in V and two are in linkage group VII. One weak glucose oxidase overproducing mutant could not be assigned to one of the linkage groups. The mutants were tested for glucose oxidase production in surface and submerged cultures, indicating a fair correspondence between those methods. From the characteristics of the mutants it can be concluded that oxygen- and carbon source dependent induction are mediated by different factors.

INTRODUCTION

Several reports have been published describing the selection of *Aspergillus niger* mutants overproducing glucose oxidase (Kundu and Das, 1985; Fiedurek *et*

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[1]This chapter is a combination of two previously published articles:

Witteveen CFB, P van de Vondervoort, K Swart and J Visser. 1990. Glucose oxidase overproducing and negative mutants of *Aspergillus niger*. Appl Microbiol Biotechnol **33**:683-686.

Swart K, PJI van de Vondervoort, CFB Witteveen and J Visser. 1990. Genetic localization of a series of genes affecting glucose oxidase levels in *Aspergillus niger*. Curr Genet **18**:435-439.

al., 1986; Markwell *et al.*, 1989). In these reports the main purpose was to find overproducing mutants. Little attention has been given to the mechanism of induction of glucose oxidase in these or other publications. Our goal was to try and find different classes of mutants which would clarify the mechanism of induction. Factors which are of importance for the induction of glucose oxidase are high glucose concentration, high oxygen levels and a pH around 5 (Röhr *et al.*, 1983). We expected these environmental effects to be transduced by different factors at a molecular level. This means that mutants might be found which have an altered induction pattern with respect to only part of the environmental factors mentioned above.

Besides selecting various overproducing strains we looked for glucose oxidase negative strains. These latter strains are of interest to apply in citric acid fermentations, where gluconate is an undesired byproduct, but they might as well throw some light on the physiological importance of the enzyme for the fungus.

The genetic analysis of the mutants supplements useful information to the phenotypical characterization. The classification into complementation groups provides a solid base for further comparison and analysis of the mutants.

MATERIALS AND METHODS

Strains. The *A. niger* wild type strain N400 (CBS 120.49) and auxotrophic mutants derived from N400 were used in the mutagenesis experiments. These mutants are: N423, short conidiophores (*csp*A1) and nicotinamide deficient (*nic*A1); N573, *csp*A1 and biotin deficient (*bio*A1); N597, *csp*A1 and p-aminobenzoic acid deficient (*pab*A1) (Bos *et al.*, 1988). N400 is described as a citric acid and gluconic acid producer. For genetic analyses we used master strains described by Bos *et al.* (1988) and Swart *et al.* (1990).

Media and growth. Medium used in agar plates contained per litre: 6 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, and traces of $MnCl_2$, $ZnSO_4$, $CuSO_4$ and $FeSO_4$, 12 g agar and one of the carbon sources indicated. The pH was adjusted to 5.8. Medium for submerged cultures contained per litre: 1.2 g $NaNO_3$, 0.5 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.5 g yeast extract and 0.04 ml of the trace metal solution described by Vishniac and Santer (1957). As carbon source 0.56 M glucose, 0.28 M fructose or 0.1 M sodium acetate was used. Where necessary the media were supplemented with 1 mg l^{-1} nicotinamide, 4 μg l^{-1} biotin or 1.37 mg l^{-1} p-aminobenzoic acid. To medium for the fermenter 0.1 ml polypropylene glycol per liter was added as antifoam. Sugars were autoclaved separately. In all experiments *A. niger* was grown at 30°C.

Conidiospores used in the genetic experiments were harvested from complete medium according to Pontecorvo *et al.* (1953), except that glucose was replaced by 2 % (w/v) sucrose. Conidiospores for the submerged cultures were harvested from potato extract, 2% (w/v) glucose medium (PDA).

Mutagenesis. A suspension of conidia (usually 10 ml, $5*10^6$ conidia ml^{-1}) in a petri dish was irradiated with U.V. (2 J m^{-2} s^{-1}) for 1.5 - 2 min. In the different experiments 33-78 % survival was found.

Mutant selection. Colonies were stained for glucose oxidase using a modification of the method described by Ramasamy *et al.*, (1985). The mutagenized spores were diluted and plated in a density of 100-1000 viable spores per petri dish. Apart from the medium described above, the plates contained 2.5 mM o-anisidine and one of the following carbon sources: 50 mM fructose, 2 or 50 mM glucose, 50 mM gluconate or 100 mM glycerol. After 40 h of colonial growth a solution containing 20 mM sodium phosphate buffer pH 7.0, 0.1 M glucose and 20 μg ml^{-1} horse radish peroxidase (Boehringer), was added. Glucose oxidase containing colonies colored brownish-red.

Acid production on agar plates. Selected mutants were tested for acid production on agar plates using a petri-dish method adapted from Bosnjak *et al.* (1986). In a petri-dish a two layer-system was used. The bottom layer contained agar plate medium with 25 g l^{-1} glucose. The top layer contained per liter: 0.2 g KH_2PO_4, 0.15 g $MgSO_4.7H_2O$, 0.3 g $(NH_4)_2HPO_4$, 25 g glucose, 9 g $CaCO_3$ and 15 g agar. The inoculated plates were incubated for 3-4 days. Acid produced during growth dissolved the $CaCO_3$.

Acid production in submerged culture. A preculture of 300 ml medium in a 1 l Erlenmeyer flask was inoculated with 5-10 * 10^6 conidiospores ml^{-1}, which were harvested from PDA medium, with a 0.8 % (w/v) NaCl, 0.05 % Tween-20 solution. After 6-8 h the preculture was added to 1.9 l medium in a 3 l jacketed Applikon fermenter with pH and dissolved oxygen control. The pH was held constant at 5.5 by automatic addition of 5 M NaOH. Approximately 0.2 vvm air was sparged in the fermenter. The dissolved oxygen tension was controled as accurately as possible by addition of pure oxygen or pure nitrogen gas. Control of oxygen levels was necessary to achieve reproducible results. Dissolved oxygen levels are expressed as the percentage of air saturation.

Complementation tests. Preliminary complementation experiments using heterokaryons had shown that staining for GOX was not uniform in heterokaryons and therefore no conclusive evidence could be obtained concerning the Gox mutations. Subsequently, tests for complementation of different Gox mutations were always done in diploids. Diploids were isolated from heterokaryons of different Gox mutants carrying different auxotrophic markers in a way described previously (Bos *et al.*, 1988). A conidiospore suspension of a diploid was inoculated on GOX staining plates (see above), mostly on different media with the different GOX induction conditions of the parental strains. In all tests it appeared that the Gox mutations behaved as recessive mutations, thus a clearcut conclusion on complementation could be obtained: for GOX^+ mutants a positive staining (comparable to one of the parents) indicates no complementation, no staining (or wild type staining) is evident for different complementation groups; for GOX^- mutants no staining is indicative of the same complementation group and wild type staining means different complementation groups.

Genetic analyses. The procedures for genetic analysis have been described in detail earlier (Bos *et al.*, 1988): somatic diploids from a mutant and a master strain were isolated and the heterozygous diploid was haploidized on CM supplemented with histidine or urea, required by the *his*D and *cnx*C mutations in the master strains respectively. To induce haploidization, benomyl was added to this medium. Benomyl was dissolved in acetone and used at a final concentration of approximately 0.25 mg/L. Segregants were isolated, purified and tested for genetic markers and GOX induction.

Analyses. Protein concentrations were estimated, after denaturation and precipita-

tion of protein with sodium deoxycholate and trichloroacetic acid (Bensadoun & Weinstein, 1976), by the microbiuret method (Itzhaki and Gill, 1964) using serum albumin as standard.

Cell-free extracts were prepared as described (Witteveen *et al.*, 1989) except that 20 mM sodium phosphate pH 6.0 was used as extraction buffer.

Glucose oxidase was measured spectrophotometrically in cell-free extracts of mycelium harvested 24 h after inoculation. The following assay mixture was used: 20 mM sodium phosphate buffer pH 6.0, 0.16 g l^{-1} o-dianisidine, 100 mM glucose and 20 μg ml^{-1} horse radish peroxidase. The reaction was followed at 450 nm. An extinction coefficient of 8.3 mM^{-1} cm^{-1} was used to calculate the activity.

Nitrate concentration in the medium was measured enzymatically using the Boehringer nitrate determination kit.

RESULTS

Using the colony staining method the wildtype strain N400 colored very intensly when grown on concentrations of glucose higher than 25 mM, demonstrating strong glucose oxidase induction under these conditions. When low glucose concentrations were used (<2 mM), no induction was found.Table 2. Glucose oxidase levels in mycelium grown in liquid cultures of *A. niger* wild type and glucose oxidase mutants. 2-Deoxyglucose was also able to induce glucose oxidase when it was added to the medium, whereas fructose gave only a very weak induction. Other substrates did not induce glucose oxidase. The auxotrophic strains N423, N573 and N597, which were also used in the mutagenesis experiments, did not differ from N400 in glucose oxidase induction.

Screening for glucose oxidase overproducing mutants could only be succesfully performed under weakly or non-inducing conditions, using fructose, glycerol, gluconate or low glucose (<2 mM) as carbon source, since the parental strains stained already too strongly under inducing conditions to discriminate overproducers. Mutants which showed glucose oxidase synthesis on fructose or low glucose were found with high frequency (10^{-3}) whereas producing mutants on gluconate or glycerol were rare (10^{-5}). The induction characteristics of the mutants on agar plates are given in Table 1. For five of the mutants and the wild type strain the relative acid production on $CaCO_3$ plates was tested (Table 1).

All mutants described in Table 1 were further characterized in complementation tests. In some cases this required first the introduction of another auxotrophic marker gene in a *gox* mutant by somatic recombination in order to be able to make a heterokaryon and to isolate a heterozygous diploid from two mutants which had originally the same background. Generally, the complementation tests gave clearcut results as summarized in Table 1. In this Table the mutations are given a capital letter for locus identification and a numerical code for allele identification. The Gox-negative mutants comprise two complementation groups (*gox*C and *gox*D) whereas the Gox-positive mutants are classified into seven

Table 1. Phenotypic and genotypic description of *A. niger* wild-type (N400) and glucose oxidase mutants.

strain	50 mM glucose[a]	2 mM glucose	50 mM fructose	50 mM gluconate	100 mM glycerol	pH 3.5[b]	$CaCO_3$ test	linkage group	allele
N400 (wt)	++++	+	+	-	-	-	4		
NW101	-	-	-	-	-	-	5	II	*gox*C17
NW102	++++	+++	+++	+++	+++	+++	1	II	*gox*B12
NW103	++++	+++	+++	++	++	++		II	*gox*B21
NW104	++++	-	++	-	-	-		II	*gox*F29
NW107	++++	+	+	+	-	+		III	*gox*I38
NW108	++++	+	++	-	-	-		?	*gox*H7
NW109	++++	++	++	-	-	+		?	*gox*H27
NW110	++++	+	+	-	-	+		V	*gox*G20
NW111	++++	++	++	-	-	-		V	*gox*G22
NW112	++++	+++	+++	-	-	-		V	*gox*G32
NW113	+++	-	+/-	-	-	-	6	V	*gox*D30
NW114	++++	+++	++	-	-	++		VII	*gox*A1
NW115	++++	++	+++	-	-	+		VII	*gox*A11
NW116	++++	++	+++	-	-	+		VII	*gox*A28
NW117	++++	++++	++++	-	-	-	3	VII	*gox*A31
NW118	++++	++	++	-	-	-		VII	*gox*A41
NW119	++++	++++	++++	-	-	-		VII	*gox*A42
NW120	++++	++++	++++	+++	-	-		VII	*gox*E8
NW121	++++	++++	++++	+++	-	-		VII	*gox*E10
NW122	++++	++++	++++	+++	-	-		VII	*gox*E13
NW123	++++	++++	++++	+++	-	-		VII	*gox*E14
NW124	++++	++++	++++	+++	-	-		VII	*gox*E15
NW125	++++	++++	++++	+++	++	+		VII	*gox*E39
NW126	++++	++++	++++	+++	++	+	2	VII	*gox*E40

[a] Relative glucose oxidase levels in mycelium grown on the indicated carbon source, determined with the colony staining method; ++++ = very good, +++ = good, ++ = poor, + very poor, - = no staining.

Table 2. Glucose oxidase levels in mycelium grown in liquid cultures of *A. niger* wild type and glucose oxidase mutants.

Strain	Glucose[a]	Fructose[b]	Acetate[c]
N400 (wt)	7.6	0.3	0
NW101	0.0	0	0
NW113	1.2	0	0
NW117	8.4	5.8	0
NW126	7.4	3.8	0
NW102	13.5	14	0.8

[a] 0.56 M glucose, oxygen level 40 % air saturation
[b] 0.28 M fructose, oxygen level 70 % air saturation
[c] 100 mM sodium acetate, oxygen level 70 % air saturation
During the whole period of growth in the fermenter the oxygen tension was kept at the indicated level. Activities are measured in cell-free extracts of mycelium grown for 24 h after inoculation and given in μmol min^{-1} (mg protein)$^{-1}$.

complementation groups (*gox*A, *gox*B, *gox*E, *gox*F, *gox*G, *gox*H and *gox*I).

From each complementation group at least one mutant was chosen to allocate the relevant *gox*-gene to one of the eight linkage groups of *A. niger* (Bos *et al.*, 1988; Debets *et al.*, 1990). The results of these experiments are summarized in Table 1. All but one *gox* gene-loci could be allocated to one of the linkage groups. The negative results for *gox*H are explained by the fact that this mutation only slightly elevates the glucose oxidase expression. With the small contrast in this case, it appeared very difficult to discriminate between positives and negatives in the GOX staining procedure, even more because staining was found to be influenced by growth properties of the colonies and in some instances by supplements in the media (e.g. histidine). Moreover the staining can be influenced by tester strain markers, e.g. strains with the *cnx*C marker give always a weaker staining compared to wild type.

Glucose oxidase induction in submerged culture was tested in a fermenter. First we did some experiments with the wild type strain to determine the importance of various parameters for glucose oxidase induction in submerged culture. Apart from the presence of glucose it turned out that the dissolved oxygen concentration had a large influence on the level of glucose oxidase formed. At low oxygen concentrations (< 5 % air saturation), which did still give good growth, no glucose oxidase was formed in the wild type, although further conditions were optimal for induction (pH 5.5 and a high glucose concentration *viz* 0.56 M). Increasing the oxygen concentration resulted in higher glucose oxidase levels when N400 was grown on glucose. The same effect of oxygen was observed with the mutant NW117 using fructose as carbon source (Fig 1). Looking at the time curve of the glucose oxidase level, an increase was found until approximately 24 h after inoculation and this level remained constant. The biomass concen-

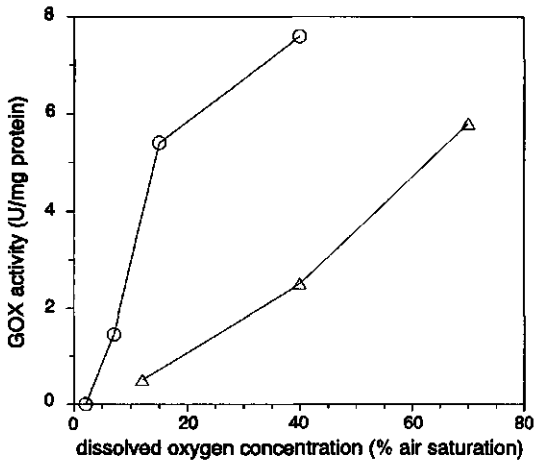

Fig 1. Influence of oxygen level on glucose oxidase levels. Glucose oxidase activity in cell-free extracts of mycelium grown for 24 h after inoculation at different oxygen levels. The oxygen level is expressed as the percentage of air saturation. O = N400, 0.56 M glucose. Δ = NW117, 0.28 M fructose.

tration at that point was 1-2 g l^{-1}. There was still nitrate available in the medium at this moment and the biomass did still increase.

Five mutants with different genotypes (see Table 1) were tested in the fermenter for glucose oxidase formation when grown on inducing (glucose) and weakly or non-inducing (fructose and acetate) substrates and using high oxygen concentrations (Table 2). N400 shows strong induction on glucose, a low activity on fructose but absolutely no activity on acetate. No activity could be detected in mutant NW101. The low producing mutant NW113 gives only low activities of glucose oxidase on glucose. The *gox*B mutant NW102 was the only mutant tested which showed some activity on acetate. On glucose and fructose this mutant showed strong overexpression (about a factor 2 compared with the wild type strain on glucose). The *gox*A and *gox*E strains NW117 and NW126 show moderately strong induction on fructose but did not show significant overproduction on glucose.

DISCUSSION

The colony staining method which is based on localization of glucose oxidase in the fungal cell wall (Mischak *et al.*, 1985; Witteveen *et al.*, 1992), turned out to be very sensitive. It can be performed in a stage where the colonies are still small so that a large number of colonies (100-1000) can be screened on a single plate. This is necessary because enrichment procedures cannot be applied and therefore large numbers of colonies have to be tested. The staining is dependent on glucose and peroxidase, so we consider it glucose oxidase specific.

The phenotypical characterization did not result in a clear classification and no

conclusions could be drawn about the number of genes involved. The genetic analysis was therefore very valuable in the analysis of the mutants. The large number of complementation groups is an indication of a complex regulation of induction. However, a number of the mutants show only an increase of induction under weakly inducing conditions and the effect of these mutations on glucose oxidase expression might be indirect. The weaker staining in the *cnx*C mutants is an indication of this. Furthermore a glycerol kinase mutant isolated by us (Witteveen *et al.*, 1990) also showed a phenotype comparable to some of the weak overproducers.

We tested the production of glucose oxidase in a fermenter with oxygen and pH control. Our experience was that this gave more reliable results than Erlenmeyer cultures in the presence of $CaCO_3$, which are normally used, and where no control of the dissolved oxygen tension is possible (Kundu and Das, 1985; Fiedurek *et al.*, 1986; Markwell *et al.*, 1989). The importance of a good oxygenation of the medium has been described before by Zetelaki (1970) and is confirmed by the results in Fig.1. Other factors like pH and medium composition were less important when the oxygen concentration was kept high, e.g. an excess nitrate (6 g $NaNO_3$ l^{-1}) or ammonium did not influence the glucose oxidase induction strongly (data not shown) and glucose oxidase induction was observed in an experiment where the pH was kept at 2.8, although the glucose oxidase synthesis started much later. Gluconate formation at low pH has been observed before (Heinrich and Rehm, 1982).

A few results are worth emphasizing when Tables 1 and 2 and Fig. 1 are considered. 1) NW101 completely lacks glucose oxidase in submerged culture. This was the only absolute negative mutant we have found. It is remarkable that in the $CaCO_3$ plate test this mutant acidifies the medium more than mutant NW113 which has still some residual glucose oxidase activity (Table 1). Also in submerged culture this mutant acidifies the medium strongly, indicating that other acids are excreted by the mycelium. 2) Especially in the mutants NW113 and NW126 the relative glucose oxidase expression seems lower in submerged culture when compared to the plate data. This may be caused by a better oxygen availability on plates. 3) NW117 synthesizes glucose oxidase in almost equal amounts on fructose as on glucose. So the effect of the carbon source on induction has been changed, but the oxygen inducibility is still intact (Fig. 1). 4) NW102 is able to make glucose oxidase on all carbon sources, though not in equal amounts. This mutant has another unique characteristic, since it is also synthesizing glucose oxidase under conditions of low oxygen. NW103, the other *gox*B mutant, showed the same phenotype. None of the other mutants produced glucose oxidase under these conditions. The very clear oxygen inducibility of glucose oxidase as shown in Fig. 1 and the behaviour of the *gox*B mutants (oxygen independent) emphasizes the importance of this parameter for glucose oxidase induction. Glucose oxidase levels in NW102 are still dependent on the carbon source but the constitutivity with respect to oxygen induction is apparent-

ly able to overrule in part the effect of the carbon source. A mutant like NW117 which is still oxygen dependent (Fig. 1), but shows good induction on fructose, demonstrates that the carbon source effect is caused by a different factor.

Since the glucose oxidase gene has recently been cloned (Kriechbaum *et al.*, 1989), the well defined mutants described here become particularly valuable to understand the regulation of the glucose oxidase gene expression.

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Chapter 4

Induction of glucose oxidase, catalase and lactonase in *Aspergillus niger*.[1]

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SUMMARY

The induction of glucose oxidase, catalase, and lactonase activities was studied both in wild-type and in glucose oxidase regulatory and structural mutants of *Aspergillus niger*. The structural gene for glucose oxidase was isolated and used for Northern analysis and in transformation experiments using various *gox* mutations. Wild-type phenotype could be restored in the glucose oxidase-negative mutant (*gox*C) by transformation with the structural gene. We conclude, therefore, that the *gox*C marker which is located on linkage groupt 2 represents the structural gene of glucose oxidase. Glucose and a high oxygen level are necessary for the induction of all three enzyme activities in the wild-type strain and it was shown that both glucose and oxygen effects reflect regulation at the transcriptional level. The *gox*B mutation results in constitutive expression of all three activities although modulated to some extent by the carbon source. The *gox*E mutation only has an effect on lactonase and glucose oxidase expression and does not relieve the necessity for a high oxygen level. Catalase and lactonase could not be induced in the glucose oxidase-negative strain (*gox*C). Addition of H_2O_2 resulted in the induction of all three enzymes in the wild-type without glucose being present. The H_2O_2 induction is probably mediated by the *gox*B product. Besides the H_2O_2 induction there is still an effect of the carbon source on the induction. A model for induction of glucose oxidase, catalase, and lactonase in *A.niger* is discussed. Transformation of wild- type and *gox*C strains with the *gox*C gene resulted in only a 3-4 fold increase of glucose oxidase activity relative to the wild type even though more than 25 copies of the structural gene were present. Transformation of the *gox*B strain gave higher activities but resulted in poor growth. *Aspergillus nidulans* does not have a glucose oxidase activity, but could be transformed with the *A.niger gox*C gene to a glucose oxidase-producing strain. Induction in these transformants was comparable to that in *A. niger* with respect to the carbon source dependency, but

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[1]Accepted for publication in Current Genetics

there was no oxygen dependency of induction. The glucose oxidase produced by the *A. nidulans* transformants was kinetically indistinguishable from the *A. niger* enzyme, but it showed small differences in glycosylation pattern.

INTRODUCTION

Aspergillus niger is used commercially to produce organic acids such as citric and gluconic acid. We have been particularly interested in *A. niger* strain breeding for gluconic acid production. Although the practical aspects of gluconic acid formation have been frequently reported, little information is available on the molecular principles governing the regulation of this process in *A. niger*. Three activities are essential for the production of gluconic acid: glucose oxidase (EC 1.1.3.4), catalase (EC 1.11.1.6), and lactonase (EC 3.1.1.17).

For an efficient induction of glucose oxidase the following three environmental factors are required: (1) the presence of high levels of glucose (Zetelaki and Vas 1968), (2) high dissolved oxygen levels (Zetelaki 1970), and (3) a pH around 5.5. Besides these factors, the presence of manganese and low nitrogen were found to have a positive influence on the induction (Röhr *et al.* 1983; Müller 1986). However, Mischak *et al.* (1985) showed that manganese is not required for glucose oxidase induction at all, and although the nature and the level of the nitrogen source may have an influence on induction it was concluded that a low nitrogen level is not required for induction (Witteveen *et al.* 1990). Furthermore, we concluded that glucose and oxygen are the main parameters and that the pH requirement is less strict and may be only indirectly involved in induction (Witteveen *et al.* 1990). Therefore, we have now investigated the effects of oxygen and glucose on the induction in more detail. Since it is to be expected that glucose oxidase, catalase, and lactonase are regulated coordinately, we studied the induction of all three enzymes.

In previous studies we isolated and genetically characterized mutants with an altered regulation of glucose oxidase induction (Witteveen *et al.* 1990, Swart *et al.* 1990), so facilitating the study of the mechanisms of regulation. The mutants with the clearest phenotype are a glucose oxidase-negative mutant (*gox*C), a mutant which produces glucose oxidase on all carbon sources tested but is still oxygen dependent (*gox*E), and a more or less constitutively overproducing mutant which also produces glucose oxidase under low oxygen conditions (*gox*B). Mutants from these three complementation groups are studied in more detail here.

Witteveen *et al.* (1992) showed that there are at least four different catalases formed by *A. niger*. Two of these are constitutive, whereas two others are induced under conditions when glucose oxidase is induced as well. Two of the four catalases are cell-wall bound (one constitutive and one inducible) and two are intracellular (one constitutive and one inducible). The induction behaviour of

the four catalases is studied here in the glucose oxidase mutants.

For a more detailed analysis of the regulation of glucose oxidase a molecular genetic approach is required. We therefore isolated the structural gene. Other groups have reported the isolation and the sequence of the glucose oxidase gene (Kriechbaum *et al.* 1989; Frederick *et al.* 1990; Whittington *et al.* 1990).

We also analyzed the regulation of expression of glucose oxidase in *A. nidulans*. As this fungus does not have the glucose oxidase gene, it is of interest to obtain information on the specificity of the regulatory system.

MATERIALS AND METHODS

Strains and plasmids. *A. niger* N400 (CBS 120.49) was used as wild-type strain and was the source of the glucose oxidase gene. The *A. niger* glucose oxidase mutants NW101 (*gox*C17, *csp*A1, *pab*A1), NW102 (*gox*B12, *csp*A1, *pab*A1), NW103 (*gox*B21, *csp*A1, *pab*A1), NW125 (*gox*E39, *csp*A1, *bio*A1) were used in the induction experiments. *Aspergillus tubigensis* NW756 (Kusters-van Someren *et al.* 1991) was used in the experiments for comparison of the glucose oxidases. The *A. nidulans* strain G191 (*pyr*G89, *paba*A1, *fw*A1, *ua*Y9; Ballance and Turner, 1985) and the *A. niger* strain N593 (*pyr*A6, *csp*A1; Goosen *et al.* 1987) were used as recipient strains in transformation experiments as were *A. niger* NW127 (*csp*A1, *pyr*A6, *nic*A1, *fwn*A1, *gox*B21) and NW128 (*csp*A1, *pyr*A6, *nic*A1, *fwn*A1, *gox*C17) which were made by recombination of respectively NW103 and NW101 with N593 and master strains described by Bos *et al.* (1988). In the co-transformation experiments pGW635 (containing the *A. niger* orotidine-5'-phosphate decarboxylase gene) was used (Goosen *et al.* 1989) to complement the *pyr*A (*A. niger*) and the *pyr*G (*A. nidulans*) mutations. pUC9 (Vieira and Messing, 1982) was used for subcloning. Plasmids were propagated in *E.coli* JM109 and λ phages were plated on *E.coli* LE392.

DNA manipulation. Plasmid DNA isolation, subcloning, Southern analysis, Northern analysis, RNA isolation for the Northern analysis (the guanidinium/cesium chloride method), and gel electrophoresis of RNA in the presence of glyoxal, were all performed using standard methods as described by Maniatis *et al.* (1982). *Aspergillus* chromosomal DNA was isolated as described by de Graaff *et al.* (1988). Sequencing of double-stranded plasmid DNA was carried out using the T7 sequencing kit (Pharmacia LKB, Uppsala, Sweden).

Construction of a cDNA library of glucose oxidase-induced mycelium and immunochemical screening. The isolation of mRNA from *A. niger* N400 for the construction of the cDNA library was done from mycelium grown on minimal medium (Witteveen *et al.* 1989) with 5% glucose as carbon source. Medium (300 ml) was inoculated with 5 x 10^6 spores/ml and pregrown in a 1-l Erlenmeyer flask for 7 h. Subsequently the culture was added to 1.7 l of medium in a fermenter and the mycelium was grown for 14 h under low aeration conditions (a dissolved oxygen concentration of 5-10% air saturation). Induction of glucose oxidase was then realised by increasing the dissolved oxygen level to 30-50% air saturation (Witteveen *et al.* 1990). The mycelium was harvested 5 h after the start of induction, filtered, washed

with demineralised water and frozen in liquid nitrogen. The frozen mycelium was stored at -70°C until it was used for RNA isolation. Total RNA was isolated as described by Cathala *et al.* (1983), the poly A^+ fraction of this RNA was isolated by oligo dT affinity chromatography, essentially as described by Maniatis *et al.* (1982). cDNA was synthesized from this poly A^+ RNA using BRL's cDNA synthesis kit according to the manufacturer's instructions. *Eco*RI linkers (Promega) were ligated to *in vitro* methylated cDNA before the cDNA was cloned in *Eco*RI-digested Lambda Zap II (Stratagene), resulting in a cDNA library of 10^5 primary plaques. The cDNA library was screened for *gox*-expressing phages essentially as described by Young and Davis (1983). Polyclonal antibodies raised against glucose oxidase (Witteveen *et al.* 1992) were used for screening the library. *In vivo* excision of the positive clones was performed according to the manufacturer's description resulting in the pBluescript SK(-) phagemid with the cDNA inserts.

Isolation of the genomic clone. The genomic clone of the glucose oxidase gene was isolated from a N400 genomic library in EMBL4 (Harmsen *et al.* 1990) by homologous hybridisation with the pIM502 glucose oxidase cDNA clone.

Transformation of *A. niger* and *A. nidulans*. Transformation of *A. niger* and *A. nidulans* was performed as described by Goosen *et al.* (1987) using the *A. niger pyr*A gene as a selection marker. For obtaining high copy number transformants pIM503 was added 30:1 relative to pGW635 whereas for obtaining low copy number transformants equal amounts were added (1 μg pGW635 DNA was added to 10^7 protoplasts per transformation).

Media and growth conditions for induction experiments in submerged culture. All mycelial cultivation and plate incubations were performed at 30°C. Unless indicated we used the medium as described by Witteveen *et al.* (1990) in submerged cultures. In plates the medium described by Witteveen *et al.* (1989) was used, unless stated otherwise. Carbon sources were used as indicated in the different experiments. Submerged cultures were done in 3-l fermenters with pH and oxygen control (Applikon BV, Schiedam, The Netherlands). The pH was kept constant by titrating with 5 M NaOH whereas the oxygen level was controlled by sparging with oxygen or nitrogen gas. We chose three growth conditions to analyze the carbon source and the oxygen effect on the induction of glucose oxidase, catalase, and lactonase: (**1**) Low oxygen conditions. The dissolved oxygen concentration was kept below 10% air saturation. The carbon source was 10% (w/v) glucose. (**2**) High oxygen conditions. The dissolved oxygen concentration was kept above 30% air saturation. The carbon source was 10% (w/v) glucose. (**3**) With 2% D-xylose as carbon source. The dissolved oxygen concentration was kept above 30% air saturation. In all cases the pH was kept at 5.5. The mycelium was harvested 24-27 h after inoculation. For the first 7 h the spore suspension (approximatelly 5×10^6 ml^{-1}, 300 ml) was incubated in an Erlenmeyer flask which was subsequently added to a fermenter with 1.8 l of medium. From that moment on the oxygen and pH conditions were kept as indicated. Condition 2 was also used for the determination of the enzyme activities in the transformants. For demonstrating the effect of the carbon source on the glucose oxidase mRNA levels in N400 and NW103 (*gox*B21) we used mycelia grown as described in conditions 2 and 3 above. The effect of oxygen on the glucose oxidase mRNA level in the wild-type strain (N400) was demonstrated in the following experiment. *A. niger* was grown under

conditions optimal for glucose oxidase induction (10% glucose, pH 5.5, and aeration above 30% air saturation during the whole growth period). After 20 h a mycelial sample was taken and the dissolved oxygen concentration was lowered to approximately 7% air saturation. Samples were taken during a 6 h period after which the dissolved oxygen concentration was elevated again to more than 30%. Two more samples were taken 1 and 6 h after the increase of the oxygen concentration. The samples were analysed on a Northern blot which was hybridized with a glucose oxidase-specific probe and a pyruvate kinase-specific probe (de Graaff *et al.* 1992a). In another experiment the oxygen effect on glucose oxidase mRNA levels in the *gox*B21 mutant (NW103) was compared to that in N400. This experiment was done in essentially the same way as in the previous experiments except that the mycelium was kept under low oxygen conditions for 12 h and the level was not increased again. The glucose oxidase mRNA levels in the *gox*C mutant (NW101) were also tested under these conditions. We studied the effect of H_2O_2 on the induction of glucose oxidase, lactonase, and catalase activities in the following experiment. N400 was grown on 3% D-xylose for 24 h under high oxygen conditions at pH 5.5. Subsequently we started to continuously add H_2O_2 from a 2 M stock solution. The amount of H_2O_2 was gradually increased during the experiment (see Fig. 3). At regular intervals samples were taken to determine enzyme activities and mycelial dry weight (dw).

Glucose oxidase levels on plates. Glucose oxidase production by mycelium grown on agar plates was visualized by the detection of H_2O_2 formation with peroxidase as previously described (Witteveen *et al.* 1990).

Enzyme assays. *Glucose oxidase*. The activity of glucose oxidase was determined by two different methods which gave essentially the same result. In the first of these the activity was determined by measuring the rate of H_2O_2 formation (Witteveen *et al.* 1990). In the second method the activity was determined by measuring the decrease in oxygen concentration in an air-saturated 100 mM sodium phosphate buffer pH 6.0 with 100 mM of glucose and 100 μg of beef liver catalase at 30°C. Since the glucose oxidase is found both in the mycelium and the medium in varying and non-constant ratios, the measurements were done in samples of the culture containing both medium and mycelium, which was sonicated to break down the mycelial pellets. The levels were related to the dry weight (dw) of the mycelium which was determined after washing a mycelial sample with distilled water and subsequent lyophilization. For the kinetic measurements, glucose oxidase from *A. nidulans* transformants, and from wild-type *A. niger* and *A. tubigensis* were partially purified on a DEAE column as described for the yeast-derived glucose oxidase by Frederick *et al.* (1990). The activity was determined by measuring the decrease in oxygen concentration (see above). Variable amounts of glucose in the case of the K_m (glucose) measurement and 100 mM in the case of the K_m (O_2) determination were used. Different oxygen concentrations were realised by mixing air- and oxygen-saturated solutions. The dissolved oxygen concentration was measured just before the start of the assay. The glucose K_m determinations were performed in air-saturated solutions. The data were fitted to the Michaelis Menten equation using the program Enzfitter by E. Leatherbarrow (Elsevier-BIOSOFT).

Lactonase. Since activity was found in both the culture fluid and the mycelial extract, most likely because the enzyme remains partly in the cell wall, we determined

the activity in unfiltered samples as described for glucose oxidase activity measurements. The activity was measured in 50 ml of 15 mM sodium acetate buffer, pH 5.4, at 30°C. The reaction was started by adding 0.45 g of solid glucono-δ-lactone (50 mM) to the buffer, to which an appropriate amount of a sample was added. The pH was kept at pH 5.4 by titration with 0.5 M NaOH using a titrator (Titrilab. Radiometer, Copenhagen). The activity was calculated from the rate of NaOH consumption between 1 and 2 min after adding the glucono-δ-lactone. By relating the activity to the dry weight present in the culture a specific activity was calculated. The activities were corrected for spontaneous hydrolysis of glucono-δ-lactone.

Catalase. The activity was determined by measuring the decrease in H_2O_2 concentration. This was done by determining the decrease in A_{240} (Aebi, 1974) in crude cell extracts (Witteveen *et al.* 1989), for which 20 mM of sodium phosphate (pH 6.0) was used as extraction buffer.

Protein concentrations were estimated, after denaturation and precipitation of protein with sodium deoxycholate and trichloroacetic acid (Bensadoun and Weinstein, 1976), by the microbiuret method (Itzhaki and Gill, 1964) using bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis. Non-denaturing polyacrylamide-gel electrophoresis for the detection of different catalase forms was performed as described previously (Witteveen *et al.* 1992).

Isoelectric focussing. Extracts of *A. niger* N400, *A. niger* transformant G35, *A. nidulans* transformant D42 and *A. tubigensis* NW756, all grown under glucose oxidase inducing conditions, were analysed by isoelectric focussing using Servalyte 3-5 (Serva). After reaching equilibrium the gels were stained for glucose oxidase activity by incubation of the gel in a solution containing 50 mM of sodium phosphate buffer, pH 6.0, 100 mM of glucose, 50 mg l^{-1} of phenazine methosulphate and 250 mg l^{-1} of nitroblue tetrazolium.

RESULTS

Isolation of the glucose oxidase gene.

From 400 cDNA clones screened, two gave a positive signal upon incubation with the glucose oxidase antibodies. These clones appeared to have inserts of respectively 1.7 and 1.9 kb. After *in vivo* excision of the pBluescript plasmid with inserts from the Lambda Zap II vectors this resulted in the plasmids which were named pIM501 and pIM502 respectively. Approximately 300 bp of the 5' end of the cDNA insert of pIM502 have been sequenced. This sequence started 15 bp after the ATG start codon and was 100% identical to previously published sequences (Kriechbaum *et al.* 1989; Frederick *et al.* 1990; Whittington *et al.* 1990).

Screening of a genomic library of *A.niger* N400 (Harmsen *et al.* 1990) under homologous hybridisation conditions using pIM502 as a probe resulted in 18 positive phages. Restriction analysis of five of these clones showed that they contained the same hybridizing fragments. From these phages a 5.5-kb *Hin*dIII

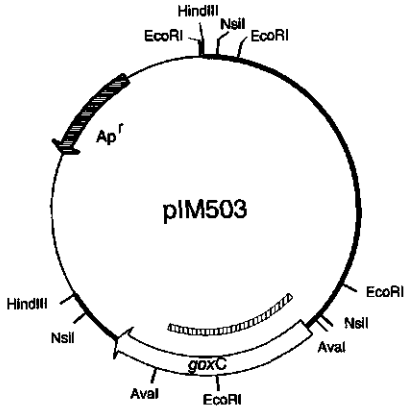

Fig. 1. pIM503 is a 5.5-kb *Hin*dIII fragment, containing the *gox*C gene, inserted in pUC9. Also indicated is the *Ava*I fragment which was used as a probe in the Northern blotting experiments. The *thick line* indicates *Aspergillus* DNA, the *thin line* pUC9 DNA.

fragment containing the complete functional gene encoding glucose oxidase was subcloned. This fragment contains approximately 3 kb upstream of and 700 bp downstream from the coding sequence. The fragment was ligated in the *Hin*dIII site of pUC9 giving pIM503 (Fig. 1).

Oxygen and carbon source effects on the induction of glucose oxidase, catalase, and lactonase activities

We investigated these activities in mycelia of four strains grown under three different conditions, which allow for separation of the pH and oxygen effect on induction. The four strains were: N400 (wild-type), NW103 (*gox*B21 mutant), NW125 (*gox*E39 mutant), NW101 (*gox*C17 mutant). The two *gox*B mutants

Table 1. Glucose oxidase, catalase and lactonase activities in *A. niger* wild-type, *gox*B, C and E mutant strains grown under low oxygen, high oxygen (both with glucose as carbon source), or D-xylose as carbon source (under high oxygen conditions).

	Glucose oxidase[1]			Catalase[2]			Lactonase[1]		
Strains	Low O_2	High O_2	D-xylose	Low O_2	High O_2	D-xylose	Low O_2	High O_2	D-xylose
N400 (wt)	0	1.5	0.02	19.6	107	2.3	<5	41	11
NW103 (*gox*B)	1.6	2.9	0.85	320	543	81	56	99	33
NW125 (*gox*E)	0	2.0	0.5	12.9	71	4	6	56	41
NW101 (*gox*C)	0	0	0	9.9	11.6	ND[3]	ND[3]	15	11

[1] in μmol (mg dry wt)$^{-1}$ (min)$^{-1}$.
[2] in μmol (mg protein)$^{-1}$ (min)$^{-1}$
[3] ND = not detected.

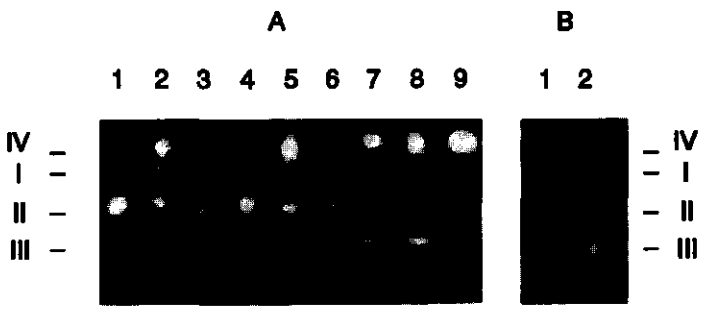

Fig 2A,B. Extracts separated on a 7.5% polyacrylamide gel and stained for catalase activity. **A:** lanes 1-3: N400 (wt) - low oxygen (1), high oxygen (2), D-xylose (3); lanes 4-6: NW125 (*gox*E) - low oxygen (4), high oxygen (5), D-xylose (6). lanes 7-9: NW102 (*gox*B) - low oxygen (7), high oxygen (8), D-xylose (9). **B:** extracts of the H_2O_2 induction experiment (See Fig. 3): lane 1, before H_2O_2 addition; lane 2, after 4.5 h of H_2O_2 induction.

available (NW102 and NW103) showed the same induction characteristics with respect to glucose oxidase, catalase and lactonase. Two *gox*E mutants (NW125 (*gox*E39) and NW126 (*gox*E40)) also showed the same induction characteristics with respect to these three enzymes. The activities of the three enzymes in mycelia grown under the conditions described are given in Table 1. When the pH is kept at 5.5 there is always a basic level (approximately 10 U/mg dw) of lactonase, independent of the carbon source (xylose and glycerol were tested, data not shown). Table 1 shows that in the wild type strain induction of the three enzymes is found only when glucose is present and the oxygen level is high. The *gox*B mutant does show expression although to a different extent in all cases, and so is less sensitive to both carbon source and oxygen level. The *gox*E mutant does show wild type induction of catalase but shows less carbon source dependent induction of glucose oxidase and lactonase, whereas the induction of all three enzymes is still oxygen dependent. The *gox*C mutant does not show induction of catalase and lactonase under any of the three conditions, indicating that glucose oxidase activity is important for the induction of these enzymes.

To distinguish between induction of the different catalases we separated extracts of mycelia grown under the three conditions described on a polyacrylamide gel and stained for catalase activity (Fig. 2A). In the case of induction the levels of the two catalases CAT III and CAT IV are strongly increased. The same catalases are also present in high levels in the *gox*B strain under non-inducing conditions.

In a separate experiment we tested whether glucono-δ-lactone was the inducer of lactonase. To N400 grown on D-xylose at pH 5.5 with a high dissolved oxygen concentration we added glucono-δ-lactone in small portions over a prolonged period, in such amounts that the average lactone concentration was approximately 5-10 mM. This did not result in an increase of the lactonase activity within 4 h.

H_2O_2 induction

We tested the effect of the addition of H_2O_2 to a D-xylose-grown culture of the wild-type strain N400 on the induction of glucose oxidase, catalase, and lactonase. The amount of H_2O_2 added was gradually increased to mimic the effect of an increasing H_2O_2 formation by glucose oxidase. The amount of H_2O_2 added is critical since a too high amount causes cell death (especially in the beginning when protective enzymes have not yet been induced) and a too low amount does not result in induction because of a rapid breakdown of the H_2O_2 by the constitutive catalases. Figure 3 shows a rapid induction of all three activities after the addition of H_2O_2. Extracts of the mycelia of $t=0$ and $t=4.5$ h after the start of H_2O_2 addition have been examined for the different catalase forms present using native polyacrylamide-gel electrophoresis followed by catalase activity staining (Fig. 2B). It shows that the same catalases are induced by H_2O_2 as those induced coordinately with glucose oxidase under normal inducing conditions (CAT III and CAT IV).

H_2O_2-induction of catalase and lactonase was also observed in a comparable experiment with the glucose oxidase-negative strain grown on glucose at pH 5.5 (data not shown).

Northern analysis of glucose oxidase expression in wild-type, *gox*B, and *gox*C A.niger strains

The effect of the dissolved oxygen level and the carbon source on glucose oxidase mRNA levels in wt *A.niger* and the *gox*B mutant were determined. Furthermore, we analyzed the mRNA levels in the glucose oxidase-negative strain (*gox*C). The effect of the dissolved oxygen level on the mRNA level was studied in two different experiments. In the first experiment mycelium of wild-type strain N400 was grown under inducing conditions and subsequently the oxygen level was lowered. After 6 h the oxygen level was increased again. Already 1 h after lowering the oxygen level no *gox* mRNA was detectable (Fig. 4) and 1 h after increasing the oxygen level again the signal was comparable to the level in the induced sample at $t=0$. As a control the Northern blot was also hybridized with a probe of the pyruvate kinase (*pki*A) gene of *A. niger*. Lowering of the oxygen level resulted in a decrease of the *pki*A mRNA level which subsequently increased during growth at low oxygen levels.

In a second experiment we compared the effect of lowering the oxygen level for a prolonged period in the wild-type strain (N400) and in a *gox*B mutant (NW103). Both strains show strong signals in the induced sample (Fig. 5). In the wild-type no mRNA was detected in samples taken 6 h and 12 h after lowering the oxygen level. In the mutant a moderately strong signal could still be detected.

The effect of the carbon source on the mRNA level in the wild-type strain and goxB mutant was studied by growing mycelium of wild-type strain N400 and *gox*B12 mutant NW102 on 2% D-xylose at pH 5.5 and with oxygen levels above 30%. In the wild-type strain no detectable levels of glucose oxidase mRNA were

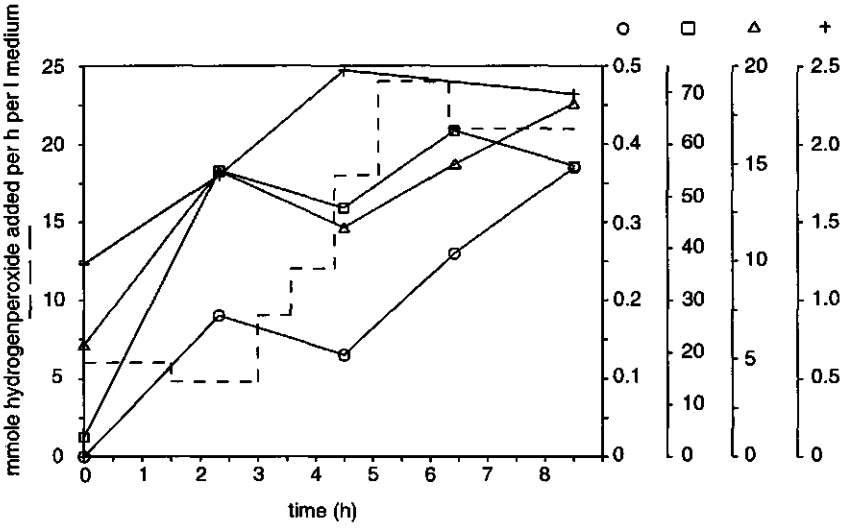

Fig. 3. The induction by H_2O_2 of glucose oxidase, catalase and lactonase activities in N400. O - glucose oxidase (U/mg dw), □ - catalase (U/mg protein), Δ - lactonase (U/mg dw), + - dry weight (g/l). The dashed line indicates the amount of H_2O_2 added per h per l of medium.

found whereas a strong signal was detected in NW102 grown under these conditions (data not shown).

In NW101 (*gox*C17) no mRNA was detected in mycelium grown under inducing conditions or with low oxygen. The control hybridization with the *pki*A probe showed strong signals, indicating that the mycelium was viable under these conditions.

Overexpression of glucose oxidase in A.niger transformants

Three different *A. niger* strains were used as acceptor strains in a co-transformation experiment. These strains all carry the *pyr*A6 mutation. With respect to glucose oxidase expression they are wild-type (N593), constitutively overproducing (NW127), or negative (NW128). pIM503 (Fig. 1) was used to co-transform these strains using the *pyr*A gene as a selection marker. Co-transformants in the NW128 background could be selected directly using the plate screening method since this strain does not produce glucose oxidase. This method also proved useful to select transformants of the other two strains since a higher level of glucose oxidase was formed when extra copies of the glucose oxidase gene were introduced. All the transformants tested had integrated multiple copies of pIM503 showing complex patterns on Southern blots. A number of high glucose oxidase-producing transformants were selected for

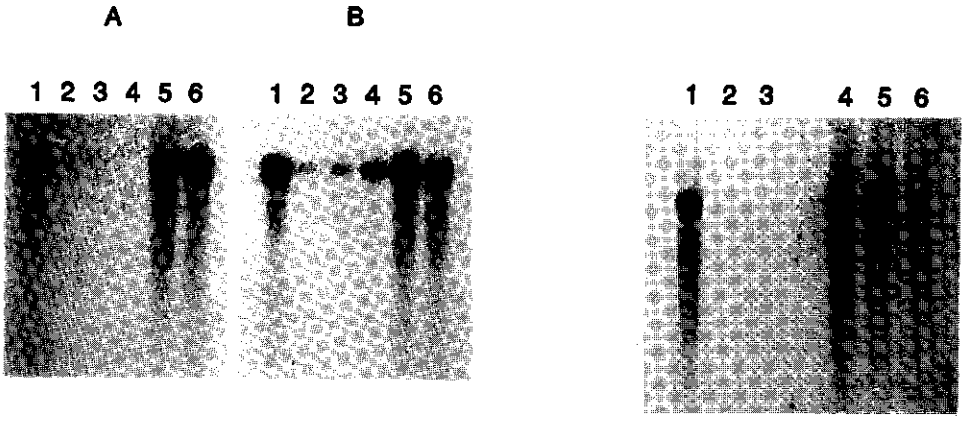

Fig 4A,B. Northern analysis of the effect of lowering and subseqently increasing the oxygen level in N400. The experiment was performed as described in the text. After growth under inducing conditions the oxygen level was kept low for 6 h and subsequently increased again. Lane 1, induced sample at t=0; 2, 1h; 3, 3h; 4, 6h; 5, 7h; 6, 12h. In **A** the *Aval* fragment as indicated in Fig. 1 was used as a probe. In **B** the *pki*A gene was used as a probe.

Fig. 5. Northern analysis of the effect of the oxygen level on glucose oxidase mRNA levels in N400 (lanes 1-3) and NW103 (lanes 4-6). The experiment was performed as described in the text. mRNA levels in induced samples (lanes 1,4) and samples of mycelia 6 (lanes 2,5) and 12 h (lanes 3,6) after lowering the oxygen level are shown. The Aval fragment indicated in Fig. 1 was used as a probe.

further analysis. Table 2 shows the glucose oxidase and catalase activities of these transformants. The highest levels reached in multicopy transformants in both wild-type and *gox*C strains were approximately 3-4 times the wild-type level under optimal induction conditions. In the *gox*B strain higher levels were reached but this resulted in poor growth of these transformants.

Furthermore, the induction pattern of transformants of the *gox*C strain was compared to that of the wild-type transformants. Induction with respect to the carbon source was comparable in both type of strains (Table 3) and the induction was still oxygen dependent (data not shown)

A. nidulans transformants

The *A. nidulans* glucose oxidase tranformants could also be stained on plates, showing strongly-stained hyphae, like in *A. niger*. The plate staining method was used to test the effect of different carbon sources on the induction of glucose oxidase (Table 3). Glucose is a very strong inducer in all transformants (data not shown) and fructose induction is comparable to that in *A. niger* transformants. One transformant showed weak induction with gluconate as the carbon source (D3). Since this was not observed in the other transformants this is probably due to a locus-specific integration.

Transformants D3 and D41 were tested for glucose oxidase induction under low and high oxygen conditions in a submerged culture in a fermenter. Under high oxygen conditions levels of respectively 2.7 and 1.7 U/mg dw were found. Remarkably, under low oxygen conditions, when no expression of glucose oxidase is found in *A. niger*, activities of respectively 3.0 and 2.5 U/mg dw were measured.

Glucose oxidase synthesized by *A. nidulans*, *A. niger* and *A. tubigensis*

The properties of the glucose oxidase formed by *A. nidulans* as a host and the enzymes from *A. niger* and *A. tubigensis* were compared by analyzing the kinetic parameters and behaviour on Western blots and IEF gels (Fig. 6). We could not measure significant differences in the K_m for oxygen (0.41 mM for *A. niger*, 0.38 mM for *A. nidulans* and 0.45 mM for *A. tubigensis*) and the K_m for glucose (14.5 mM for *A. niger*, 16.0 mM for *A. nidulans* and 12.8 mM for *A. tubigensis*). On Western blots no clear difference in molecular weight between the three proteins was found (data not shown). This is in agreement with the results of Whittington *et al.* (1990). Separation on an IEF gel (Fig. 6) shows that all three enzymes show different patterns on gel. The *A. niger* transformant shows the same pattern as the wild-type strain. Glucose oxidase analyzed on an IEF gel always

Table 2. Glucose oxidase and catalase activities and dry weights after 24 h of growth under inducing conditions in *A. niger* N400 and NW103, and in wt, *gox*C and *gox*B strains which were transformed with pIM503.

Strain nr	Glucose oxidase activity (U/mg dw)	Catalase activity (U/mg prot)	Mycelium dry weight	Strain description
N400	1.5	98	1.7	wild type
G1	4.0	111	1.2	wild type transformant, high copy no
G15	5.5	98	0.5	wild type transformant, high copy no
C35	4.5	95	1.0	*gox*C17 transformant, high copy no
C36	0.8	93	1.5	*gox*C17 transformant, low copy no
NW103	2.7	381	1.8	*gox*B21
B24	9.1	113	0.12	*gox*B21 transformant, high copy no
B7	3.9	233	0.44	*gox*B21 transformant, low copy no

Table 3. Expression of glucose oxidase on agar plates with either fructose or gluconate as carbon source. Indicated is the relative staining on agar plates.

Description	Strain no.	Expression of glucose oxidase on	
		50 mM fructose	50 mM gluconate
A. niger Wild type	N400	+	-
*gox*B21	NW103	+ + +	±
A. niger	G1	+ + + +	-
Wild-type	G12	+ + +	-
Transformants	G15	+ +	-
	G23	+ + +	-
*gox*C17	C18	+	-
Transformants	C35	+ + + +	-
	C36	+	-
	C40	+ + +	-
*gox*B21	B7	+ +	±
Transformants	B9	+ + +	±
	B10	+ + +	+
	B24	+ + + +	+ +
A. nidulans	D2	±	-
Transformants	D3	+ + +	+
	D41	+	-
	D42	+ +	-

shows several bands. Hayashi and Nakamura (1981) demonstrated that this is caused by differences in glycosylation and that the peptide chains of the proteins in the different bands are the same. Apparently the differences between the *A. niger* and the *A. nidulans* enzymes are the result of differences in the glycosylation of the three enzymes. The differences detected for the *A. tubigensis* enzyme might be caused by differences in both the peptide chain and the glycosylation.

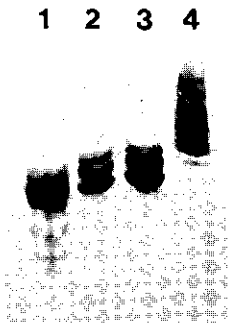

Fig. 6. Extracts of *A.tubigensis* (1), *A. niger* wild-type (2) and transformant G35 (3) and an *A. nidulans* transformant D42 (4) separated on an IEF gel and stained for glucose oxidase activity.

DISCUSSION

Both the sequence of the 5' end of the cDNA insert of pIM502 and the reactivity of the product of this cDNA clone with glucose oxidase-specific polyclonal antibodies prove that this cDNA clone codes for glucose oxidase.

Strains carrying the *gox*C mutation do not show any glucose oxidase activity and no protein is detectable on a Western blot (Witteveen *et al.* 1990). We have now shown, in addition, that no mRNA is detectable in the strain carrying the *gox*C mutation. Glucose oxidase expression, similarly regulated as in wild-type *A. niger*, can be restored by transformation of the *gox*C17 mutant with the structural gene for glucose oxidase. The small differences in regulation (e.g., a higher expression on fructose) is caused by the high copy number of the glucose oxidase gene in the transformants, since the same effect of multiple copies was shown in transformants with a wild-type background. From this we conclude that the *gox*C marker represents the structural gene of glucose oxidase. Were *gox*C to be a regulatory gene this would not have resulted in a wild-type phenotype with respect to the induction of glucose oxidase after transformation. The stability of this mutation (Witteveen *et al.* 1990) , the easy and very sensitive colony staining method, and the availability of the structural gene for glucose oxidase, makes this a very promising reporter system for the study of *Aspergillus* promoters and promoter elements as already described by De Graaff *et al.* (1992b).

We have previously reported that the carbon source and the oxygen level were the two main environmental factors determining the induction of glucose oxidase (Witteveen *et al.* 1990). Northern-blot analysis of glucose oxidase mRNA levels in the wild-type strain shows that regulation of glucose oxidase expression

by carbon-source and oxygen level takes place at the transcriptional level. The activity measurements (Table 1) show that the *gox*B mutation has an effect on the regulation of all three enzymes, and that the dependency on glucose and high oxygen levels are both diminished. In the *gox*E strain only the carbon source dependency, but not the oxygen dependency of glucose oxidase and lactonase, is changed. So, apparently, the carbon source and oxygen effect are relayed, at least partially, by different factors. These results indicate that the *gox*B gene is somehow involved in the oxygen effect.

The absence of induction of catalase and lactonase in the *gox*C strain shows that glucose oxidase activity is necessary for induction of these enzymes, and probably for the induction of glucose oxidase itself as well. Gluconate or glucono-δ-lactone are not inducers of these enzymes, so the induction by H_2O_2 was investigated. All three enzymes are induced by H_2O_2 even though no glucose is present. Since H_2O_2 also induced catalase and lactonase in the glucose oxidase-negative strain, in which we were not able to induce these enzymes by other means, it is very likely that the H_2O_2 which arises from the glucose oxidase reaction is responsible for the dependency of the glucose oxidase induction on glucose oxidase activity itself. Thus H_2O_2 plays a central role in the induction of glucose oxidase and is able to overrule, at least in part, the carbon source effect. The factors necessary for optimal induction, the presence of (high) glucose and high oxygen levels, are also conditions for the optimal activity of glucose oxidase and thus H_2O_2 production. This also explains why 2-deoxyglucose, which is a substrate of glucose oxidase, is an inducer (Mischak *et al.* 1985). It is rather unusual that a product of a reaction induces the enzyme catalyzing the reaction. Here, because of the parallel induction of two catalases, which degrade the H_2O_2 formed, there is a feedback preventing overinduction.

This model can also explain the behaviour of the *gox*B mutants, assuming that the *gox*B product mediates the H_2O_2 induction. The fact that the oxygen effect is diminished in these mutants and that they are the only *gox* mutations that have an effect on the catalase induction confirm this interpretation. The activity levels on D-xylose in this mutant are still lower than on D-glucose. On substrates like acetate and galacturonate the levels of glucose oxidase formed by the *gox*B mutants are even much lower (Witteveen *et al.* 1990). Therefore, besides H_2O_2 induction there is also an effect of the carbon source on induction. The *gox*E gene seems to be involved in this, but other genes might also be involved, considering that we isolated mutants in nine different complementation groups having an effect on glucose oxidase formation. A schematic view of the induction dependencies is given in Fig. 7.

In the multiple-copy transformants with a wild-type or *gox*C background only a 3-4 fold increase in glucose oxidase activity relative to wild-type was observed. Higher levels were present in transformants with a *gox*B background. The poor growth of these strains, might be caused by the high H_2O_2 production. The results of Whittington *et al.* (1990), who also transformed *A. niger* with the

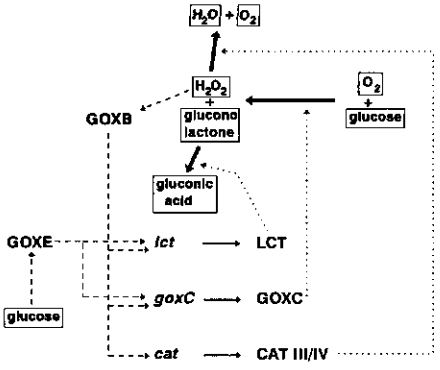

Fig. 7. Scheme for the induction of the glucose oxidase system. lct, goxC, cat indicate the structural gene (in italics) or gene product (capitals) of lactonase, glucose oxidase, and catalase respectively. The *dotted lines* connect the gene products with the reactions they catalyze, indicated by *thick arrows*. The *hyphenated lines* indicate signal transduction between the different components.

glucose oxidase gene, are comparable although a direct comparison cannot be made because of different culturing conditions and different methods of measuring the glucose oxidase levels. There is no straightforward explanation for the lower catalase levels in the *gox*B transformants. However, it might be an indication of the binding of a positive regulator of both the glucose oxidase and catalase genes to the multiple copies of the glucose oxidase gene.

No glucose oxidase has been found in *A. nidulans* (Whittington *et al.* 1990). Studying the induction of glucose oxidase in the *A. nidulans* transformants allows us to obtain information on the specificity of the factors involved in the regulation of the glucose oxidase gene. The transformants show an induction pattern of glucose oxidase similar to the induction in *A. niger* wild-type transformants with respect to glucose dependency. Apparently a more general mechanism is involved. The oxygen dependency, however, seems to be absent in *A. nidulans* transformants, suggesting that this regulatory system (*gox*B) is not present in *A. nidulans*. It also confirms that the oxygen and glucose effects are relayed separately in *A. niger*. The strong staining of the hyphae of the *A. nidulans* transformants using the plate staining method was comparable to that observed in *A. niger*, indicating that a large fraction of the secreted protein is retained in the cell wall.

Comparison of the glucose oxidases formed by *A. niger*, *A. nidulans* and *A. tubigensis* only showed some differences when the proteins were separated by IEF-gel electrophoresis. Hayashi and Nakamura (1981) demonstrated that the different bands of glucose oxidase after separation on an IEF gel are caused by differences in glycosylation. So the most likely explanation for our results is that

there are some differences in glycosylation between the glucose oxidase of *A. niger* and *A. nidulans*. *A. tubigensis* is closely related to *A. niger* (Kusters-van Someren *et al.* 1991). Since we only found some small differences on the IEF gel this protein is presumably very similar to the *A. niger* enzyme, although there might be some minor differences in the peptide chain and/or the glycosylation of the protein. Since the differences in properties were very small we have not investigated this further.

Acknowledgements.
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Chapter 5

Characterization of a glycerol kinase mutant in *Aspergillus niger*[1]

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SUMMARY

A glycerol-kinase-deficient of *Aspergillus niger* mutant was isolated. Genetic analysis revealed that the mutation is located on linkage group VI. The phenotype of this mutant differed from that of a glycerol kinase mutant of *Aspergillus nidulans* in its ability to utilize dihydroxyacetone (DHA). The weak growth on glycerol of the glycerol kinase mutant showed that glycerol phosphorylation is an important step in glycerol catabolism. The mutant could still grow normally on DHA because of the presence of a DHA kinase. This enzyme, probably in combination with an NAD^+-dependent glycerol dehydrogenase, present only in the mutant, is resposible for the weak growth of the mutant on glycerol. Enzymic analysis of both the mutant and the parental strain showed that at least three different glycerol dehydrogenases are formed under different physiological conditions: The NAD^+-dependent described above, a constitutive $NADP^+$-dependent enzyme, and a D-glyceraldehyde specific enzyme which is induced on D-galacturonate. The glycerol kinase mutant showed impaired growth on D-galacturonate.

INTRODUCTION

Glycerol plays a central role in the physiology of many organisms. Its role in the regulation of the osmotic potential of the cell is generally recognized (Brown, 1976 and 1978). The function of glycerol as an osmoticum has also been demonstrated in filamentous fungi (Hocking and Norton, 1983; Beever and Laracy, 1986). Furthermore, glycerol is involved in metabolic processes occurring during germination of fungal spores (Van Laere and Hulsmans, 1987) and is also an intermediate in the breakdown of some compounds, for example galacturonic

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Abbreviations: DHA, dihydroxyacetone; DHAP, dihydroxyacetonephosphate; GAD, D-glyceraldehyde; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide; PMS, phenazine methosulfate.

acid (Uitzetter *et al.*, 1986). Glycerol formation plays a role during citric acid production by *Aspergillus niger* (Röhr *et al.*, 1987). Legisa and Mattey (1986) suggested that glycerol accumulation initiates this process.

The catabolic pathways of glycerol utilization have been investigated in many organisms (Lin, 1976 and 1977). These studies demonstrated that there are two alternative pathways. In one, the phosphorylating pathway, glycerol is phosphorylated and subsequently oxidized to dihydroxyacetone phosphate (DHAP) by a flavin-dependent membrane bound mitochondrial glycerol-3-phosphate dehydrogenase (Gancedo *et al.*, 1968). In the other, the oxidation pathway, glycerol is oxidized to dihydroxyacetone (DHA) followed by phosphorylation to DHAP (May and Sloan, 1981; May *et al.*, 1982; Gancedo *et al.*, 1986). The first pathway is used by most organisms.

In the filamentous fungus *Neurospora crassa* glycerol is catabolized through the phosphorylating pathway (Courtright, 1975 a and b; Holm *et al*, 1976). Three classes of glycerol non-utilizing mutants have been isolated in *Aspergillus nidulans*: *glc*A, *glc*B (Payton, 1978; Uitzetter *et al.*, 1986) and *glc*C (Visser *et al.*, 1988). *glc*A and *glc*B are deficient in glycerol kinase and FAD-dependent glycerol-3-phosphate dehydrogenase respectively (J.Visser and co-workers, unpublished results), and *glc*C is a glycerol uptake mutant. This demonstrates that *A. nidulans* also uses the phosphorylating pathway.

There were three reasons for us to investigate glycerol metabolism in *A. niger*: (1) glycerol metabolism appears to be different from that in *A. nidulans* and *N. crassa*; (2) it plays an important role in osmotic regulation; and (3) it may play a role in citric acid production in *A. niger.*

METHODS

Biochemicals. DHAP, D-glyceraldehyde (GAD) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma. $NAD(P)^+$, NAD(P)H, ATP, glycerol-3-phosphate and glycerol-3-phosphate dehydrogenase were supplied by Boehringer Mannheim. Phenazine methosulfate (PMS) and DHA were from Serva. Other chemicals were from Merck. Sephadex G25 and the Mono Q column were obtained from Pharmacia.

Isolation of the mutant and genetic mapping of the mutation. The parental strain used in this investigation was the auxotrophic and morphological mutant N423, derived from *A.niger* N400 (CBS 120.49). The markers of this mutant are short conidiophores (*csp*A1) and nicotinamide deficiency (*nic*A1) (Bos *et al.*, 1988).

Mutations were induced by UV treatment. A suspension of conidia (8×10^6/ml) in a Petri dish was irradiated for 2.5 min with 2 $\mu J\ mm^{-2}\ s^{-1}$ resulting in 70 % survival (Bos, 1987). A filtration enrichment procedure was used as described essentially by Uitzetter *et al.* (1986). A sample (10 ml) of the irradiated conidia and 40 ml minimal medium, with D-galacturonate as carbon source were used. The conidial suspension was incubated at 30°C in a rotary shaker. The medium was filtered twice and renewed

once a day. The enrichment procedure was continued for 10 d after which time 1.3 x 10^6 conidia were left. The conidia were rescued on complete medium with glucose as carbon source and grown for 48 h at 30°C. The colonies obtained were tested on minimal medium with glucose, acetate or D-galacturonate as carbon source. Mutants which showed defective growth on D-galacturonate were purified for further analyses.

The linkage group of the mutation was determined by somatic recombination with a tester strain (N655) carrying a colour marker and auxotrophic markers on different linkage groups, i.e.: *fwn*A1(I); *his*D4(II); *lys*A7, *bio*A1 (III); *leu*A1(IV); *met*B1(V); *pab*A1(VI). The origin and characterization of this strain were described by Bos *et al.* (1988). Isolation of diploids and haploidization was done according to Bos *et al.* (1988). Segregants were analyzed for genetic markers and the linkage of the mutation with the markers of the tester strain was determined.

Media and growth conditions. Minimal medium contained (l^{-1}): 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 6 g $NaNO_3$, 0.1 g yeast extract, 0.9 mg $ZnSO_4.7H_2O$, 0.2 mg $MnCl_2.4H_2O$, 0.06 mg $CoCl_2.6H_2O$, 0.06 mg $CuSO_4.5H_2O$, 0.04 mg $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.29 mg $CaCl_2.2H_2O$, 0.2 mg $FeSO_4.7H_2O$. In all experiments minimal medium was supplemented with 1 mg nicotinamide l^{-1}. The carbon source was 100 mM glucose, DHA or glycerol or 50 mM galacturonate; these were added separately as membrane filter sterilized solutions. The growth temperature for all experiments was 30°C.

Conidiospores were harvested from cultures on complete medium according to Pontecorvo *et al.* (1953) except that glucose was replaced by 2% (w/v) sucrose. Mycelium was grown in 1 litre Erlenmeyer flasks containing 300 ml medium, inoculated with approximately 10^6 conidiospores ml^{-1} and incubated in a New Brunswick rotary shaker for 20 - 24 h at 280 rpm.

To measure enzyme activities induced by glycerol or galacturonate in the *A.niger* mutant NW201, mycelium grown on glucose for 24 h was transferred for 16 h to minimal medium containing either of these substrates. Growth tests were performed on Petri dishes containing minimal medium, 1.2% (w/v) agar and 50 mM of one of the carbon sources.

Preparation of mycelium and mycelial extracts for NMR measurements. Mycelia of the parental strain N423 and the glycerol mutant NW201, grown for 24 h in minimal medium with 2% (w/v) sucrose and subsequently transferred for 16 h to minimal medium containing either DHA or D-galacturonate as carbon source, were harvested and prepared for NMR measurements as described by Dijkema *et al.* (1985). Mycelia of the mutant and the parental strain grown on sucrose (2 % (w/v)) for 24 h and transferred for 16 h to glycerol were harvested by filtration, washed with distilled water and frozen in liquid nitrogen. The frozen mycelium was extracted with 10% (v/v) $HClO_4$ as follows: 1.5 g (wet wt) frozen mycelium was crushed in a mortar and added in small portions to 3 ml ice-cold 10% $HClO_4$ in a Potter-Elvejhem homogenizer and homogenized. This suspension was frozen in liquid nitrogen, allowed to thaw and vortexed. The freeze-thaw procedure was repeated twice to allow quantitative liberation of the cell metabolites (Weibel *et al.*, 1974). This extract was centrifuged in the cold for 10 min at 10,000 x g. The supernatant was neutralized with $KHCO_3$ after which the precipitate was removed by centrifugation. The supernatant was used for NMR measurements.

NMR measurements. ^{13}C NMR spectra of mycelia and mycelial extracts were

obtained at 75.46 MHz on a Bruker CXP-300 NMR spectrometer using 10 mm NMR tubes. Mycelial spectra were acquired by accumulation of 7200 transients with a 60 ° flip angle and 0.5 s recycle time (1 h), applying broadband proton noise decoupling. Spectra of mycelial extracts were acquired by accumulating 3600 transients with a 30° flip angle and 1 s recycle time (1 h), using the standard composition Waltz pulse sequence for proton decoupling. In all cases the measuring temperature was kept close to 0°C.

Preparation of cell-free extracts. Mycelium was harvested by filtration, washed with 0.85% NaCl and frozen in liquid nitrogen. The frozen mycelium was disrupted in a Braun homogenizer and subsequently suspended in extraction buffer (50 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$). After 1 h the homogenate was centrifuged at 1000 x g for 30 s to remove cell debris. The supernatant was centrifuged at 10,000 x g for 10 minutes. The pellet of this high speed centrifugation was resuspended in extraction buffer and used for the measurement of FAD-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.99.5). The supernatant was used for the other enzyme measurements.

Fractionation of cell free extract. 1 ml cell-free extract was fractionated on an FPLC Mono Q column using a 0 - 0.5 M NaCl gradient in 20 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM ß-mercaptoethanol. Fractions of 0.4 ml were collected and DHA kinase and GAD kinase were measured as described below.

Enzyme assays. Enzyme activities were measured at 25°C on an Aminco DW-2 spectrophotometer in the dual wavelength mode, measuring A_{340} - A_{380}. A value of 5.33 mM^{-1} cm^{-1} was used for the absorption coefficient of NAD(P)H. The enzyme assays were done in a 1 ml volume. DHA kinase (EC 2.7.1.28) was measured in a mixture containing: 50 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM ATP, 0.2 mM NADH, 3 units of glycerol-3-phosphate dehydrogenase, 30 units of triosephosphate isomerase and 10 mM DHA. GAD kinase was determined by the same method as GAD kinase except that 10 mM GAD was used. FAD-dependent membrane--bound glycerol-3-phosphate dehydrogenase (EC 1.1.99.5) was measured in the resuspended pellet of the high speed centrifugation step (see above), using a modification of the method described by Adler *et al.* (1985). The reaction mixture contained: 50 mM HEPES/NaOH, pH 7.5, 10 mM KCN, 0.5 mM MTT, 0.2 mM PMS, 0.05% Triton X-100, 10 mM L-glycerol-3-phosphate. An absorption coefficient for reduced MTT at 550 nm of 8.1 mM^{-1} cm^{-1} was used. Glycerol dehydrogenase (EC 1.1.1.6) was measured in 100 mM glycine NaOH buffer pH 9.6, 0.4 mM $NAD(P)^+$, 100 mM glycerol. DHA reductase and GAD reductase were measured in 50 mM sodium phosphate pH6.5, 0.2 mM NAD(P)H, 10 mM DHA or GAD. Glycerol kinase (EC 2.7.1.30) was assayed by the discontinuous assay described by de Koning *et al.* (1987).

Analytical methods. Protein concentration was estimated, after denaturation and precipitation of protein with sodium deoxycholate and trichloroacetic acid (Bensadoun and Weinstein, 1976), by the microbiuret method (Itzhaki and Gill, 1964) using bovine serum albumin as standard.

RESULTS

Isolation of the mutant and genetic mapping of the mutation

After the filtration enrichment 1.3 x 10^6 conidiospores were left from which 1200 colonies were rescued. Screening of these colonies revealed only one (NW201) that was unable to grow on galacturonate. The mutation in the glycerol-negative mutant was denoted *glc*A1.

Upon haploidization of a diploid obtained from a heterokaryon of NW201 and the tester strain N655, 105 segregants were analyzed. The frequency of recombination between *glc*A1 and *pab*A1 was found to be 8.6%, whereas this frequency with all the other markers varied between 44% and 53%. Therefore the *glc*A1 mutation is located on linkage group VI. The recombination between markers, known to be located on one linkage group was 1.9% for *nic*A1 and *met*B1 (linkage group V).

Growth Characteristics

Growth of the mutant NW201 and the parental strain N423 was tested on agar plates for a number of carbon sources. Growth on glucose, acetate and gluconate was the same in both the mutant and the parental strain. The mutant grew only slightly slower on DHA. A large effect of the mutation was seen on glycerol or D-galacturonate, since with these carbon sources only weak growth was observed in the mutant compared to the parental strain. In addition, we observed a decreased ability of the glycerol mutant to sporulate.

Enzyme activities in the wild-type and mutant NW201

Table 1 summarizes the enzyme activities related to glycerol metabolism in cell-free extracts from the wild-type and from the mutant. All the activities were measured at least twice and the mean of these values is given. The reproducibility was reasonable, activities from different mycelia not varying more than 10 - 20 %. The results show that glycerol kinase activity could not be measured in any of the mycelial extracts of the mutant NW201, while the other enzymes were present. It was thus concluded that the *glc*A1 mutation causes a glycerol kinase deficiency.

The other enzyme activities showed no significant differences between the mutant and the parental strain when grown on glucose. The other three substrates, however, seem to cause a greater induction of a number of activities in the mutant: DHA kinase, GAD kinase, the FAD-dependent glycerol-3-phosphate dehydrogenase and a NAD^+-dependent glycerol dehydrogenase. The latter activity is very low in the parental strain and seems to be relatively specific for DHA since the activity of the NADH-dependent DHA reductase is increased in the same mycelial extracts whereas the NADH-dependent GAD reductase activity is much lower.

Apart from the NAD^+-dependent glycerol dehydrogenase there are at least two

Table 1. Enzyme activities involved in glycerol metabolism in the *A. niger* glycerol kinase mutant NW201 and the parental strain N423.

	Enzyme activity [nmol min^{-1} (mg protein)$^{-1}$] in *A. niger* NW201 grown on[1]:			
Enzyme	Glucose	DHA	(transfer to) Glycerol	(transfer to) D-galacturonate
DHAP reductase	19	15	14	10
DHAP kinase	0	208	290	688
D-Glyceraldehyde kinase	0	136	195	426
Glycerol-3-phosphate deh.	3	55	95	289
Glycerol deh. ($NADP^+$)	145	19	56	189
Glycerol deh. (NAD^+)	0	6	25	62
DHA reductase (NADPH)	925	70	238	276
DHA reductase (NADH)	0	64	252	212
GAD reductase (NADPH)	625	138	277	1534
GAD reductase (NADH)	0	35	74	110
Glycerol kinase	0	0	0	0

	Enzyme activity [nmol min^{-1} (mg protein)$^{-1}$] in *A. niger* NW423 grown on[1]:			
Enzyme	Glucose	DHA	Glycerol	D-galacturonate
DHAP reductase	12	8	10	27
DHAP kinase	4	46	21	29
D-Glyceraldehyde kinase	0	30	10	12
Glycerol-3-phosphate deh.	5	18	6	13
Glycerol deh. ($NADP^+$)	111	83	60	98
Glycerol deh. (NAD^+)	0	5	0	0
DHA reductase (NADPH)	623	353	362	535
DHA reductase (NADH)	0	8	0	3
GAD reductase (NADPH)	537	403	327	1283
GAD reductase (NADH)	14	38	5	15
Glycerol kinase	9	77	40	35

[1]Mycelia were grown for 20-24 h on the carbon source indicated, except in the case of the mutant NW201 on glycerol and D-galacturonate, for which mycelium was grown for 24 h on minimal medium with glucose and subsequently transferred for 15 h to minimal medium with either glycerol or D-galacturonate.

other glycerol dehydrogenases, both $NADP^+$-dependent. One is induced on D-galacturonate and relatively specific for GAD. The other is found especially in glucose-grown mycelium and is slightly repressed by the other three substrates. This repressive effect is even stronger in the mutant. This enzyme is relatively specific for DHA and is probably the same enzyme as the one partly purified by Baliga *et al.* (1962). This enzyme has been purified and characterized in our laboratory (Schuurink *et al.*, 1990) and is relatively specific for DHA, GAD giving

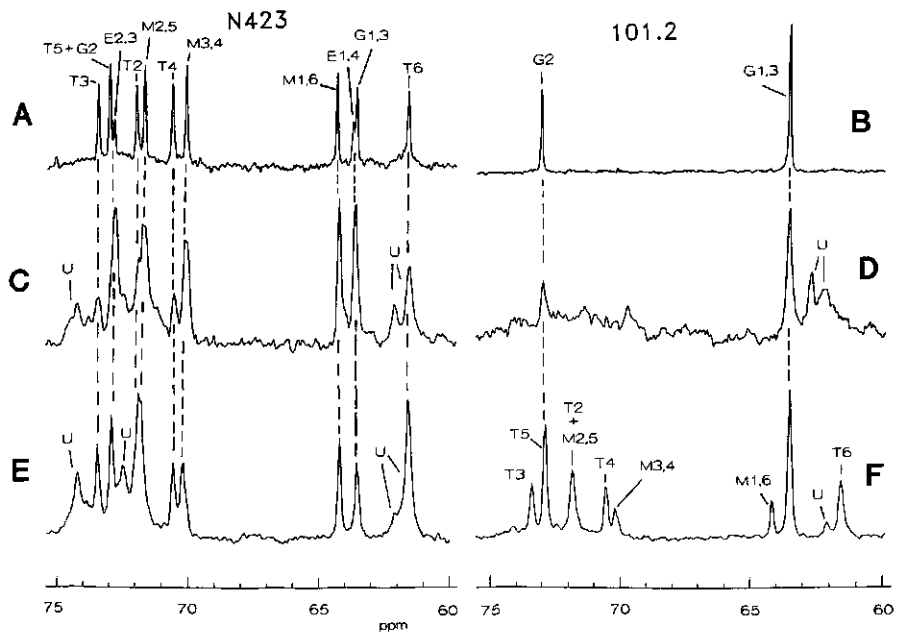

Fig. 1. ^{13}C NMR spectra of extracts of mycelia (A, B) and of mycelia (C, D, F) of the *A. niger* parental strain N423 (A, C, E) and the glycerol kinase mutant NW201 (B, D, F). Spectra were determined 16 h after transfer of mycelia to glycerol (A, B), D-galacturonate (C, D) or DHA (E, F). Details are given in Methods. M, mannitol; E, erythritol, G, glycerol; T, trehalose; U, unidentified resonance.

only 10 % of the DHA reductase activity.

Metabolite accumulation in the wild-type and mutant NW201

Mycelia of N423 and NW201, grown for 24 h on sucrose, were transferred for 16 h to minimal medium with DHA, D-galacturonate or glycerol as carbon source. ^{13}C NMR spectra of mycelia transferred to DHA and D-galacturonate or of extracts of mycelia after transfer to glycerol are shown in Fig.1. The spectra of the mycelia of the wildtype and the mutant grown on sucrose for 24 h were essentially the same as those shown by Witteveen *et al.* (1989), that is high levels of mannitol and to a lesser extent of erythritol were accumulated. After transfer to glycerol, D-galacturonate or DHA, the parental strain accumulated especially glycerol and mannitol and the disaccharide trehalose (Fig. 1A, C, E). These metabolites are usually seen in mycelium grown on substrates whose metabolism is associated with glycerol, as in *Aspergillus nidulans* (Dijkema *et al.*, 1985). The period of 16 h was long enough for the mycelium to adapt to the new carbon sources and to grow on them. The mutant mycelia transferred to glycerol or D-galacturonate accumulated only glycerol in the mycelium (Fig. 1B, D). These results demonstrate that D-galacturonic acid is converted to glycerol, which cannot be further metabolized by the mutant, and also that the mutant can

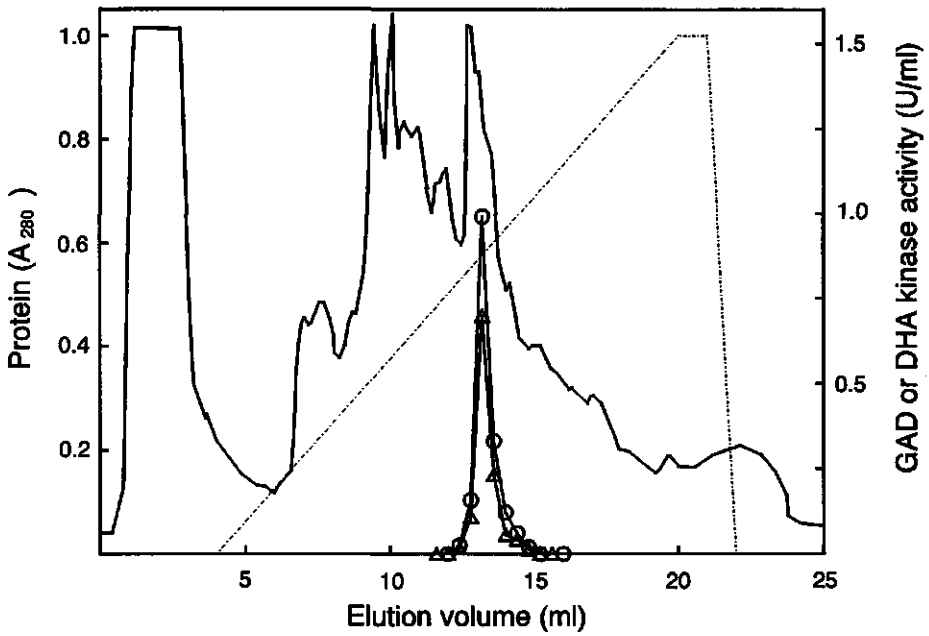

Fig. 2. Fractionation of cell-free extract of mycelium of the glycerol kinase mutant NW201. The mutant was grown for 24 h on glucose and subsequently transferred for 15 h to minimal medium with D-galacturonate. An FPLC MonoQ column was used. Elution was with a gradient (---) of 0 - 0.5 M NaCl in 20 mM Tris/HCl, pH 7.5; fractions of 0.4 ml were collected. Protein eluting from the column was determined as the A_{280} (——). DHA kinase (○) and GAD kinase (△) activities were measured in all fractions; only the levels in fractions containing detectable kinase activities are indicated.

take up glycerol. The mannitol and erythritol pools were depleted by the mutant under these conditions, since no other substrates are available. Transfer of the mutant to DHA (Fig. 1F) gave high levels of glycerol, trehalose and mannitol, although lower than those observed on the transfer of the parental strain to DHA (Fig. 1E). Thus DHA can still be metabolized by the mutant.

DHA kinase and GAD kinase

The results in Table 1 suggest that phosphorylation of DHA and of GAD is catalysed by a single enzyme. Since this is of importance for interpretation of the induction characteristics to test this further, we fractionated an extract of the mutant mycelium, grown for 24 h on sucrose and subsequently transferred to D-galacturonate for 16 h, on an FPLC Mono Q Column. All the fractions were tested for DHA and GAD kinase activities. The two activities eluted siultaneously (Fig. 2), a strong indication that both are catalysed by the same enzyme. Only those fractions showing some phosphorylating activity are shown in Fig. 2.

These partly purified preparations were also used for some preliminary kinetic measurements. By varying the DHA or GAD concentrations we found that the concentration required to obtain half of the maximum velocity was approximately 5-10 μM for DHA and 30-100 μM for GAD.

DISCUSSION

The phenotype of the glycerol kinase mutant demonstrates that glycerol catabolism in *A.niger* occurs by the phosphorylation of glycerol to glycerol-3-phosphate and subsequent oxidation by an FAD-dependent glycerol-3-phosphate dehydrogenase, the same pathway as used by *Neurospora crassa* (Courtright, 1975a, b) and *A. nidulans* (unpublished results from our laboratory).

The fact that the glycerol kinase mutant grows only weakly on glycerol even though glycerol dehydrogenase and DHA kinase are present, can be explained by the very unfavourable equilibrium for oxidation of glycerol to DHA (McGregor *et al.*, 1974). It is remarkable though, that in the glycerol kinase mutant, where the phosphorylation route is blocked, an NAD^+-dependent glycerol dehydrogenase is induced. We postulate this enzyme to be responsible for the weak growth which is still observed on glycerol. Organisms which catabolize glycerol via the oxidation of glycerol also use an NAD^+-dependent glycerol dehydrogenase (Lin, 1976; May and Sloan, 1981). The lower catabolic reduction charge in the cell compared to the anabolic reduction charge favours the NAD^+-dependent oxidation of glycerol. From this point of view the results of St Martin *et al.* (1975) are of interest. From an *E. coli* parental strain with defects in both the glycerol kinase and in the FAD-dependent glycerol 3-phosphate dehydrogenase genes, and thus not able to grow on glycerol, a mutant was selected which could utilize glycerol. This mutant overproduced an NAD^+-dependent glycerol dehydrogenase, enabling the utilization of glycerol via the oxidative route.

The ability of the glycerol kinase mutant to grow on DHA and the presence of DHA kinase is an indication that the major degradation pathway for DHA in wild-type mycelium is by direct phosphorylation and not via reduction to glycerol. This, however, may be an oversimplification. In mutant mycelium grown on DHA the level of DHA reductase is strongly repressed while the level of DHA kinase is much higher than in the parental strain. Furthermore, the NMR spectra of the mycelia grown on DHA show a different metabolite accumulation pattern as compared to the parental strain grown on DHA. Therefore, it may very well be that in the parental strain DHA is still largely catabolized via glycerol.

Assuming a catabolic pathway of D-galacturonic acid leading to GAD and pyruvate (Uitzetter *et al.*, 1986), it is not clear why the mutant is not able to grow on D-galacturonate since pyruvate can still be metabolized and GAD can be reduced to glycerol, a nontoxic compound. The inability of the glycerol mutant to grow on D-galacturonate, the NMR spectra of mycelia transferred to D-galacturo-

nate and the induction of a number of enzymes from glycerol metabolism by D-galacturonate confirm that D-galacturonate is catabolized, in part, via glycerol. High levels of GAD kinase are present are present in the mutant. With the situation for DHA in mind, it must be considered why GAD formed from D-galacturonate is not directly phosphorylated thus allowing the mutant to grow on D-galacturonate. The situation is different from that of DHA catabolism at two points. Firstly, a strong induction of a GAD reductase takes place, stimulating a fast reduction of GAD to glycerol. Secondly, the affinity of the enzyme catalyzing both DHA and GAD phosphorylation, is much higher for DHA than for GAD. These two characteristics might result in phosphorylation of DHA, while GAD is preferably reduced. The position of the equilibrium far in the direction of glycerol prevents further metabolism.

Under the different growth conditions at least three different glycerol dehydrogenases are apparent, all with different functions. The highest levels of the NADPH-dependent enzyme which is relatively specific for DHA are found in glucose-grown mycelium. This enzyme might function in the synthesis of glycerol which accumulates in this fungus under certain conditions or in the detoxification of DHA formed in the cell. The repression of this enzyme by glycerol, DHA and D-galacturonate indicates that it does not *per se* have a role in catabolism of these substrates. The second NADPH dependent enzyme is induced by D-galacturonate and is involved in a step of the degradation route of this substrate. Considering the relative levels of NADPH-dependent GAD reductase and DHA reductase, this particular enzyme is very likely to be also present when *A.niger* is grown on one of the other substrates. This enzyme might be comparable to the glycerol dehydrogenase isolated from *Neurospora crassa* by Viswanath-Reddy *et al.* (1978). The NADH-dependent glycerol dehydrogenase, which we could measure in significant amounts only in the glycerol kinase mutant, might be involved in the oxidation of glycerol as an alternative for the phosphorylation pathway. Perhaps this enzyme has a role when the route via the membrane bound mitochondrial glycerol-3-phosphate dehydrogenase is inhibited. The ability to produce at least three different glycerol dehydrogenases bears some similarity with the situation in *Schizosaccharomyces pombe*. Kong *et al.* (1985) found four different glycerol dehydrogenases in this yeast, which have characteristics comparable to the enzymes described here.

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Chapter 6

L-arabinose and D-xylose catabolism in *Aspergillus niger*[1]

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SUMMARY

A mutant of *Aspergillus niger* unable to grow on D-xylose and L-arabinose has been isolated. Genetic analysis revealed that the mutation was located on linkage group IV. Enzymic analysis revealed a deficiency in D-xylulose kinase activity. After transfer of growing mycelium to a medium containing either D-xylose or L-arabinose, the mutant accumulates large amounts of arabitol and xylitol, as shown by ^{13}C NMR spectroscopy. These data and an analysis of enzyme activities induced by D-xylose and L-arabinose in the wild type strain led to the following catabolic pathway for D-xylose: D-xylose - xylitol - D-xylulose - D-xylulose-5-phosphate; and for L-arabinose: L-arabinose - L-arabitol - L-xylulose - xylitol - D-xylulose - D-xylulose-5-phosphate. The reduction steps of the sugars to the corresponding polyols are all NADPH dependent. The oxidation steps of the polyols to the sugars are all NAD^+ dependent. Fractionation of cell-free extracts gave information about the specificity of the enzymes and showed that all reactions are catalysed by different enzymes.

INTRODUCTION

Pentose metabolism in filamentous fungi has received little attention since the early reports on this subject by Chiang and coworkers (Chiang *et al.*, 1958; Chiang and Knight, 1959, 1960a, b, 1961). Later Hankinson (1974) studied in *A. nidulans* two classes of mutants, *ppp*A and *ppp*B, which were disturbed in the pentose phosphate pathway. These mutants showed no or decreased growth on D-xylose and L-arabinose. D-xylose metabolism has been studied intensively in yeasts, especially in relation to the production of ethanol under anaerobic conditions (Jeffries, 1985). L-arabinose catabolism, however has received little attention.

Most organisms convert both D-xylose and L-arabinose to D-xylulose-5-phos-

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phate, although different pathways are used by different organisms . A remarkable difference between bacteria and most fungi is the way in which they convert D-xylose to D-xylulose. Bacteria usually use an isomerase, whereas fungi reduce D-xylose to xylitol which is subsequently oxidized to D-xylulose (Jeffries, 1983). For L-arabinose breakdown several pathways have been described. The reduction-oxidation pathway described for *Penicillium chrysogenum* by Chiang and Knight (1961) contains the following steps: an NADPH dependent reduction to L-arabitol, oxidation to L-xylulose (NAD^+), reduction to xylitol (NADPH) and oxidation to D-xylulose (NAD^+). The principle of this catabolic sequence is comparable to the D-xylose pathway found in fungi (Chiang and Knight, 1960b).

Aspergillus niger grows very well on pentose substrates and it can also hydrolyse polymers, such as hemicelluloses, which contain these sugars. Pentoses are presumably important substrates for this fungus, and since for *Aspergillus* little information is available on this subject, this study on catabolism of L-arabinose and D-xylose was initiated.

METHODS

Biochemicals. The pent(ul)oses, pentitols and D-xylulose-5-phosphate were purchased from Sigma. $NAD(P)^+$, NAD(P)H, ATP, glucose-6-phosphate, 6-phosphogluconate, phosphoenolpyruvate and enzymes were supplied by Boehringer Mannheim. Other chemicals were from Merck. Sephadex G25 and the Mono Q column were obtained from Pharmacia. Matrex Red A was from Amicon.

Isolation of the mutant and genetic mapping of the mutation. The parental strain used in this investigation was an auxotrophic and morphological mutant, N423, derived from *A.niger* N400 (CBS 120.49). The markers of mutant N423 are short conidiophores (*csp*A1) and a nicotinamide deficiency (*nic*A1) (Bos *et al.*, 1988).

Further mutations were induced by UV treatment of strain N423. A suspension of conidia (10^7 ml^{-1}) in a Petri dish was irradiated for 2 min with 2 μJ mm^{-2} s^{-1} resulting in 93 % survival (Bos,1987). A filtration enrichment procedure was employed essentially as described by Uitzetter *et al.*, (1986), using 10 ml of the irradiated conidia and 40 ml minimal medium, with xylitol as carbon source and supplemented with nicotinamide(1 mg l^{-1}). The conidial suspension was incubated at 30°C in a rotary shaker. The filtration and refreshment of the medium was carried out twice a day and the enrichment procedure was continued over 96 h at which time 3 x 10^4 conidia were left. The conidia were rescued on complete medium with glucose as carbon source and grown for 48 h at 30°C. The colonies obtained were tested on minimal medium supplemented with nicotinamide and different carbon-sources : glucose, gluconic acid, acetic acid, D-xylose and L-arabinose. Mutants which showed defective growth on xylose were purified for further analyses.

The linkage group of the mutation was determined by somatic recombination with a tester strain (N655, Bos *et al.*, 1988) carrying a colour marker and auxotrophic markers on different linkage groups, viz. *fwn*A1(I); *his*D4(II); *lys*A7, *bio*A1 (III); *leu*A1(IV); *met*B1(V); *pab*A1(VI). Isolation of diploids and haploidization was performed according

to Bos *et al.* (1988). Segregants were analysed for genetic markers and the linkage of the mutation with the markers of the tester strain was determined.

Media and growth conditions. Minimal medium contains per litre: 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 2.1 g urea, 0.1 g yeast extract, 0.9 mg $ZnSO_4.7H_2O$, 0.2 mg $MnCl_2.4H_2O$, 0.06 mg $CoCl_2.6H_2O$, 0.06 mg $CuSO_4.5H_2O$, 0.04 mg $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.29 mg $CaCl_2.2H_2O$, 0.2 mg $FeSO_4.7H_2O$, and 1 mg nicotinamide. The carbon source was 20 g l^{-1} sucrose or 100 mM D-xylose, L-arabinose or D-glucose; these were added separately as membrane filter sterilized solutions.

Conidiospores were harvested from complete medium according to Pontecorvo *et al.*(1953) except that glucose was replaced by 2% (w/v) sucrose.

Mycelium was grown in Erlenmeyer flasks containing 300 ml medium and incubated in a New Brunswick rotary shaker for 24 h at 280 rpm and 30°C.

To permit measurements of pentose-catabolism-related enzyme activities in the pentose mutant N572, this strain was grown on minimal medium with sucrose for about 24 h and subsequently transferred to minimal medium containing either L-arabinose or D-xylose for 16 h to induce the pentose catabolism related activities. The same procedure was used to grow mycelium for the NMR measurements.

Growth tests were performed on Petri dishes containing minimal medium, 1.5% (w/v) agar and 50 mM of one of the carbon sources.

Preparation of mycelium for NMR measurements. NMR sample preparation and NMR measurements were done as previously described (Dijkema *et al.*, 1985).

Preparation of cell-free extracts. Mycelium was harvested by filtration, washed with 0.85% NaCl and frozen in liquid nitrogen. The frozen mycelium was disrupted in a Braun dismembrator and subsequently suspended in extraction buffer (20 mM Bis-Tris pH 6.5, 5 mM $MgCl_2$, 1 mM ß-mercaptoethanol). After 1 h the homogenate was centrifuged at 10,000 x g for 10 min. Low molecular weight compounds were removed from the supernatant by passage through a 15 ml Sephadex G25 column. This preparation was used for enzyme assays and fractionation experiments.

Fractionation of cell free extract. A 1 ml portion of cell-free extract purified over a Sephadex G25 column was fractionated on a FPLC Mono Q column using a gradient of 0 - 0.5 M NaCl in 20 mM Bis-Tris pH 6.5, 5 mM $MgCl_2$ and 1 mM ß-mercaptoethanol.

Enzyme assays. Enzyme activities were measured at 25°C on an Aminco DW-2 spectrophotometer in the dual wavelength mode, measuring A_{340} - A_{380}. A value of 5.33 mM^{-1} cm^{-1} was used for the absorption coefficient of NAD(P)H.

Enzyme assays were performed in a 1 ml volume. The assay mixtures for the enzymes were as follows. Pentitol dehydrogenases: 100 mM glycine buffer pH 9.6, 0.4 mM $NAD(P)^+$, 100 mM xylitol, L-arabitol, D-arabitol or ribitol; pentose reductases (EC 1.1.1.21): 50 mM sodium phosphate buffer pH 6.5, 0.2 mM NAD(P)H, 100 mM L-arabinose or D-xylose; pentulose reductases were assayed as the pentose reductases with 5 mM D-xylulose, L-xylulose or D-ribulose; D-xylulose kinase(EC 2.7.1.17): 250 mM glycylglycine pH 7.4, 5 mM $MgSO_4$, 0.2 mM NADH, 1 unit pyruvate kinase, 3 units lactate dehydrogenase, 1.5 mM phosphoenolpyruvate, 5 mM D-xylulose, 1 mM ATP. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.44) and fructose-1,6-bisphosphatase (EC 3.1.3.11) were measured as described by Bruinenberg *et al* (1983b). D-Xylulose-5-phosphate phosphoketolase (EC 4.1.2.9) was measured by the two methods described by Evans and Ratledge

Table 1. *Enzyme activities involved in pentose metabolim in A. niger*

		Specific activity[1]		
		Carbon source:		
Enzyme	Cofactor	D-glucose	L-arabinose	D-xylose
D-Xylose reductase	NADH	0	0	0
	NADPH	10	245	934
L-Arabinose reductase	NADH	0	0	0
	NADPH	26	619	786
D-Ribose reductase	NADH	0	0	0
	NADPH	5	142	516
D-arabinose reductase	NADH	0	0	0
	NADPH	4	3	12
L-Xylulose reductase	NADH	5	240	49
	NADPH	22	287	71
D-Xylulose reductase	NADH	31	1137	700
	NADPH	14	67	53
D-Ribulose reductase	NADH	21	365	114
	NADPH	60	72	78
Xylitol dehydrogenase	NAD^+	21	852	494
	$NADP^+$	8	118	40
D-arabitol dehydrogenase	NAD^+	12	15	8
	$NADP^+$	18	19	23
L-arabitol dehydrogenase	NAD^+	6	279	68
	$NADP^+$	0	16	16
Ribitol dehydrogenase	NAD^+	14	583	274
	$NADP^+$	3	12	10
D-xylulose kinase[2]		0	430	230

[1] *A. niger* N423 was grown for 21 h on D-glucose, L-arabinose or D-xylose (each 100 mM) as carbon source. Activities are exrpessed in nmol min^{-1} (mg protein)$^{-1}$.
[2] Activities could not be determined accurately (see text).

(1984). D-Xylose isomerase (EC 5.3.1.5) was determined as described by Callens *et al.* (1986).

To enable measurement of D-xylulose kinase in crude cell free extract we either fractionated the mycelium on an FPLC Mono Q column (for analysis of the mutant) or partially purified the extract by passing 1 ml over a 0.3 ml Matrex Red A affinity column, which results in removal of most of the D-xylulose reductase activity, thus enabling the kinase assay to be performed. The last method was used for the data shown in Table 1.

Analytical methods. Protein concentration was estimated, after denaturation and

precipitation of protein with sodium deoxycholate and trichloroacetic acid (Bensadoun and Weinstein, 1976), by the microbiuret method (Itzhaki and Gill, 1964) using bovine serum albumin as standard.

RESULTS

Isolation and mapping of the mutant

After the filtration enrichment 420 colonies were rescued. Screening of these colonies revealed only three mutants : 1 pentose negative mutant (N572) and 2 auxotrophic mutants. The mutation in the pentose negative mutant is denoted *xki*A1.

Upon haploidization of a diploid obtained from a heterokaryon of N572 and N655, 130 segregants were analysed. The frequency of recombination between *xki*A1 and *leu*A1 was found to be 20.0%, whereas this frequency with all the other markers varied between 45% and 60%. Therefore the *xki*A1 mutation is located on linkage group IV. The recombination between markers known to be located on one linkage group was 10.0% for *bio*A1 and *lys*A7 (linkage group III) and 5.4% for *nic*A1 and *met*B1 (linkage group V). These data demonstrate the high frequency of mitotic recombination in *A.niger* relative to *A.nidulans*.

Growth characteristics

The growth of strain N423 and the pentose mutant N572 on a range of carbon sources was compared. The mutant did not grow at all on D-xylose, L-arabinose or xylitol; the slight growth which *A.niger* normally shows on agar medium without carbon source is prevented in the mutant by these compounds. The parental strain grew well on these three substrates. On the other pentoses and pentitols tested, the parental strain grew weakly, and the mutant very weakly. Thus the effect of the mutation on growth was more severe on good substrates. The *xki*A mutant grew normally on substrates other than pentoses (D-glucose, acetate, D-glucoante, D-glacturonate, D-glucuronate, meso-inositol). The utilization of nitrogen sources, including nitrate, was the same in the mutant and the parental strain.

Polyol accumulation in the *xki*A1 mutant and its parent

Information on the *in vivo* effect of a mutation can be derived from the accumulation patterns of metabolites. *In vivo* ^{13}C NMR spectroscopy is a useful tool for such measurements. We used this technique to study metabolite accumulation in the parental strain N423 and the pentose mutant N572, both grown for 24 h on minimal medium with sucrose and subsequently transferred for 16 h to minimal medium with D-xylose or L-arabinose. The results are shown in Fig. 1. Only that part of the spectrum in which the polyol resonances are visible is shown. The resonance positions of erythritol, mannitol and arabitol are as des-

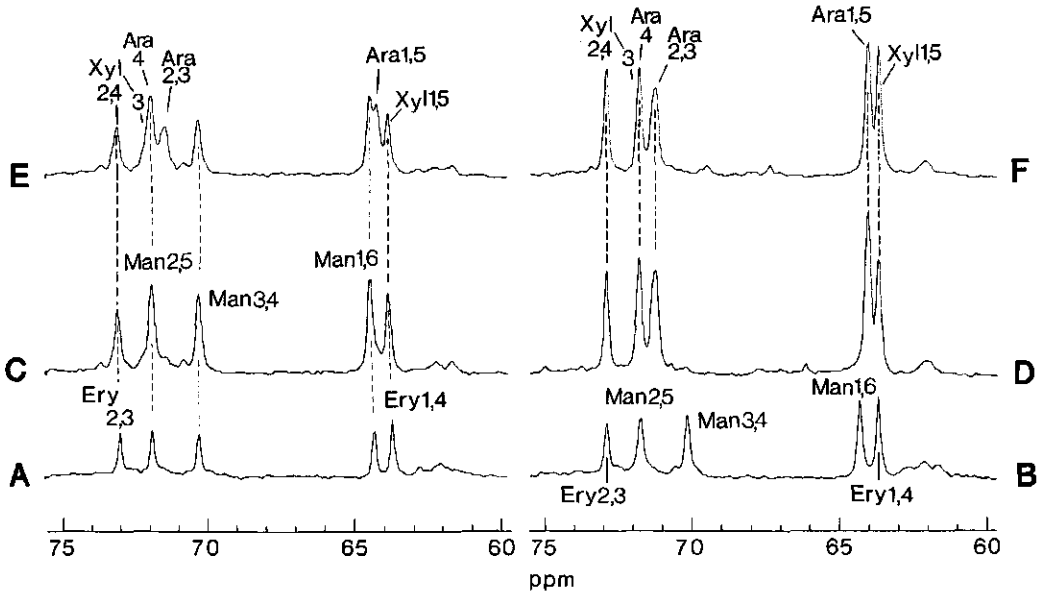

Fig 1. Natural-abundance ^{13}C NMR spectra of the polyol resonance region of *A. niger* N423 (parental strain; spectra A,C and E) and N572 (*xki*A1 mutant; spectra B,D and F). A,B, spectra taken after 24 h growth on 50 mM sucrose; C,D, spectra 16 h after transfer to D-xylose (0.1 M); E, F, spectra 16 h after transfer to L-arabinose (0.1 M).

cribed in Dijkema *et al.* (1985); xylitol shows resonances at 63.6 (C1 and C5), 71.9 (C3) and 72.9 (C2 and C4) ppm. The 24 h cultures of both strains grown on sucrose showed the usual metabolite accumulation pattern for *A. niger*, namely high levels of mannitol and erythritol. Growth of N572 on sucrose is normal and no difference between the mutant and the parental strain was seen in these spectra (Fig. 1, A and B). Transfer of these sucrose-grown mycelia to media containing D-xylose and L-arabinose followed by 16 h incubation resulted in large differences between parent and mutant. On D-xylose the parent accumulated, in comparable amounts, the same polyols as observed on sucrose (Fig 1C). Transfer to L-arabinose resulted in accumulation of arabitol and xylitol as well as mannitol and erythritol (Fig. 1E). The erythritol resonances coincide with two of the xylitol resonances. Integration of the spectrum showed that the relative amounts of arabitol and xylitol are approximately 3:1. Transfer of mutant mycelium to either D-xylose or L-arabinose resulted in accumulation of about equal amounts arabitol and xylitol (Fig. 1, D and F). The polyols mannitol and erythritol had been metabolized by the mycelium because no external carbon source was available. The strong similarity between the mutant spectra on L-arabinose and D-xylose indicates a connection between the two catabolic pathways prior to the step affected by the mutation. Since it is impossible to discriminate between L-arabitol and D-arabitol with ^{13}C NMR we do not know which isomer of arabitol is accumulated in the mycelium.

Determination of D-xylulose kinase deficiency

In crude cell-free extracts it was impossible to measure D-xylulose kinase activity with the pyruvate kinase/lactate dehydrogenase coupled assay because of high NADH dependent D-xylulose reductase and ATP-ase activities. These interfering activities were separated from the kinase activity by fractionation of the cell-free extract on an FPLC Mono Q column. The elution of the D-xylulose kinase activity from the column is shown in Fig. 2(a). At the position where in N423 extracts D-xylulose kinase eluted, only a residual activity (<5% of the parental value) was found in extracts of N572. Other enzyme activities of the pentose catabolic pathway were found at normal or even higher levels in mutant mycelium compared to induced parental mycelium (data not shown). Thus induction of the pathway did take place in the mutant with the procedure we used. In all other fractions no D-xylulose kinase activity was found (data not shown). This proves that N572 is a D-xylulose kinase mutant.

Induction of enzymes specific to the D-xylose and L-arabinose pathway specific enzymes

We measured the activities of a number of relevant enzymes to establish the catabolic pathways which *A.niger* utilizes to degrade pentoses. In addition, the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and fructose-1,6-bisphosphatase were measured to see whether the pentose phosphate pathway is involved in the regeneration of NADPH needed for the pentose reduction.

The pentose related enzyme activities (Table 1) were found to be dependent on the age of the mycelium, showing a gradual decrease with time. However, the relative values were comparable. The activities for D-xylulose kinase could not be determined accurately. The partial purification on a Matrex Red A column may have caused some loss of activity and the high background activities, even after partial purification, decreased the precision of the measurement.

In agreement with the catabolic pathway described for Penicillium by Chiang and Knight (1961), growth of *A. niger* on D-xylose induced D-xylose reductase (NADPH), xylitol dehydrogenase (NAD^+) and D-xylulose kinase, while growth on L-arabinose inducted L-arabinose reductase (NADPH), L-arabitol dehydrogenase (NAD^+), L-xylulose reductase (NADPH), xylitol dehydrogenase (NAD^+) and D-xylulose kinase (Table 1). However, besides these expected activities we measured a few activities which are not involved in the catabolic pathway described by Chiang and Knight (1961). In the first place relatively large amounts of NAD^+-dependent ribitol dehydrogenase and D-ribulose reductase were found, especially in L-arabinose grown mycelium. To find out whether this was due to lack of specificity of one of the enzymes or to induction of other enzymes, we fractionated a cell free extract of L-arabinose grown mycelium (Fig. 2b). A protein binding weakly to the Mono Q column contained NAD^+-dependent xylitol

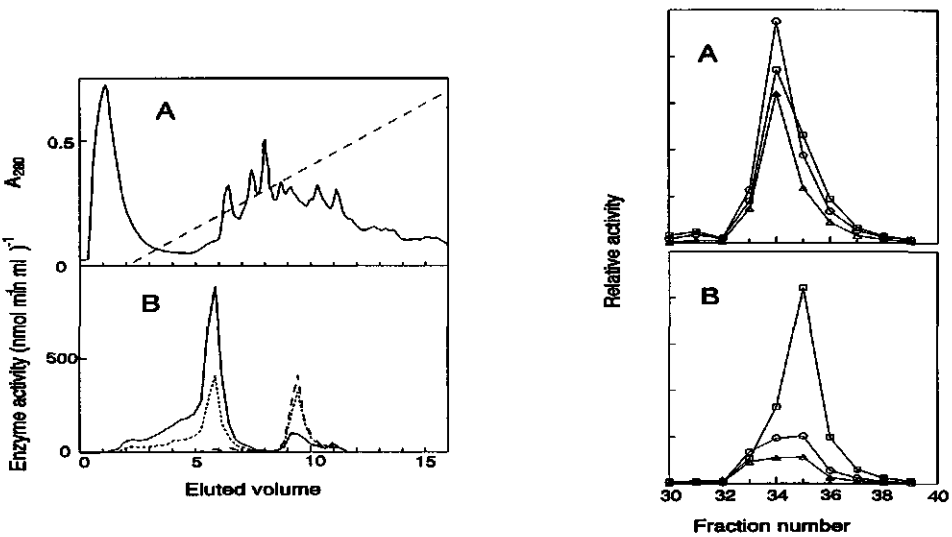

Fig 2. Fractionation by FPLC Mono Q chromatography of cell-free extract of *A. niger* N423 grown on L-arabinose. (a) Absorption at 280 nm of column eluate. The applied salt gradient (0-0.5 M NaCl in 20 mM Bis-Tris pH 6.5) is indicated (---). The elution position of the D-xylulose kinaseactivity is indicated by the arrow. (b) Activity profile of NAD^+-dependent pentitol dehydrogenase, upon fractionation. ————, xylitol dehydrogenase; ..... ,ribitol dehydrogenase; ----, L-arabitol dehydrogenase.

Fig 3. NADPH-dependent pentose reductase activities in cell-free extracts of *A. niger* N423 grown on D-xylose (1) and L-arabinose (b) fractionated by FPLC Mono Q column chromatography. Fractions of 0.3 ml were collected and assayed for the following enzyme activities: O, D-xylose reductase; □, L-arabinose reductase; ▵, D-ribose reductase.

and ribitol dehydrogenase activity (relative activity of xylitol:ribitol is 1 : 0.43). The second activity peak shows NAD^+-dependent L-arabitol, xylitol and ribitol dehydrogenase activity (1 : 0.35 : 0.8, respectively). NADH-dependent L-xylulose and D-xylulose reduction was measured in this second activity peak. The first activity was also found in D-xylose-grown mycelium whereas the second could only be measured in L-arabinose-grown mycelium. These data prove that the high ribitol dehydrogenase activity is due to lack of specificity of both the L-arabitol and xylitol dehydrogenases.

A second observation that needs further explanation is the induction of D-ribose reductase and L-arabinose reductase on D-xylose and the induction of D-ribose reductase and D-xylose reductase on L-arabinose (Table 1). These data suggest that there are at least two enzymes involved which might have a broad substrate spectrum. To investigate the properties of pentose reductases in

D-xylose- and L-arabinose-grown mycelium we separated the activities on an FPLC Mono Q column (Fig. 3). These results show that two different enzymes exist which elute very close to each other. The first is induced in particular by D-xylose and is relatively nonspecific. From these and other data we estimated a relative activity for this general pentose reductase towards D-xylose, L-arabinose and D-ribose of 1 : 0.78 : 0.66, respectively. This enzyme is also found in L-arabinose-grown mycelium, together with an L-arabinose reductase with a relatively high specificity towards L-arabinose. The relative activity of this latter enzyme towards D-xylose, L-arabinose and D-ribose was estimated to be 0.2 : 1 : 0.1, respectively. In later stages of growth on L-arabinose the activity of the general pentose reductase increased relative to the L-arabinose reductase, giving about equal activities of both enzymes (data not shown). We have subsequently purified another L-arabinose pathway specific enzyme, the NADPH-dependent L-xylulose reductase. This enzyme is quite specific towards L-xylulose as substrate and is only induced by L-arabinose. The detailed results are described in Chapter 7.

We conclude from the data presented that all the steps in the two catabolic pathways are catalyzed by different enzymes. We were not able to measure significant amounts of D-xylose isomerase, the enzyme catalyzing the isomerization reaction between D-xylose and D-xylulose.

Especially for growth on L-arabinose, large amounts of NADPH are required. We expected therefore that growth on L-arabinose would require increased activity of the pentose phosphate pathway. To test this hypothesis we measured the fructose-1,6-bisphosphatase, glucose-6-phosphate-dehydrogenase and 6-phosphogluconate dehydrogenase activities (Table 2). The activities of the two dehydrogenases were much higher in mycelia grown on the two pentoses relative to glucose, suggesting an increased flux through the pentose phosphate pathway.

Finally we investigated whether D-xylulose-5-phosphate phosphoketolase was present, which would be able to convert xylulose-5-phosphate in a phosphoroclastic reaction into acetylphosphate and glyceraldehyde-3-phosphate. We were not able to measure significant amounts of this enzyme. This makes it very likely that only the non-oxidative pentose phosphate pathway is involved in D-xylulose-5-phosphate conversion.

DISCUSSION

The mutant N572 is only defective in growth on pentoses or pentitols. Its growth is normal on other substrates, including those which are most probably catabolized by pathways which lead to the pentose phosphate pathway; this is regardless of the nitrogen source used. This indicates that the mutant is defective in a pentose-specific pathway rather than in the pentose phosphate

Table 2. Enzyme activities *A. niger* N423 grown for 21 h on 100 mM D-glucose, L-arabinose or D-xylose with urea as nitrogen source.

	Specific activity[1]		
	Carbon Source		
Enzyme	D-Glucose	L-Arabinose	D-Xylose
Glucose-6-phosphate dehydrogenase	436	928	839
6-Phosphogluconate dehydrogenase	103	185	236
Fructose-1,6-bisphosphatase	12	9	17

[1] Activities are in nmol min^{-1} (mg protein)$^{-1}$.

pathway like the mutants described by Hankinson (1974).

Chiang and coworkers proposed for *Penicillium chrysogenum* a series of reduction and oxidation steps for the conversion of L-arabinose and D-xylose to D-xylulose which is subsequently phosphorylated to D-xylulose-5-phosphate (Chiang and Knight, 1959 and 1961). The phenotype of the mutant N572, the ^{13}C NMR data and the enzyme activity measurements show that this pathway is also used by *A.niger*.

Chiang and Knight (1961) assumed that D-xylose and L-arabinose are reduced by a single NADPH-dependent enzyme and also that L-arabitol and xylitol oxidation is catalysed by a single enzyme. Our data clearly show that this is not the case in *A.niger*. Every catabolic step is catalyzed by a separate enzyme, although some of the enzymes show a broad substrate specificity and may, in part, take over each other's function. For example, the general pentose reductase, the enzyme catalyzing D-xylose reduction, also has a high L-arabinose reductase activity and is present in large amounts during growth on L-arabinose, especially in the later stages.

The induction of the dehydrogenases of the pathway and the lack of xylose isomerase activity confirm the assumption that the isomerase reaction does not play a role in most fungi (Jeffries, 1983). Considering all the metabolic intermediates involved, one can conclude that all the enzymes necessary for growth on D-xylose are also involved in L-arabinose catabolism. However, the reverse is not true. This has some implications for the kind of mutants one might expect to find, using the filtration enrichment method as we did. A mutant growing on L-arabinose and not growing on D-xylose cannot be obtained. Our method to isolate a mutant not growing on xylitol logically resulted in the isolation of a D-xylulose kinase mutant. The other possibility, a xylitol dehydrogenase mutant, is not likely to be found, since the conversion of xylitol to D-xylulose is also catalyzed by L-arabitol dehydrogenase. This activity is probably sufficient to enable some growth and prevent selection by the filtration enrichment method. The strong correlation between the two catabolic pathways has already been descri-

bed by Barnett (1976), who studied the growth of a large number of yeasts on several substrates.

There are a few other examples in the literature of fungal mutants in D-xylose metabolism. Maleszka et al. (1983) described a xylitol dehydrogenase mutant in *Pachysolen tannophilus*, unable to grow on D-xylose or xylitol. This proves that in this organism no isomerase is involved. McCracken and Gong (1983) isolated a number of mutants in a *Candida* sp. with abnormal product formation from D-xylose. One mutant, showing higher D-xylose conversion, lower xylitol formation and an increased ethanol production under aerobic conditions, was characterized more extensively. Higher levels of xylitol dehydrogenase and D-xylulose kinase were found in this mutant. This is an indication that the xylitol dehydrogenase and D-xylulose kinase levels are rate-limiting in the parental strain.

Since the reduction steps all use NADPH and the oxidation steps NAD^+ as cofactor there is a large need for NADPH, especially during growth on L-arabinose. Calculations by Bruinenberg *et al.* (1983a) of the total need of the yeast cell for NADPH on different substrates illustrate this very well. The induction of both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogaenase by growth on pentoses (Table 2) suggests that the oxidative pentose phosphate pathway supplies this NADPH as it does in yeast (Bruinenberg *et al.* 1983b).

For the final conversion of D-xylulose-5-phosphate there are two possibilities. The first one is formation of fructose-6-phosphate and glyceraldehyde-3-phosphate by the enzymes of the nonoxidative pentose phosphate pathway. The second is phosphoketolase-catalysed conversion to acetylphosphate and glyceraldehyde-3-phosphate. The latter reaction is known to occur in e.g. *Lactobacillus plantarum* (Heath *et al*, 1958) and is claimed to play an important role in some yeasts (Evans and Ratledge 1984). We were not able, however, to detect significant phosphoketolase activity. Therefore the most likely pathway in *A. niger* is the pentose phosphate pathway.

The sequence of four alternating reduction and oxidation steps in L-arabinose catabolism with the thermodynamic equilibrium far in the direction of the polyols is potentially a rate-limiting factor in the catabolic sequence. The fact that the pentitols of the pathway are visible in the ^{13}C NMR spectra of wild-type grown on L-arabinose confirms that a considerable accumulation of, in particular, arabitol is necessary to obtain a forward flux. The cofactor use of the enzymes involved stimulates the flux in the direction of D-xylulose, assuming that the anabolic reduction charge [ARC = $NADPH/(NADP^+ + NADPH)$] is larger than the catabolic reduction charge [CRC = $NADH/(NAD^+ + NADH)$]. It is not possible to determine the real cofactor levels in the cytoplasm because different compartments are present and unknown proportion of the pyridine nucleotides are bound to enzymes. However, total concentrations have been measured in several organisms and these results are in agreement with the above statement (Führer *et al.*, 1980; Voordouw *et al.*, 1983, Gancedo and Gancedo, 1973, Sáez and Lagunas, 1976). The values measured for *A.niger* as determined by Führer *et al.*

(1980) are: ARC = 0.37 and CRC = 0.1. Apart from consequences due to the specific cofactor use as discussed above, the extra high level of the D-xylulose kinase in L-arabinose grown mycelium (Table 1) would stimulate the flux through the pathway as well.

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Chapter 7

Purification and characterization of two xylitol dehydrogenases from *Aspergillus niger*[1]

Cor FB Witteveen, Frans Weber, Ronald Bussink and Jaap Visser.

SUMMARY

An NADPH-dependent L-xylulose reductase (Xylitol:$NADP^+$ 4-oxidoreductase (EC 1.1.1.10) (LXDH)) from *Aspergillus niger* was purified and characterized. It is an octamer with a monomeric M_r of 32 kDa showing high specificity for L-xylulose, xylitol and NADP(H). The K_m values for L-xylulose and xylitol are relatively high (16.5 and 925 mM, respectively). An NAD^+-dependent xylitol dehydrogenase (Xylitol:NAD^+ 2-oxidoreductase (EC 1.1.1.9) (DXDH)) was partially purified from the same fungus. It has a monomeric M_r of 40 kDa and shows a high specificity for D-xylulose, xylitol and NAD(H). The K_m values for D-xylulose and xylitol are relatively low (approximately 4 and 50 mM respectively). The reactivity towards xylitol, the product c.q. substrate these enzymes have in common, confirms their role in the L-arabinose catabolic pathway.

INTRODUCTION

The first steps of L-arabinose catabolism in *A. niger* and *Penicillium chrysogenum* are 4 alternating reduction and oxidation reactions (Fig. 1) (Witteveen *et al.* 1989, Chiang and Knight 1961). The equilibria of these reversible redox reactions are far in the direction of the polyols L-arabitol and xylitol, so two thermodynamically unfavourable steps have to be taken before phosphorylation occurs and D-xylulose-5-phosphate enters the pentose phosphate pathway. To investigate the question what the mechanisms are which still allow an efficient flux to occur in the direction of D-xylulose-5-phosphate seems therefore important. The phosphorylation of D-xylulose causes a net flux in the direction of D-xylulose. Witteveen *et al.* (1989) concluded that the cofactor specificity of the 4 dehydrogenases also results in an increase of the efficiency of the flux towards

[1] Submitted for publication

D-xylulose, since the ARC (Anabolic Reduction Charge = [NADPH] / ([NADP$^+$] + [NADPH])) is higher than the CRC (Catabolic Reduction Charge = [NADH]/([NAD$^+$]+[NADH]) (Führer *et al.* 1980). There is another possibility for the cell to influence the efficiency of the flux towards D-xylulose and that is by differences in the specific kinetic properties of the enzymes involved. The affinity of the enzymes might have an important effect on the efficiency of the flux towards D-xylulose. We studied this possibility by analyzing the two xylitol dehydrogenases of this route: L-xylulose reductase (NADPH-dependent)(LXDH) and xylitol dehydrogenase (NAD$^+$-dependent) (DXDH). They catalyze two nearly identical reactions, just differing in the stereoisomer converted in the reduction step. In the oxidative reaction they have xylitol as a common substrate (Fig. 1). We purified the proteins and analyzed whether their properties were somehow adapted to their opposite physiological function.

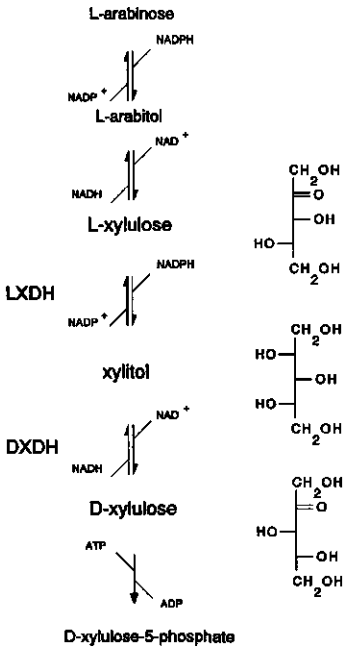

Fig.1 Schematic representation of the first five reactions of the L-arabinose pathway

METHODS

Materials. FPLC equipment, Sephacryl S300 and the Mono Q column were from Pharmacia LKB. Procion Red HE3B agarose (Matrex Red A) was obtained from Amicon. L-arabinose, L-xylulose and D-xylulose were from Sigma. The nicotinamide cofactors were from Boehringer. All other chemicals were from Merck and were of analytical grade.

Strains and culture conditions. For the isolation of the two xylitol dehydrogenases *A. niger* N400 (CBS 120.49) mycelium was grown for 21 to 24 h on minimal medium (Witteveen *et al.* 1989) at 30°C with 50 mM L-arabinose (for the NADP$^+$-dependent enzyme) or with 50 mM D-xylose (for the NAD$^+$-dependent enzyme) as a carbon source. The mycelium was harvested by filtration and stored at -20°C until further use. Mycelium for the determination of intracellular xylitol and arabitol concentrations was grown as follows. N400 spores (5 x 10^6 ml^{-1}) were precultured in an Erlenmeyer flask

for 8 h in the same medium as described above with 50 mM L-arabinose as carbon source and subsequently transferred to a fermenter containing the same medium. Incubation was continued for another 10 h. In this period the culture was well-aerated (dissolved oxygen > 30% air saturation) and the pH was kept at 4.5.

Preparation of cell-free extract. Extracts of mycelium for the purification of the enzymes were prepared either using a Waring Blender (Schuurink *et al.* 1990) or a Braun dismembrator (Witteveen *et al.* 1989). A 20 mM potassium phosphate buffer pH 6.5 containing 5 mM $MgCl_2$ and 1 mM ß-mercaptoethanol was used as extraction buffer.

Determination of xylitol and arabitol concentration in mycelium. N400 mycelium grown on L-arabinose for 18 h as described above was harvested by filtration and frozen in liquid nitrogen. The mycelium was not washed since this resulted in considerable losses of the intracellular polyols (up to 60%). The frozen mycelium was lyophilized. The weight of the lyophilized mycelium was taken as the dry weight. It was extracted by adding 10% perchloric acid (approximately 2 ml per 50-100 mg mycelium) and subsequent shaking for 30 min at 0 - 4°C. Then 2 ml water was added and the mycelium was spinned down (15000 x g, 15 min). The supernatant was neutralized using a concentrated $KHCO_3$ solution. The $KClO_4$ precipitate was removed by centrifugation and the polyol content of the supernatant was determined on a Dionex system with a CarboPac MA1 column using isocratic elution with 0.48 M NaOH.

Protein determinations. Protein concentrations were estimated, after denaturation and precipitation of protein with sodium deoxycholate and trichloroacetic acid (Bensadoun and Weinstein, 1976), by the microbiuret method (Itzhaki and Gill, 1964) using bovine serum albumin as standard.

Electrophoresis and isoelectric focusing. Electrophoresis in 7.5% or 10% (w/v) polyacrylamide gels containing 0.1% SDS were done according to Laemmli (1970). The molecular weight of the denatured proteins on these gels was determined by comparison with the Serva marker kit protein standards: carbonic anhydrase (29 kDa), ovalbumine (45 kDa), BSA (67 kDa), phosphorylase B (92.5 kDa). Analytical thin layer isoelectric focusing was done in the pH range 3-7 on a FBE-3000 apparatus (Pharmacia) using the focusing and staining conditions stipulated by the manufacturer.

Native molecular weight determination by gel filtration. To determine the native molecular weight of L-xylulose reductase we applied a mixture of proteins with known molecular weight to a 150 x 2.6 cm Sephacryl S300 column. The column was eluted with MonoQ buffer (see below) with addition of 25 mM NaCl. From the retention volumes a calibration curve was made. The marker proteins were: horse ferritin (M_r = 450 kDa), bovine catalase (M_r = 240 kDa), rabbit aldolase (M_r = 160 kDa), BSA (M_r = 67 kDa), chicken egg albumin (M_r = 45 kDa) and horse myoglobin (M_r = 17.8 kDa).

Purification of L-xylulose reductase. 150 ml extract from 50 g mycelium (wet weight) was applied to a 25 ml Matrex RedA column which retained the activity. After washing the column with approximately 100 ml of the extraction buffer the enzyme was eluted using a 100 ml linear gradient of 0 to 1.5 mM $NADP^+$ in extraction buffer.

The fractions containing activity were pooled and applied to a MonoQ column. The MonoQ column was eluted with a gradient of 0 to 0.5 M NaCl in a 20 mM bis-Tris/HCl pH 6.5 containing 5 mM $MgCl_2$ and 1 mM ß-mercaptoethanol (MonoQ buffer). Active fractions from the MonoQ column were diluted 4-fold with MonoQ buffer and reloaded on the MonoQ column. The same salt gradient was used for elution. The active fractions were loaded to a Sephacryl S300 column (150 cm in length, 2.6 cm diameter) which was equilibrated in MonoQ buffer with 25 mM NaCl. For elution the same buffer was used.

Purification of NAD^+-dependent xylitol dehydrogenase. The first step in the purification of this enzyme was an ammoniumsulphate precipitation applied to 100 ml extract obtained from 40 g mycelium. The enzyme precipitated between 40% - 60% ammoniumsulphate saturation. The pellet was collected by centrifugation (10000 x g, 10 min) dissolved in 25 ml 50 mM Tris/HCl pH 7.5 and subsequently this fraction was applied to a DEAE-A50 column. The enzyme did not bind to the column under these conditions. Fractions with activity were collected and then applied to an 8 ml Phenyl-Sepharose CL-4B column. This column was washed with 20 ml 50 mM Tris/HCl buffer pH 7.5 and with 20 ml 20% (v/v) ethyleneglycol in Tris/HCl buffer. The enzyme was finally eluted using 40% ethyleneglycol in 50 mM Tris/HCl pH 7.5. The fractions containing activity were applied to a DEAE-Sepharose A50 column equilibrated in the same Tris buffer with 20% ethyleneglycol, which was washed and subsequently eluted with 0.1 M ammoniumsulphate in the same buffer. This preparation was used for further characterization of the enzyme and for a kinetic analysis.

Kinetic measurements. All kinetic measurements were performed in 100 mM sodium phosphate buffer pH 7.0 at 30°C. The initial velocities were measured spectrophotometrically by measuring the rate of change in the absorbance difference between 340 and 380 nm, using an Aminco DW-2 UV/VIS spectrophotometer ($\epsilon_{340\text{-}380} = 5.33$ mM^{-1} cm^{-1}). The kinetics of the enzymes were studied by varying one substrate and keeping the other substrate constant. The data were fitted to the Michaelis-Menten equation using the program Enzfit (Elsevier Biosoft). The activity measurements in the presence of product were performed by keeping one substrate constant and varying the other. This was done at a few different constant concentrations of the product. The inhibition parameters K_{is} (slope effect) and K_{ii} (intercept effect) were determined from replots of the slopes and intercepts respectively of double reciprocal plots against the inhibitor concentrations.

The pH dependency of the enzyme activities was measured in McIlvaine buffer (pH 5-8) (Dawson *et al.* 1986) and in 100 mM glycine NaOH (pH 8.5-11).

RESULTS

Purification and characterization of LXDH.

The results of the purification of LXDH on the Matrex RedA column were not

Table 1. Substrate specificity of the NAD^+-and $NADP^+$-dependent xylitol dehydrogenases of *A. niger*.

substrate	relative activity[a]		substrate	relative activity[a]	
	LXDH	DXDH		LXDH	DXDH
D-Xylulose	4.1	100	Xylitol	100	100
L-Xylulose	100	0	L-Arabitol	0	0
L-Arabinose	0	0	D-Arabitol	0	0
D-Xylose	0	0	Ribitol	0	9
D-Fructose	0	0.8	D-Sorbitol	16	20
D-Erythrose	2.8	ND[b]	Erythritol	0	3
Dihydroxyacetone	2.1	6	Glycerol	0	0

[a] LXDH was assayed with $NADP^+$ (polyols) or NADPH (sugars) as cofactor wheras for DXDH NAD^+ (polyols) or NADH (sugars) was used. The reduced cofactor was used in a concentration of 0.2 mM and the oxidized cofactor in a concentration of 0.5 mM. The polyols and sugars were added in a concentration of 50 mM except for L-xylulose which was added in a concentration of 2.5 mM. The sugar reduction reactions were measured in 100 mM potassium phosphate buffer pH 6.5, whereas the polyol oxidation reactions were measured in 200 mM glycine-NaOH buffer pH 9.6.
[b] ND = not detected.

fully reproducible. The efficiency of elution of the enzyme using an $NADP^+$ gradient was influenced by factors which were not completely controled. However, since this step resulted in a very good purification (approximately 20-fold) we have maintained it. The subsequent steps on the FPLC could be done with high efficiency (90%). The overall recovery of the enzyme after the Sephacryl S-300 purification step was 50-60% in the best cases, but usually lower (20-40%). The protein eluted from the Sephacryl S-300 column as a separate and symmetrical peak which upon SDS-PAGE showed a single band with a M_r of 32 kDa. From the Sephacryl S-300 elution volume of the enzyme and from those of the calibration proteins we determined a native M_r of 250 kDa. This indicates that the enzyme is composed of 8 identical subunits. The isoelectric point is 6.3. The enzyme is stable in a 20 mM bis-Tris/HCl pH 7.0 buffer at 4°C for at least several weeks. The pH optima of the L-xylulose reduction and xylitol oxidation reactions are given in Fig. 2. The pH optimum for the reductase reaction is around 6.5 and rather broad whereas the xylitol oxidation proceeds most

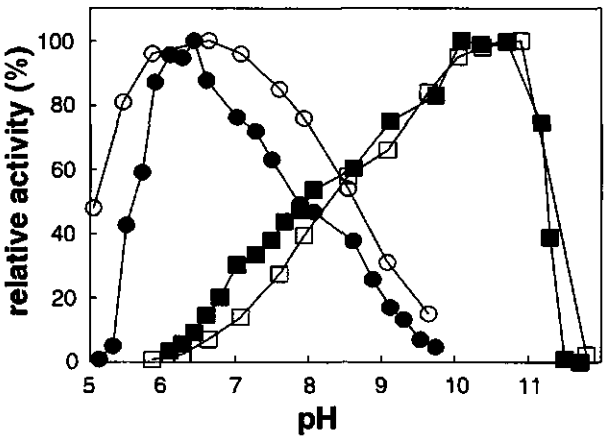

Fig. 2 pH dependencies of LXDH (open) and DXDH (filled) reductive (circles) and oxidative (squares) reactions.

efficiently at high pH values above 10. The enzyme is highly specific for L-xylulose as the sugar substrate and in the oxidative direction for xylitol although some activity was measured using other substrates (Table 1). No activity was determined when NAD(H) was used as cofactor instead of NADP(H).

Partial purification and characterization of the DXDH

A complication in the purification of this protein was the instability of the enzyme as experienced in most column chromatographic steps, especially later in the purification where the protein concentration is lower. We have therefore tried to select a dye affinity column for the purification of the protein by testing 96 different dye affinity materials as described by Hondmann and Visser (1990) but only very low recoveries were found, due to loss of activity. The procedure finally used is described in the methods section and resulted in a preparation which consisted for approximately 80-90% of the NAD^+-dependent xylitol dehydrogenase. The presence of ethyleneglycol in the final DEAE-Sepharose purification step was essential to prevent inactivation. Recovery from the ammonium sulphate step was about 70% and from the column chromatography steps 90% or more. The M_r determined by SDS-PAGE was approximately 40 kDa. The final preparation was stable both at 4°C and -20°C in a 50 mM Tris/HCl pH 7.5 buffer containing 20% ethyleneglycol. The enzyme was not stable on a gel filtration column, not even in the presence of ethyleneglycol. The enzyme is highly specific for D-xylulose and xylitol, but shows activity with some other substrates as well (Table 1). The pH optimum for the reduction of D-xylulose is 6.5 and for the oxidation of xylitol 10 (Fig. 2).

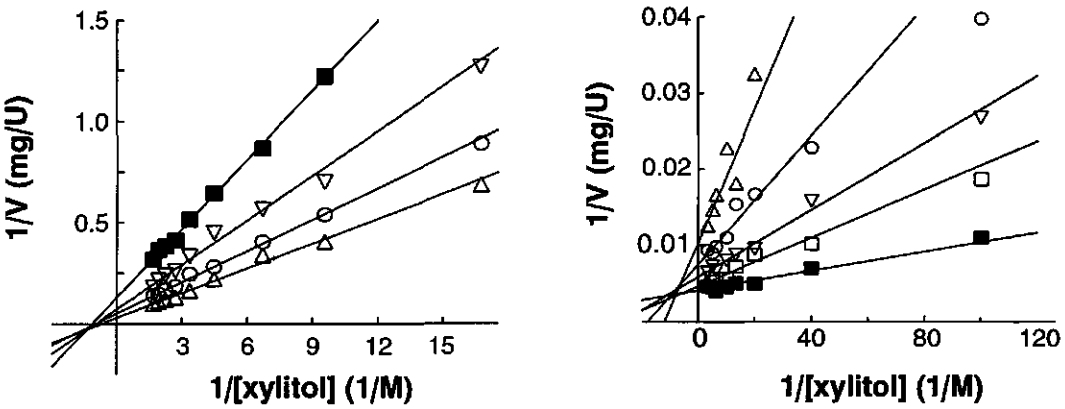

Fig 3. Double reciprocal plot of velocity of LXDH reaction versus xylitol concentration at different fixed $NADP^+$ concentrations: ■, 0.04 mM; ▽, 0.1 mM; ○, 0.2 mM; △, 0.5 mM.
Fig 4. Double reciprocal plot of velocity of DXDH reaction versus xylitol concentration at different fixed NAD^+ concentrations: △, 0.025 mM; ○, 0.05 mM; ▽, 0.1 mM; □, 0.2 mM; ■, 0.3 mM.

Kinetics of LXDH

The initial rate measurements resulted in double reciprocal plots with a series of intersecting lines. The points of intersection of all four double reciprocal plots (forward and reverse reactions, both substrates) were all situated on or just below the horizontal axis. Fig. 3 shows the plot with varying xylitol concentrations at different constant $NADP^+$ concentrations. The intercepts and slopes were replotted against the reciprocal of the fixed substrate concentration resulting in linear relationships. This result is consistent with a sequential mechanism. The lines of the different primary double reciprocal plots intersect very close to the X-axis meaning that the apparent K_m for one substrate is not strongly influenced by the concentration of the other substrate. In Table 2 the apparent K_m's are given. The V_{max} values were calculated from the secondary plot of the intercepts against the reciprocal of the constant substrate.

We also performed a series of measurements to study the product inhibition. In case the lines intersected on the reciprocal velocity axis it was concluded that the inhition was competitive (no change in V_{max}). In all other cases the lines intersected somewhere left of this axis in which case the inhibition was non-competitive. NADPH showed competitive inhibition relative to $NADP^+$ and vice versa (Table 3). The inhibition by xylitol was very low or not detectable at concentrations below 1 M. Furthermore, the accuracy of the data of inhibition by

Table 2. Apparent K_m and V_{max} values of the NAD^+-specific (DXDH) and $NADP^+$-specific xylitol dehydrogenases of *A. niger*.

DXDH			LXDH	
K_m (NADH)		0.03 mM	K_m (NADPH)	0.03 mM
K_m (D-xylulose)		4 mM	K_m (L-xylulose)	17 mM
V_{max}		1500 U/mg	V_{max}	200 U/mg
K_m (NAD^+)	([xylitol] = 200 mM)	0.1 mM	K_m $NADP^+$	0.13 mM
	([xylitol] = 25 mM)	0.3 mM		
K_m (xylitol)	([NAD^+] = 0.8 mM)	30 mM	K_m (xylitol)	925 mM
	([NAD^+] = 0.1 mM)	70 mM		
V_{max}		270 U/mg	V_{max}	33 U/mg

L-xylulose was too low to determine whether the inhibition was competitive or not.

Kinetics of DXDH.

It was difficult to obtain accurate kinetic data of the NAD^+-dependent enzyme. One of the problems was that the activity was strongly dependent on the order by which the substrates were added to the enzyme, the reaction being inhibited when xylitol or D-xylulose were added to the enzyme before the cofactor. Most activity measurements were performed by starting the reaction by adding the enzyme to the mixture. The low accuracy of the data made interpretation difficult. Double reciprocal plots of the forward and reverse reactions with different substrates gave lines which intersected more or less at one point. All the points of intersection were above the horizontal axis. In the oxidation of xylitol the apparent K_m was dependent on the concentration of NAD^+ and vice versa (see Fig. 4), whereas in the reduction of D-xylulose the apparent K_m values were only to a small extent dependent on the second substrate. In Table 2 the values for the apparent K_m's and V_{max} values are given.

The product inhibition studies showed a competitive inhibition pattern of NADH relative to NAD^+ and vice versa (Table 3). Competitive inhibition was as well observed by xylitol when D-xylulose was varied. In all other cases non-competitive inhibition was observed except in the case of inhibition by NAD^+ when D-xylulose was varied where uncompetitive inhibition was observed. However the

Table 3. Kinetic parameters of product inhibition in the reactions of the NAD^+- (DXDH) and $NADP^+$-dependent (LXDH) xylitol dehydrogenases.

Variable substrate	inhibitor	constant substrate	inhibition pattern[a]	K_{is}[b]	K_{ii}[b]
LXDH					
$NADP^+$	NADPH	xylitol (600 mM)	C	0.02	
$NADP^+$	L-xylulose	xylitol (600 mM)	?[d]	3.7	
xylitol	NADPH	$NADP^+$ (0.5 mM)	NC	0.3	0.1
xylitol	L-xylulose	$NADP^+$ (1 mM)	?[d]	6.6	
NADPH	$NADP^+$	L-xylulose (2.5 mM)	C	0.08	
L-xylulose	$NADP^+$	NADPH (0.02 mM)	NC	0.16	0.37
DXDH					
NAD^+	NADH	xylitol (160 mM)	C	0.01	
NAD^+	D-xylulose	xylitol (160 mM)	NC	0.4	2.5
xylitol	NADH	NAD^+ (0.25 mM)	NC	0.006	0.06
xylitol	D-xylulose	NAD^+ (0.25 mM)	?[d]	0.7	
NADH	NAD^+	D-xylulose (2.5 mM)	C	0.8	
NADH	xylitol	D-xylulose (2.5 mM)	NC	340	1170
D-xylulose	NAD^+	NADH (0.025 mM)	UC[c]	0.064	
D-xylulose	xylitol	NADH (0.05 mM)	C	310	

[a] C = competitive, NC = noncompetitive, UC = uncompetitive. [b] Expressed in mM. The meaning of K_{is} and K_{ii} is explained in the methods section. [c] Parallel lines were observed but the accuracy of the data was not high enough to exclude interception of the lines outside the plot. [d] The accuracy of the data was too low for conclusions about the type of inhibition.

accuracy of the data was not high enough to exclude the possibility that the lines do intercepte far from the origin.

Polyol concentrations in L-arabinose grown mycelium.

The arabitol and xylitol concentrations in mycelium grown on L-arabinose were 20-30 and 200 μmol (g dw)$^{-1}$ respectively. For the calculation of the polyol concentration in mycelium from these values a conversion factor is needed. Not much data are available with regard to this. Lester *et al.*(1962) calculated a

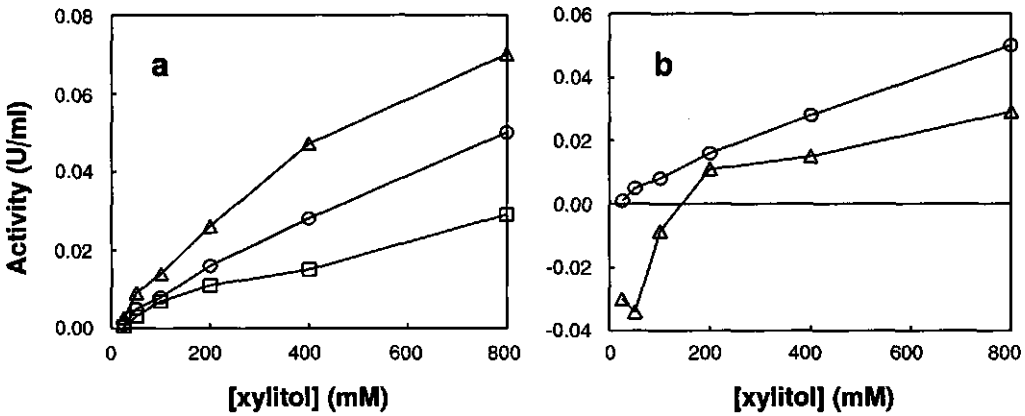

Fig 5. Dependency of the rate of xylitol oxidation catalyzed by LXDH on the ARC (**a**) (ARC = 0 (△), 0.1 (○), 0.2 (□)) and at a ARC of 0.1 in the presence of 0 (○) or 1 (△) mM L-xylulose (**b**).

factor of 2.45 and Slayman and Tatum (1964) a factor of 2.54 for the calculation of the intracellular concentration of a metabolite from the amount given in mole per gram dry weight. Using these conversion factors it can be calculated that if grown on L-arabinose the xylitol concentration in mycelium is in the range of 8-15 mM. Fig. 5 and 6 show the activities of the two enzymes dependent on the xylitol concentration in the presence and absence of either L-xylulose or D-xylulose and with different anabolic and catabolic reduction charges, assuming a total NAD(H) or NADP(H) pool concentration of 1 mM which is in the range to be expected in the cell (Führer *et al.* 1980). In the range of 10 to 15 mM xylitol hardly or no activity can be measured for the $NADP^+$-dependent enzyme whereas the the NAD^+-dependent enzyme shows activity up to 15-30% of its V_{max} (calculated from the data in Fig. 6a).

From Fig 5b and 6b equilibrium constants at pH 7.0 at 30°C can be calculated using the following equation:

$$Keq = \frac{[NAD(P)H][D(L)\text{-}xylulose][H^+]}{[NAD(P)^+][xylitol]}$$

This gives for DXDH 4 x 10^{-11}M and for LXDH 6 x 10^{-11}M. These data are comparable with previously published values (Rizzi *et al.* 1989b and Ditzelmüller *et al.* 1984)

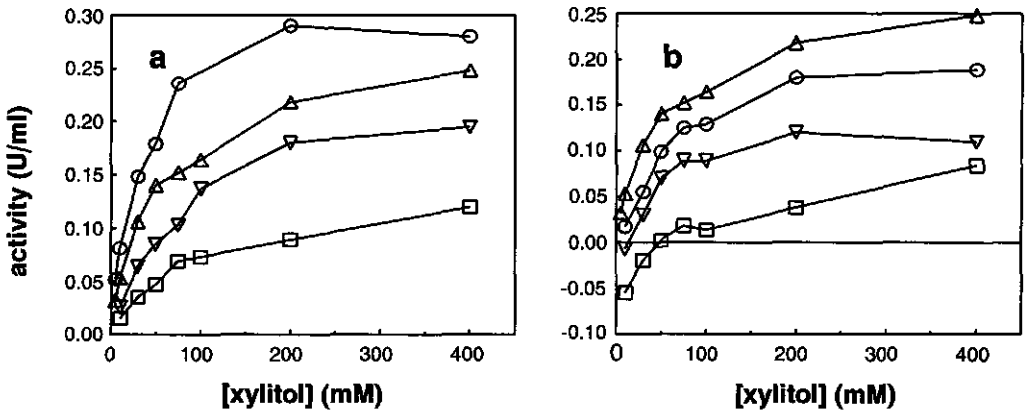

Fig 6. Depencency of the rate of xylitol oxidation catalyzed by DXDH on the CRC (**a**) (CRC= 0 (○), 0.02 (△), 0.05 (▽), 0.1 (□)) and at a CRC of 0.02 in the presence of different D-xylulose concentrations (**b**) (D-xylulose = 0 (△), 0.1 (○), 0.3 (▽), 1 mM (□)).

DISCUSSION

Only a few descriptions of L-xylulose reductase have been published. Hollmann and Touster (1957) for instance have described the presence of an NADPH-dependent L-xylulose reductase in guinea pig liver. To our knowledge no purifications of such an enzyme has been described. In contrast to this several NAD^+-dependent xylitol dehydrogenases have been isolated from different organisms, for example *Pachysolen tannophilus* (Ditzelmüller *et al.*, 1984) and *Pichia stipitis* (Rizzi *et al.*, 1989).

The two enzymes have comparable pH optima. However, LXDH shows a broader pH optimum for the reductive reaction and has relatively high activity at physiological pH (around 7). The oxidative reaction is catalyzed more efficiently by DXDH at physiological pH. So when the reactions around this pH are considered each of the two enzymes seems to catalyze the reaction which is *in vivo* its most important one more efficiently than the other enzyme.

The position of the intersection points of the double reciprocal plots of LXDH on or just below the horizontal axis for both forward and reverse reactions indicate that a random mechanism is operative (Fromm, 1975). The results of the product inhibition experiments are in line with this. The position of the intersection points of DXDH above the horizontal axis does not allow to discriminate between an ordered or random mechanism (Fromm, 1975). The product inhibition studies, showing competitive inhibition of NAD^+ towards NADH and vice versa and competitive inhibition of xylitol towards D-xylulose seems to indicate that for

DXDH also a random order mechanism is operative. However, as stated in the results, the accuracy of the data is low so further analysis is necessary to elucidate the details of the mechanism.

With respect to the product inhibition data it should be kept in mind that the K_{ii} and K_{is} values are dependent on the concentration of the substrate that is kept constant. This makes it difficult to compare the inhibition constants between the two enzymes. Nevertheless it can be concluded that the inhibition by xylulose and xylitol is much stronger in DXDH than in LXDH. This is in line with the higher affinity of this enzyme for these substrates.

The most striking difference between the two enzymes is the much higher affinity of DXDH for xylitol. Furthermore DXDH has a much higher V_{max} for the reductive reaction. In order to compare the reactivity of the enzymes toward xylitol for the reactions under more physiological circumstances, we made some measurements with substrates and products in the physiological range. Figs. 5a and 6a show the activity of the enzymes when the xylitol concentration is varied under conditions of different anabolic and catabolic reduction charge. For the total cofactor concentration we have chosen 1 mM which is in the physiological range (Führer *et al.* 1980). A realistic estimate of the anabolic and catabolic reduction charge is more difficult. Führer *et al.* (1980) reported relatively high values for both the ARC (0.38) and the CRC (0.15-0.18). In our laboratory lower values for the CRC have been determined ($\leq$0.1) and more variation in the ARC (0.2-0.5) (unpublished results). The concentration of xylitol in L-arabinose growing hyphae is in the range of 8-15 mM. Calculated for 15 mM substrate the activity would be 32% (CRC = 0) to 14% (CRC = 0.1) of the V_{max} of DXDH under these conditions. For LXDH these values are much lower: $\leq$1.5% of the V_{max}. These values are in the absence of D- or L-xylulose. When the activity of DXDH is measured in the presence of D-xylulose at a value of 0.02 for the CRC (Fig. 6b) one measures only a net xylitol oxidation (xylitol = 15 mM) when the D-xylulose concentration is in the range of 0.1 mM or lower. From this it can be concluded that both the D-xylulose and the CRC must be very low, in the range of 0.1 mM and 0.02 respectively or lower. This means that the CRC has to be much lower than the 0.15 reported by Führer *et al.* (1980) otherwise higher xylitol levels would accumulate during growth. Since the reaction by which xylitol is oxidized to L-xylulose is catalyzed only with a very low efficiency by LXDH at relatively low ARC values and in the absence of L-xylulose, the reaction will occur even less efficient in the presence of L-xylulose and at higher ARC values.

In Figs. 5b and 6b it can be seen that the presence of 1 mM xylulose has a much stronger effect on DXDH than on LXDH even though the CRC is much lower than the ARC. This is because of the higher affinity of DXDH for D-xylulose compared to the affinity of LXDH for L-xylulose and because of the stronger

product inhibition by D-xylulose. This means that *in vivo* the D-xylulose concentration must be low, probably far below 1 mM in order to reach an efficient flux towards D-xylulose. This implicates as well that the D-xylulose kinase must have a high affinity for D-xylulose in order to achieve such a low D-xylulose concentration.

Acknowledgements

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Chapter 8

Polyol pools in *Aspergillus niger*.

Cor FB Witteveen, Cor Dijkema and Jaap Visser.

SUMMARY

The accumulation of polyols and their excretion was investigated under various growth conditions and at different stages of the *Aspergillus niger* life cycle. Glycerol was found to be the major solute in osmotic adjustment of the hyphae. Conidiospores contain large amounts of mannitol which are metabolized quickly and then lead to accumulation of glycerol. Glycerol is the major compound in young mycelium whereas in older mycelium mannitol and erythritol are the major polyols. In all experiments we found that the polyols were excreted as well. The mechanism and function of this process is unknown, but it might be a way of controling the levels of the intracellular polyol pools. The polyols are rapidly taken up again upon starvation. In a glycerol kinase mutant the synthesis of glycerol is unaffected but the excretion level of the polyol is higher. This glycerol is taken up again upon starvation where it accumulates intracellularly as it can not be metabolized further. Long fermentations (more than 3 days) under low oxygen conditions lead to an increased excretion of the polyols as shown for *A. niger* N400. In the later stage approximately 45% of the glucose consumed by the fungus is converted into extracellular polyols. The function and mechanism of regulation of the polyol pools are discussed.

INTRODUCTION

Amongst the polyols that accumulate in fungi, mannitol and glycerol are most frequently found but also the presence of arabitol, erythritol and occasionally ribitol have been reported. (Jeffries 1984, Blumenthal 1976). Of the possible functions that have been proposed for these polyols we mention: 1. A contribution to the osmotic balance. 2. A role as carbon storage compounds. 3. A role in balancing the anabolic and catabolic reduction charge. 4. Transport of metabolites through the hyphae.

The polyols are present in very high concentrations (up to hundreds of mM), so they make a significant contribution to the osmotic potential of the cell. A relevant question seems whether they play a role in the adaption of the cell to changes in the water activity of the environment. In many fungi glycerol has a role in regulating the osmotic potential of the cell (Brown 1978, Blomberg and

Adler 1992).

Mannitol was reported to be present in conidia of *Aspergillus oryzae* where it was metabolized in a very early stage of germination (Horikoshi *et al.* 1965). This, together with the observation that polyols are metabolized under starvation conditions (Dijkema *et al.* 1985) justifies the conclusion that they can be considered as storage compounds.

It is difficult to say whether the polyols play a role in the regulation of either the catabolic or the anabolic cofactor reduction charge. A mannitol cycle has been postulated which has a function in the formation of NADPH at the cost of an NADH and an ATP (Hult and Gatenbeck 1978). Singh *et al.* (1988) concluded that the mannitol cycle does not play a role in NADPH generation in *A. nidulans* because there was no correlation between enzyme activities of the enzymes involved and the need of the cell for NADPH. No convincing evidence for an active mannitol cycle has been published. However, it is very well possible that under certain conditions the synthesis of polyols contributes to the regeneration of oxidized cofactors.

A transport function of polyols through the hyphae is very well possible but no evidence is available for that.

In this study we have investigated the composition of the polyol pools in *A. niger* under diverse physiological conditions to address the question which polyols are specifically involved in the various functions mentioned. We have given special attention to the changes of the polyol composition during the various phases of fungal growth since changes could be related to developmental processes. Little attention has been given to this aspect in previous reports where usually measurements have been made at a single time point. The availability of a glycerol kinase mutant (Witteveen *et al.*, 1990b) allowed us to study how glycerol accumulation is affected in a strain which is not able to catabolize the compound. Extracellular polyol accumulation upon fungal growth is known to occur but has not been investigated in detail. *Aspergillus candidus* is capable of excreting large amounts of mannitol into the medium (Smiley *et al.* 1967). For *A. niger* it has been reported that small amounts of polyols are excreted into the medium during early phases of the citric acid production process, whereas in later phases they are metabolized again (Röhr *et al.* 1987). Glycerol formation has been suggested to play an important role in the initiation of citric acid accumulation (Legiša and Mattey, 1986). In addition to the investigation of the intracellular polyol pools we have studied the potential of *A. niger* to accumulate large amounts of polyols in the medium.

METHODS

Strains. *A. niger* N400 (CBS 120.49) was used as wild type strain. NW101 (*gox*C17, *csp*A1, *pab*A1) is a glucose oxidase negative mutant derived from N400

(Witteveen *et al.* 1990a). NW201 (*glc*A1, *csp*A1, *nic*A1) is a glycerol kinase negative mutant (Witteveen *et al.* 1990b) and is also derived from N400.

Culturing conditions. Conidiospores were grown on complete medium with 2% sucrose as described by Pontecorvo *et al.* (1953). Minimal medium, as described by Witteveen *et al.* (1989) was used in all experiments except in the experiment where ammonium was used instead of nitrate. There $NaNO_3$ was replaced by an equimolar amount of NH_4Cl (70 mM). To all cultures 0.05 % (w/v) yeast extract and 0.04 ml per l medium of the trace metal solution described by Vishniac and Santer (1957) was added. 2% (w/v) D-glucose was used as a carbon source unless stated otherwise. The effect of the osmotic value of the medium on the polyol pool composition was studied in mycelium grown in minimal medium to which 0, 0.2, 0.5 or 1.0 M NaCl was added.

Mycelium was grown in fermenters or in Erlenmeyer flasks. In case of growth in fermenters the conidiospores were preincubated in an Erlenmeyer flask for 6-8 h in the same medium and subsequently added to a 3 l Applikon fermenter (Schiedam, The Netherlands) with pH and oxygen control. Oxygen and pH were not controled during the pregrowth period. Unless stated otherwise the pH was kept at 4.0 in all experiments. In case of low oxygen conditions the oxygen concentration was kept below 8% air saturation (100% = air saturated solution) whereas in case of high oxygen conditions this was above 30%. The growth temperature was 30°C in all cases. Erlenmeyer flask cultures were incubated in a rotary shaker (200 rpm).

Extraction of polyols from the mycelium. Mycelium samples were harvested by filtration except for the experiment in which conidiospores were examined. These were harvested by centrifugation. No washing was done since this resulted in considerable losses of the intracellular polyols (up to 60%). The mycelium was frozen in liquid nitrogen and lyophilized. The weight of the lyophilized mycelium was taken as the dry weight. The lyophilized mycelium was extracted by adding 10% perchloric acid (2 ml per 50-100 mg dry mycelium) and shaking it for 30 min at 0 - 4°C. 2 ml water was added and the mycelium was spinned down (15000 x g, 15 min). The supernatant was neutralized using a concentrated $KHCO_3$ solution. The $KClO_4$ precipitate was spinned down and the polyol content of the supernatant was determined on an HPLC (Dionex) with a CarboPac MA1 column using isocratic elution with 0.48 M NaOH.

^{13}C glucose labeling. To analyze the formation of newly synthesized polyols in response to a shift in the dissolved oxygen concentration, randomly labeled ^{13}C glucose was used. Mycelium was grown under low oxygen conditions. After 22 h part of the mycelium was transferred to a small fermenter (0.5 l) with oxygen and pH control. Approximately 2 mM glucose was supplied and the mycelium was allowed to adapt to the new conditions for two hours. Just before the glucose was exhausted randomly labeled ^{13}C glucose (20% enriched) was added to a final concentration of 2.4 mM and samples were taken every 15 minutes. The samples were quickly filtered on a glass filter, washed with a small amount of glucose free medium and frozen in liquid nitrogen. After 45 min the oxygen supply was increased so that the dissolved oxygen concentration rose above 50% of air saturation and sampling was continued.

After perchloric acid extraction ^{13}C NMR spectra of the extracts were recorded at 75.47 MHz on a 300 MHz Bruker-CXP spectrometer equipped with a 10 mm probe head. 3 ml of the extract contained 10% D_2O (v/v) to provide a lock signal. For each extract 7200 free induction decays (FID's) were accumulated, using 16 k data points; a

Table 1. Amounts of polyols accumulated in mycelium of *A. niger* grown in the presence of different NaCl concentrations[a]. The polyol amounts are given in μmole (g dw)$^{-1}$.

	Polyol levels[b]				
	0 M[c] 16 h[d]	0.2 M[c] 16 h[d]	0.5 M[c] 16 h[d]	1.0 M[c] 16 h[d]	1.0 M[c] 28 h[d]
glycerol	461	856	1505	2095	2017
erythritol	304	223	200	88	408
arabitol	10	8	7	4	14
trehalose	36	29	21	13	4
mannitol	179	157	122	45	209
dry weight (g/l)	2.1	1.2	1.2	0.9	2.85

[a] The carbon source was 2% glucose in all cases. The cultures were harvested after 16 or 28 h incubation. [b] polyol levels are in μmole (g dry weight)$^{-1}$. [c] NaCl concentration in culture medium. [d] moment of harvesting of the mycelium.

45° pulse angle was applied and a spectral window of 20.000 Hz was used. The interpulse time was 1 s. Extract spectra were obtained after Fourier transformation of 7200 FID's using zero-filling to 32 k and applying Lorenzian line broadening of 3 Hz. The resonance intensities of the polyols were determined by peak surface measurement, and corrected for nuclear Overhauser (NOE) and relaxation effects by comparing the spectra with spectra of polyol mixtures with known concentrations and also with spectra of the enriched glucose. From these data amounts of newly formed polyols were calculated by assuming that they all originated from the enriched glucose.

RESULTS

Influence of osmotic value of the medium on the polyol pools

Aspergilli grow very well on media with high sugar or salt concentrations as is for example illustrated by organic acid fermentations wich are performed with high sugar concentrations or in the studies of Beever and Laracy (1989) using high salt. In Table 1 the influence of increasing salt (NaCl) concentrations on the pool size of the polyols in *A. niger* is given. The mycelium was grown in Erlenmeyer flasks and was harvested after 16 h or 28 h. These data show very clearly that glycerol is the polyol that plays a major role as an osmoticum under these conditions. In the 16 h samples the levels of mannitol, erythritol and trehalose

decreased with increasing salt concentration. The amounts of the polyols were higher again in the 28 h sample of mycelium grown on 1 M NaCl. The arabitol levels were low in all cases. Comparable studies with increasing (up to 1 M) glucose concentrations were also performed. These gave essentially the same results (data not shown).

Variation in polyol pool composition during fungal growth

Variation in polyol pool composition during growth was studied in two independent experiments, each of which covered a different growth phase of *A. niger*. In the first experiment the polyol pools were studied as they develop during germination (Fig. 1) and in the second one the development of the pools during active growth and upon glucose exhaustion was followed (Fig. 2). Fig 1 shows that conidiospores contain mainly mannitol which concentration exceeds the amount of trehalose and glycerol present approx. 5-6 fold. The glycerol content varied, presumably because its formation already started during harvesting of the spores. During the first hours of incubation the mannitol and trehalose pools were metabolized. The glycerol pool was build up very quickly in the first hour but leveled off to a lower, stationary level. After about 8 h of incubation the first hyphae were formed. Only minor changes in the polyol pools were then observed.

Fig. 2a shows how the intracellular polyol composition changes with time after 22 h. In the period between 13 and 22 h glycerol is the main polyol and only minor amounts of erythritol and mannitol are observed. The mycelium was grown in a fermenter under well aerated conditions at pH 4.0 with 2% glucose as carbon source. The data are from one experiment. However duplicate experiments gave essentially the same result. It is clear that in mycelium of approximately 22 h, which is still fast growing, glycerol is the major polyol. In a later phase this pool size decreased with a concomitant increase of the mannitol and

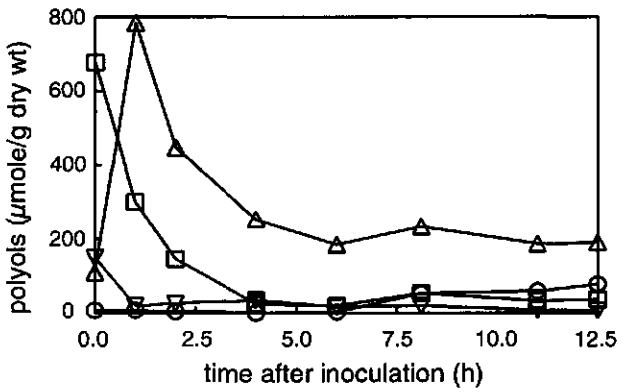

Fig. 1. Intracellular polyol amounts in germinating spores of *A. niger* N400 as a function of time after inoculation. △ = glycerol, □ = mannitol, ▽ = trehalose, ○ = erythritol.

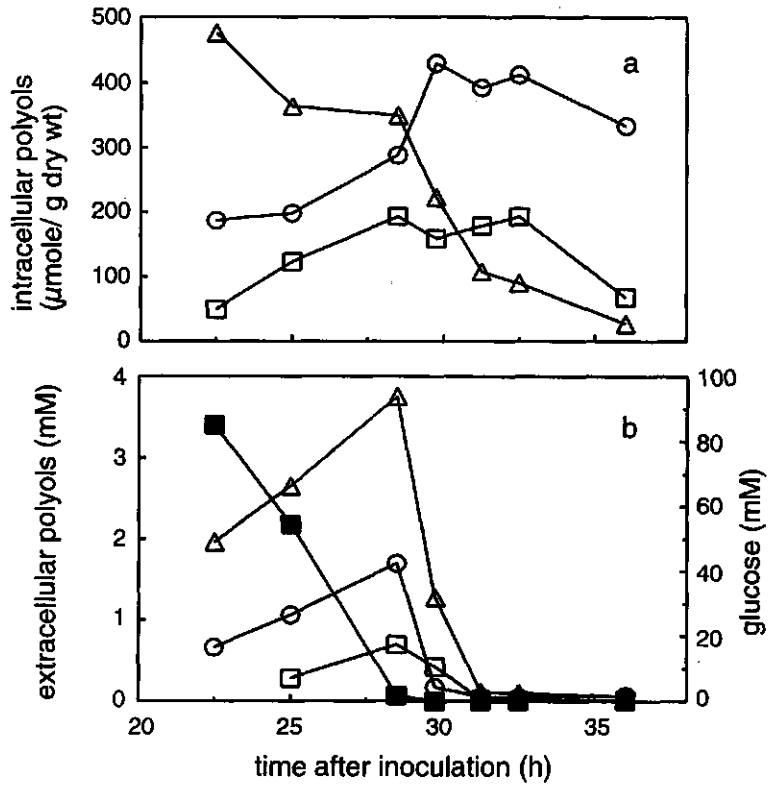

Fig. 2. Intra- (a) and extracellular (b) polyols in a N400 culture grown at pH 4.0 with 2% glucose as carbon source and high oxygenation. △ = glycerol, □ = mannitol, ○ = erythritol, ■ = glucose.

the erythritol pools. In the medium (Fig. 2b) an increasing amount of all three polyols was observed. After exhaustion of the glucose all three polyols were rapidly taken up again by the mycelium. Upon starvation the polyol pools were metabolized. The intracellular glycerol pool decreased very rapidly once glucose was exhausted. The mannitol pool was degraded more slowly whereas the erythritol pool initially even increased, apparently because uptake from the medium was faster than metabolization. This compound was degraded only very slowly.

Effect of oxygenation, nitrogen source and pH.

In Table 2 the polyol pool compositions in mycelia grown under different conditions are given 24 h after inoculation. Low oxygen levels resulted in approximately 50% reduction of the erythritol concentration, and in a 3-fold increase of the mannitol and trehalose concentrations in comparison to the high oxygen level culture. Arabitol which is low anyhow also increased 2-fold. The effect of the N-source was tested under conditions where high oxygen levels

Table 2. Levels of polyol pools in mycelia of *A. niger* harvested 24 h after inoculation.

	polyol levels[a]			
strain:	N400	N400	N400	NW101
oxygen level:	low	high	high	high
N-source:	$NaNO_3$	$NaNO_3$	NH_4Cl	$NaNO_3$
pH:	4.0	4.0	4.0	6.0
glycerol	400	414	467	331
erythritol	115	258	163	250
arabitol	18	9	5	5
trehalose	55	16	41	6
mannitol	265	84	223	101
dry weight (g/l)	1.6	4.6	6.2	3.1

[a] The data are the mean values of two experiments and are given in μmole (g dry weight)$^{-1}$. In all cases the 2% glucose was used as carbon source.

were applied. Ammonium resulted in less erythritol accumulation and in a pronounced increase of trehalose and mannitol when compared to the nitrate grown culture. The effect of the pH was tested under conditions of high oxygenation. In these eperiments a glucose oxidase negative mutant (NW101, Witteveen *et al.*, 1990a), was used to prevent fast oxidation of the glucose by glucose oxidase. The results obtained are comparable to a high oxygen culture at pH 4, only the glycerol level was lower.

Polyol pools in the glycerol kinase mutant

In Fig. 3 the results are shown of an experiment that was essentially the same as that given in Fig. 2 except that a glycerol kinase mutant (NW201) was used. The data shown originate from one experiment, but duplicate experiments gave essentially the same results. The polyol accumulation pattern in the medium was comparable with that of the wild type but the intracellular pools turned out to be very different. The intracellular glycerol pool is much smaller compared to the wild type. After the exhaustion of glucose all polyols, including glycerol, are taken up again from the medium. The amounts of intracellular mannitol and erythritol decreased in a comparable way as in the wild type but the glycerol pool increases very strongly. When the total amount of glycerol intra- and extracellularly formed glycerol is considered it is clear that it increased until the glucose had disappeared from the medium. After that the total amount remained the same but the localization of the glycerol changed. The total amount (intra- plus extracellular) of glycerol formed at any time before glucose was exhausted is nearly identical to the amount formed in the wild type. Therefore, glycerol

synthesis is not affected by the mutation. Also the growth of the mutant was comparable with that of the wild type.

^{13}C labeling experiment

In Table 2 it was shown that there were differences in the erythritol and mannitol pools when the mycelium was grown under low and high oxygen conditions. We also investigated how polyol synthesis was affected in mycelium being initially pregrown under conditions of low oxygenation, that was subsequently exposed to higher dissolved oxygen levels. In order to be able to determine newly formed polyols sufficiently accurate we used ^{13}C enriched glucose. The results of this experiment are shown in Fig.4 . The first 45 min after addition of the label low oxygen conditions were maintained. Mannitol was formed and only small amounts of glycerol and erythritol. Subsequently, the dissolved oxygen level was increased (arrow in Fig. 4). After an initial decrease of the levels of all the pools, a strong increase of glycerol and erythritol levels was observed, whereas the mannitol pool remained stationary.

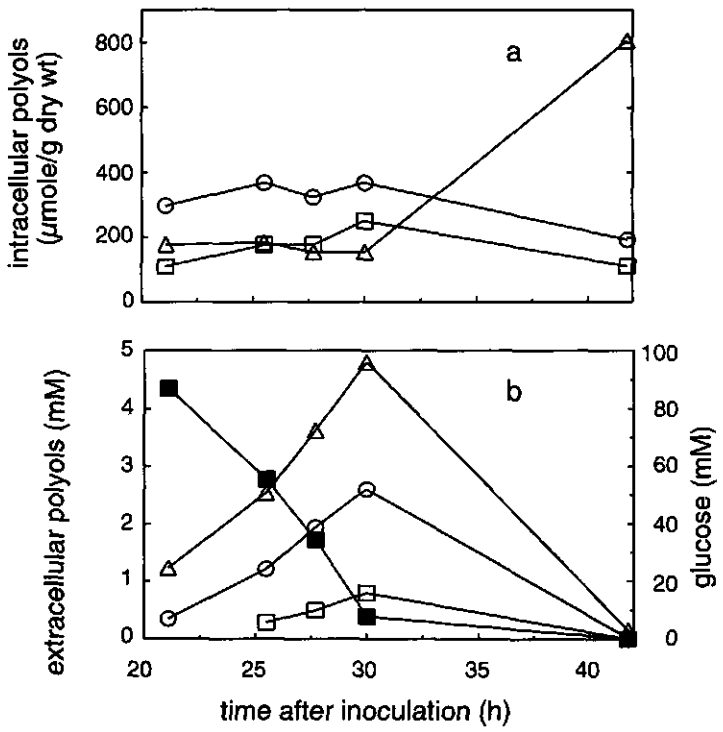

Fig. 3. Intra- (a) and extracellular (b) polyols in a culture of the glycerol kinase mutant NW201 grown at pH 4.0 with 2% glucose as carbon source and high oxygenation. △ = glycerol, □ = mannitol, ○ = erythritol, ■ = glucose.

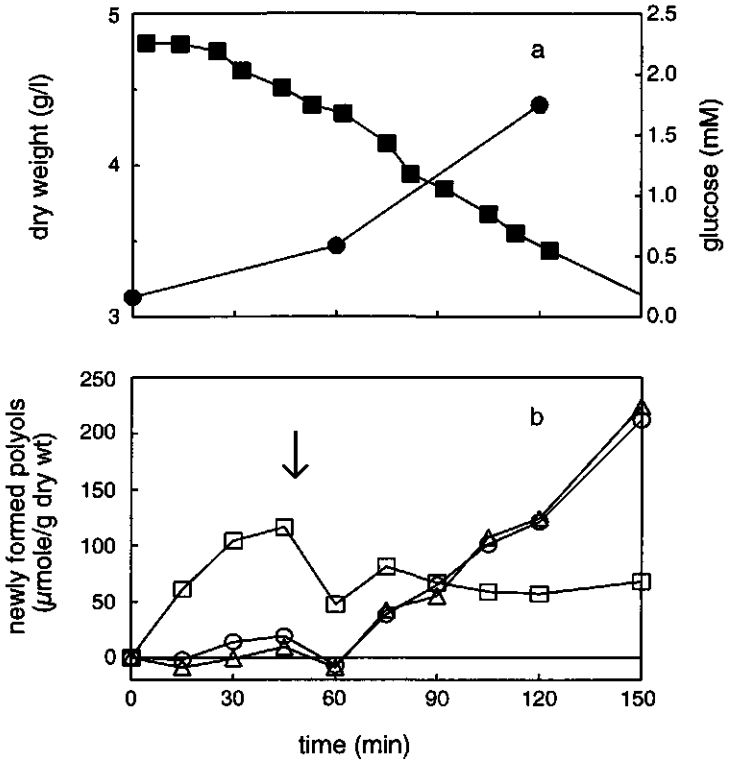

Fig 4. Amounts of newly synthesized intracellular polyols as determined by incorporation of randomly ^{13}C labeled glucose (a). The glucose concentration and the mycelium dry weight are indicate Fig 4b. The mycelium was pregrown under low oxygen conditions. At t=0 the glucose label was added. The low oxygen conditions were maintained for 45 min at which moment the oxygenation was strongly increased (arrow in the figure). △ = glycerol, □ = mannitol, ○ = erythritol, ■ = glucose. ● = dry weight.

Long term polyol accumulation

In analogy to the experiments of Smiley *et al.* (1967) and Hendriksen *et al.* (1988), we studied the polyol accumulation in the medium upon prolonged cultivation. Mycelium was grown under low oxygen conditions at pH 4.0 starting with 2% glucose. After 72 and 97 h more glucose was added to the culture. The fermentation was continued for 5 days. In Fig. 5a the quantities of the polyols that had accumulated in the medium are shown. Mannitol levels up to 70 mM and glycerol levels up to 45 mM were measured. Especially in a later phase when growth had slowed down (after about 60 h) a considerable part of the glucose consumed (approx. 45%) is converted into polyols. In Fig. 5b the intracellular polyol levels are shown and it is clear that the polyols that were excreted in the medium are a reflection of the intracellular pools except for glycerol which is excreted in relatively high amounts.

To show that the phenomenon is not dependent on D-glucose we studied the

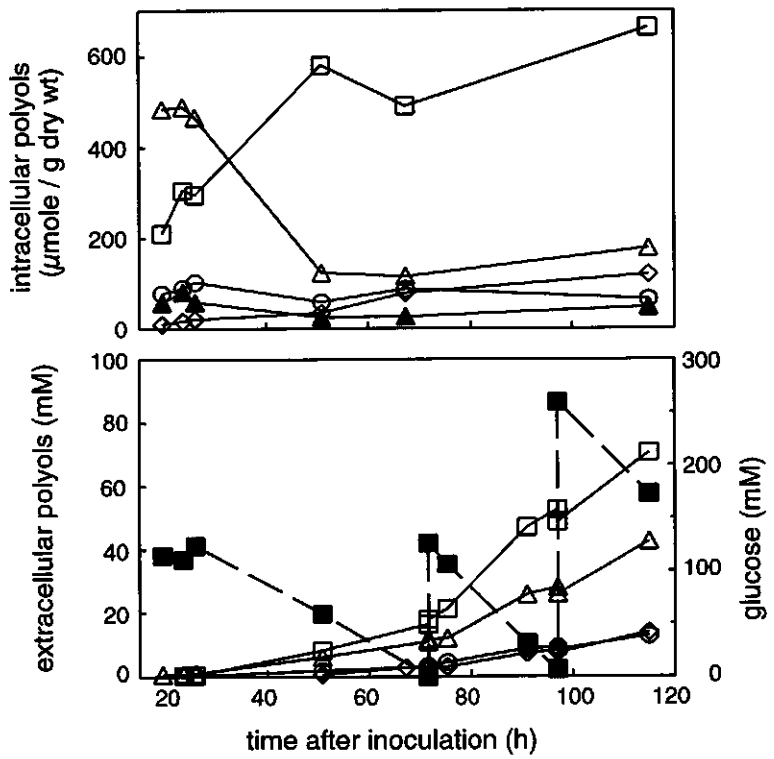

Fig. 5. Intra- (a) and extracellular (b) polyols in a N400 culture grown at pH 4.0 with low oxygenation. The culture was started with 2% glucose. At t = 72 and 97 h glucose was added to the culture. △ = glycerol, □ = mannitol, ○ = erythritol, ▲ = arabitol, ◇ = trehalose, ■ = glucose.

accumulation of polyols with D-xylose as carbon source. For this fermentation we used low oxygenation conditions and pH 4 as well. The initial concentration of D-xylose was 180 mM and at 69 h 150 mM extra D-xylose was added. In Table 3 the polyols accumulated in the culture medium after a 5 day fermentation are shown. Especially xylitol (45 mM) accumulated, and in addition relatively large amounts of arabitol (16 mM).

DISCUSSION

The major role of glycerol as an osmoticum (*cf* Table 1) is common in many fungi (Brown 1978, Blomberg and Adler 1992). The decreasing amounts of the other polyols with increasing salt concentration might be related to a difference in growth stage of the mycelia as high salt concentrations slow down fungal growth. Therefore the high salt mycelia may be in an earlier developmental stage in which other polyols are less abundant. This is confirmed by the changes in

Table 3. Polyol accumulation in medium of a *A. niger* N400 culture grown for 5 days on D-xylose[a].

	concentration (mM)
glycerol	10.6
erythritol	5.3
xylitol	45.3
arabitol	16.4
mannitol	24.2

[a] The starting concentration of D-xylose was 180 mM and after 2 days an extra 150 mM was added. After 5 days 200 mM of the substrate was consumed and the dry weight was 6.7 g/l.

polyol content of mycelium grown in the presence of 1 M NaCl and harvested after 28 h. Beever and Laracy (1986) studied the osmotic adjustment in *A. nidulans* and concluded that glycerol and erythritol are the most important solutes. Our experiments show that in *A. niger* erythritol plays only a minor role in maintaining the osmotic balance, although its contribution increased with the age of the mycelium, as is the case for mannitol. Glycerol seems to be the major osmoticum for two reasons. In the first place glycerol is the smallest polyol synthesized and this is therefore the most efficient way for the fungus to produce solutes for osmotic pressure. Secondly, glycerol has a stabilizing effect on enzymes (Blomberg and Adler 1992). The biosynthetic route of glycerol is assumed to go via reduction of dihydroxyacetonephosphate to glycerol-3-phosphate and subsequent dephosphorylation. In *Saccharomyces cerevisiae* the level of the NAD^+-dependent glycerol-3-phosphate dehydrogenase is increased in high salt media (Blomberg and Adler 1989). From the green alga *Dunaliella tertiolecta* a glycerol-3-phosphate phosphatase was characterized and it was found that an important factor for the *in vivo* activity of this enzyme was the glycerol-3-phosphate concentration because of the high K_m for glycerol-3-phosphate (Belmans and Van Laere 1988). The level of this compound is low in comparison to the K_m value but increased after an osmotic shock.

The presence of large amounts of mannitol in conidiospores indicates that this polyol has an important role as carbon storage compound. Horikoshi *et al.* (1965) also observed a decrease in mannitol in the first hour of germination of *Aspergillus oryzae* but after that the mannitol pool was replenished again. In *A. niger* we observe an almost immediate decrease in the mannitol pool but the mobilization of this pool continues in contrast to the situation described for *A. oryzae*. The high levels to which glycerol is synthesized in the first hour of incubation of the conidiospores might be related to its role as an osmoticum. The relatively dry conidia will take up a large volume of water in the first phase of germination.

Whether this hydration is stimulated by the increased glycerol content or whether the dilution of the cell interior induces glycerol formation is difficult to distinguish. Synthesis of a large amount of glycerol has also been observed during the first phase of germination of spores of *Phycomyces blakesleeanus* (Van Laere and Hulsmans 1987). It was concluded that amongst other events, increasing the glycerol concentration in the spores caused increased water uptake. A difference with the *A. niger* spores, which start germinating as soon as they are brought into medium, is that the *P.blakesleeanus* spores are constitutively dormant and that a suspension of the spores is activated by a heatshock or by adding ammonium acetate. Therefore it is certainly possible that the glycerol synthesis in *A. niger* is the result of rehydration and not the cause of it. After this initial transient glycerol accumulation, the concentration of this compound drops approximately 4-fold. However, glycerol remains the major polyol till later growth stages. The predominant role of glycerol in fast growing cells can be explained in the same terms as the glycerol accumulation during germination. It apparently provides an efficient way for fast growing cells to maintain cell turgor.

Considering mannitol there seems to be a kind of cyclic process. In spores high mannitol levels are present which are metabolized upon germination. In fast growing *A. niger* hyphae glycerol dominates whereas in a later stage mannitol accumulates again and then slightly levels off upon starvation. The relationship between glycerol accumulation and active growth combined with the late accumulation of mannitol suggests that mannitol specifically accumulates in older hyphae. In these older hyphae differentiation can occur in submerged cultures resulting in the formation of conidiophores, thus completing the cycle to conidia which contain high mannitol levels, although some other triggers than just aging are needed for conidiation in submerged culture (Ng *et al.* 1973). Erythritol formation is also higher in older mycelium. However, this polyol is not found in conidiospores. We did not analyze polyol accumulation under conditions where conidiation in submerged culture is optimal and have no information on erythritol accumulation under these conditions.

More than 50% of the polyols accumulated in the course of 24 h was found extracellularly. The question is whether this occurs because of leakage through membranes or that a transporter is involved. Leakage through the cell membrane is highly unlikely in view of the behaviour of an *A. nidulans* glycerol uptake mutant which did not grow even on high concentrations of glycerol (Visser *et al.* 1988). So apparently a transport system is necessary for the passage of the polyols over the plasma membrane. The polyols are taken up by the mycelium again within a few hours after exhaustion of glucose. This polyol uptake is against a concentration gradient so active polyol transport systems are derepressed after exhaustion of the glucose. The metabolization of the intracellular polyols, especially of mannitol and erythritol, is much slower than the uptake from the medium.

Polyol accumulation in the glycerol kinase mutant showed an unexpected

result. Rather than an increase of the intracellular glycerol pool we observed a reduction. Calculating the total amount of glycerol accumulating intra- and extracellularly showed that approximately the same amount of glycerol is formed as in wild type, but this is largely is excreted. Thus the glycerol kinase defect has no effect on the rate of glycerol formation but does lead to an increased excretion. This indicates that the excretion of the polyols is controled by the fungus and is not a result of leakage. Excretion might be the mechanism to control the polyol levels rather than catabolic metabolism. A decrease in the membrane permeability of *Saccharomyces cerevisiae* at high osmotic values is supposed to be involved in the regulation of the intracellular glycerol level (Blomberg and Adler 1989). The fact that both wild type and glycerol kinase mutant form the same amount of glycerol, although localized in different compartments, suggests that the turnover of this polyol pool is very low.

Thus far we discussed the need to synthesize polyols in terms of osmotic balance (glycerol) and the build up of storage compounds (mannitol). Dijkema *et al.* (1985, 1986) showed that environmental factors such as the nitrogen and carbon source, pH and aeration also have a strong influence on the polyol pool composition in *A. nidulans*. Table 2 shows that these factors especially influence the erythritol and mannitol pools in *A. niger*. These effects seem to be part of the fungal strategy to maintain both proper levels of key intermediates in the central carbohydrate metabolic pathways and proper balances between reduced and oxidized cofactor pools. This does not require a specialized function of any specific polyol. Thus their synthesis will depend on the availability of the central metabolic intermediates from which they are derived and on the cofactor specificity of the enzymes involved. In the case of growth on nitrate using D-glucose as a carbon source it is well known that the flux through the pentose phosphate pathway is stimulated, possibly resulting in a higher erythrose-4-phosphate level. The lower erythritol level under low oxygen conditions can be explained by the fact that the mycelium is still in an earlier growth phase (biomass 1.6 g/l versus 4.6 g/l). The higher mannitol levels under low oxygen conditions might be caused by either an accumulation of intermediates at the fructose-6-phosphate level or an increased catabolic reduction charge. Both result in an increased activity of fructose-6-phosphate reduction. For studying the pH effect we used the glucose oxidase negative mutant to prevent the effect of a very fast external glucose oxidation by glucose oxidase with presumably multiple effects on carbon metabolism. No essential differences in polyol pool composition were observed between pH 4 and 6 as shown in Table 2.

The most remarkable result of the NMR oxygen shift experiment is the identical rate of formation of the glycerol and erythritol pools under these conditions. This indicates that identical regulatory mechanisms operate in glycerol and erythritol formation under these conditions, where mycelium which is not yet adapted to a high oxygen supply has to adjust its metabolism to these new conditions. The precise nature of this mechanism is still unknown. Although in

the NMR experiment the rate of formation at a certain moment is studied and in Table 2 the accumulation over 24, it is possible to make some comparisons. The finding that mannitol formation diminished upon the increase of oxygenation is then in line with the lower mannitol levels observed under high oxygen conditions (Table 2). The increase of the glycerol and erythritol levels is not in line with the results of Table 2 and are probably due to transient effects caused by the sudden change in conditions.

In a late stage of its development, when the pH has become low and growth has ceased, *A. niger* is known to efficiently produce citric acid. For several other fungi, amongst which some *Aspergillus* species, considerable polyol excretion has been described to occur in this phase (Smiley *et al.* 1967, Hendriksen *et al.* 1988). The tendency of these fungi to overproduce organic acids or polyols in this stage might be related phenomena. The main difference in production conditions is the oxygenation of the culture: high oxygen levels promote organic acid formation whereas low oxygen levels stimulate polyol accumulation (Birkinshaw *et al.* 1931). Here we have shown that *A. niger* is also capable of accumulating polyols in the medium under low oxygen conditions (Fig. 5). Mannitol and, to a lesser extent, glycerol are the most abundant polyols to appear. Variation in the culturing conditions (increasing the oxygen level results in increased glycerol formation, data not shown) showed that the polyols excreted are a reflection of the intracellular pool distribution although glycerol is excreted to a higher extent. Apparently polyol formation in the cell continues and the levels of the polyols are controled by excretion into the medium. In later stages of the fermentation (cf. Fig. 4) about 45% of the glucose consumed was converted into extracellular polyols. This is comparable to conversion efficiencies reported in literature (Smiley *et al.* 1967). We did not thoroughly investigate whether by optimizing the conditions higher conversion efficiencies can be obtained. Also other carbon sources than D-glucose result in polyol accumulation. The polyols excreted on D-xylose (Table 3) reflect the metabolic pathway of this compound (Witteveen *et al.* 1989). D-xylose is first reduced to xylitol. This compound accumulated to very high levels in the cell in later stages of growth under low oxygen conditions, which is an indication of an elevated catabolic reduction charge since the next step (oxidation of xylitol to D-xylulose) is NAD^+ dependent. Under these conditions a considerable amount of arabitol accumulated as well. Our detection method did not allow for distinction between the L- and D-form of arabitol. In this case it might be either compound since L-arabitol can be derived from xylitol in two steps (Witteveen *et al.* 1989) and D-arabitol can be derived from D-xylulose or D-ribulose in one reduction step. The arabitol observed in other experiments, where D-glucose was used, is presumably D-arabitol since no xylitol is observed in these mycelia. This would have to be present if L-arabitol would accumulate, assuming a biosynthetic route of L-arabitol via D-xylulose, xylitol and L-xylulose.

Concluding we can state that glycerol, which is the most mobile pool, plays a major role in the osmotic adjustment of *A. niger*. Mannitol seems to fulfil a

storage function. Erythritol does not seem to have a defined role. It is present in high amounts under certain conditions but is absent in conidiospores and in early growth stages. It is also the polyol to disappear at the slowest rate from the mycelium under starvation conditions. It seems most likely that to accomplish maintenance of cofactor balances and/or to realize carbon overflow the fungal cell has to remain flexible and does not select a specific polyol to serve these functions. The nature of the polyols formed will depend on the specific environmental and nutritional conditions as those determine the availability of the various metabolic intermediates from which the polyols originate.

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Chapter 9

Summary and concluding remarks

The capacity of *A. niger* to accumulate metabolites is remarkable. Under all conditions polyols accumulate in the cell and when mycelium in later developmental stages is considered, depending on the carbon source, aeration and external pH, polyols and/or organic acids can be formed in a very efficient way. The aim of this thesis was to obtain a better understanding of the mechanisms governing the metabolism and formation of these metabolites. The first part of this thesis reports a study of gluconic acid formation and the second part involves polyol metabolism in *A. niger*.

The fungus has a general tendency to synthesize organic acids under conditions of good aeration and when sugars like D-glucose or sucrose are available. An important function of the organic acid synthesis might be the acidification of the medium. Combined with the removal of the sugars from the environment this may contribute to the competitiveness of the fungus. *A. niger* is tolerant to pH values as low as 1.5. When glucose is the carbon source and the culture is well aerated *A. niger* will produce at neutral or slightly acidic pH mainly gluconate, at very low pH values citrate formation will occur and at intermediate pH oxalate is formed. The result is that *A. niger* by the consecutive synthesis of a series of organic acids strongly acidifies the medium.

In this thesis the formation of gluconic acid was studied in more detail. In Chapter 2 evidence is provided for a cell wall localization of glucose oxidase. Furthermore, it was shown that two catalases are induced in parallel with glucose oxidase, one intracellular (CAT III) and one localized in the cell wall (CAT IV). Two other catalases, also one intracellular (CAT I) and one localized in the cell wall (CAT II), are constitutively present. About 50% of the lactonase activity was measured in the culture fluid. Therefore it was concluded that the whole glucose oxidase system is localized extracellularly, and is mainly localized in the cell wall. The induction of the intracellular CAT III may be seen as a second defense barrier, detoxifying hydrogen peroxide that diffuses into the cell.

The cell wall-localization of glucose oxidase combined with the easy detection of hydrogen peroxide produced in the enzyme reaction makes visualization of the enzyme in intact hyphae possible. This detection system was used to isolate a series of mutants with altered glucose oxidase induction. In Chapter 3 a phenotypic and genetic characterisation of these mutants is presented. The mutants were classified in 9 different complementation groups, 1 non-producing, 1 low producing and 7 overproducing mutants. From induction experiments with the wild type strain it was concluded that the carbon source and the dissolved oxygen level are main factors determining induction of glucose oxidase. One mutant was found which never synthesized glucose oxidase (*gox*C). Only one of the mutant classes was no longer dependent on oxygen for induction

(*gox*B). Several mutant groups were found with a decreased glucose dependency of induction. Some of these were quite strong (*gox*A and *gox*E) whereas others showed only a minor overproduction under conditions which are only weakly inducing in the wild type. The latter group might in fact influence glucose oxidase induction only indirectly. The genetic analysis provided the information necessary for the construction of recombinant strains containing different *gox* mutations and other genetic markers. This is essential for further analysis and also an important step in further strain breeding.

In Chapter 4 the induction mechanism of glucose oxidase, lactonase and the catalases was analyzed in more detail. For this we used beside the wild type strain *gox*B, *gox*C and *gox*E mutants. These mutants had a clear and pronounced phenotype. It was shown that in a wild type strain induction of all three activities is found only when glucose is present and the culture is well aerated. Induction of all three activities was effected by the *gox*B mutation. Neither glucose nor high oxygen levels were required for induction. The glucose dependency of glucose oxidase and lactonase induction was affected by the *gox*E mutation. In this mutant catalase was unaffected and high oxygen was still required. Thus with the *gox*B and *gox*E mutants the effects which oxygen and glucose have on induction could be partly separated. None of the activities was induced in the *gox*C mutant. This mutant could be transformed to a wild type phenotype using the structural gene of glucose oxidase, thus indicating that the mutation concerns the structural gene. The glucose oxidase structural gene was isolated via antibody screening of a cDNA expression library and subsequent homologous screening of a genomic library using the cDNA clone as a probe. Northern blotting showed that both the oxygen and carbon source effects were influencing induction at the transcriptional level. No glucose oxidase mRNA was observed in the *gox*C mutant. The absence of induction of all three activities in this mutant indicates that glucose oxidase activity is required for induction. It could be shown that hydrogen peroxide, besides gluconate a product of the glucose oxidase reaction, is inducing both catalase and lactonase in this mutant. It was concluded that hydrogen peroxide is the main factor required for the induction of the three activities and that the *gox*B gene product is involved in mediating this effect. It explained the requirement of high oxygen levels for induction because glucose oxidase has a high K_m for oxygen ($K_m = 0.48$ mM at 27°C, Gibson *et al.*, 1964). Even the glucose requirement for induction can be explained this way but the presence of the *gox*E mutant and the fact that the carbon source was still affecting the level of glucose oxidase in the *gox*B mutant, which is supposed to be involved in the transduction of the hydrogen peroxide signal, indicates that the induction process is more complicated. Using the plasmid pIM503 which carries the structural gene for glucose oxidase multicopy transformants were made. Only a relatively small increase in glucose oxidase activity was observed (3 fold) even though more than 50 copies of the gene were integrated. Transformation of *A. nidulans*, a fungus that does not have the glucose oxidase gene itself, resulted in

strains which produced glucose oxidase. In these strains glucose but not a high oxygen concentration was required for induction. This is indicating that a *gox*B-like gene is not present in *A. nidulans* but that the glucose requirement is presumably transduced by a more general system which is not specific for glucose oxidase.

Chapters 2,3 and 4 are contributions to a better understanding of how the glucose oxidation system functions and of the molecular mechanism of its induction. Thus far it is the only coordinately regulated enzyme system in *A. niger* of which regulatory mutants have been isolated and which has been worked out in some detail. However, the understanding of the mechanism is still incomplete, especially the way by which the carbon source is affecting the induction is still unclear. No explanation is yet available why on fructose or D-xylose a basal level of glucose oxidase is found, whereas on acetate, gluconate or glycerol no activity is detected. Somehow the system senses the presence of an easy metabolisable carbon source and low-level induction occurs.The mechanism behind this phenomenon is probably not glucose oxidase-specific since in the *A. nidulans* transformants still glucose is required for induction. The *gox*B system is probably more glucose oxidase specific and therefore no oxygen (H_2O_2) effect is found in *A. nidulans*. For a better understanding of the factors involved in glucose oxidase expression, a detailed analysis of the *gox*A and *gox*E mutants and of the functioning of the promoter of glucose oxidase is required. An important consequence of the regulatory mechanism hypothesized in Chapter 4 is a build-in feedback control. Catalase is not only induced by hydrogen peroxide but degrades this inducer as well, thus diminishing the induction. Ever increasing amounts of especially oxygen will be required to cause induction to continue. This will not happen because oxygen is quite soon the limiting factor in gluconate fermentation processes. The feedback mechanism of preventing overinduction is absent in *gox*B mutants. Therefore these mutants might be valuable in industrial processes.

The function of the polyols is different from that of the organic acids. This is already clear from their presence in the fungus during all phases of the life cycle. Organic acids are formed only in late stages of development. Furthermore, the organic acids are excreted whereas the polyols beside being excreted also accumulate in large amounts in the mycelium. Information on carbon metabolism and more specifically polyol metabolism in *A. niger* was scarce at the start of this project. Therefore it was decided to analyze some metabolic pathways which directly relate to polyol metabolism.

In Chapter 5 the characterization of a glycerol kinase mutant is described. Glycerol is one of the main polyols accumulating in *A. niger* and it was shown that glycerol kinase is involved in the degradation of glycerol. It could be demonstrated that the degradation pathway of glycerol in *A. niger* is largely the same as in *A. nidulans* (Hondmann *et al.*, 1990) and *N.crassa* (Courtright 1975). First phosphorylation to glycerol-3-phosphate occurs and this is followed by its

oxidation to dihydroxyacetonephosphate by a mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase. However, there were some differences with the pathway in *A. nidulans*. Whereas in the latter fungus dihydroxyacetone was catabolized via glycerol, in *A. niger* a dihydroxyacetone kinase was present enabling growth of the glycerol kinase mutant on dihydroxyacetone. Combined with an NAD^+-dependent glycerol dehydrogenase converting glycerol into dihydroxyacetone this formed an escape route for glycerol catabolism in the glycerol kinase mutant. Growth on D-galacturonate was strongly affected in the glycerol kinase mutant thus demonstrating a D-galacturonate degradation pathway via glycerol.

Pentose metabolism is described in Chapter 6. The isolation of a D-xylulose kinase mutant played an essential role in this work. It could be demonstrated that L-arabinose and D-xylose are catabolized via a series of reduction and oxidation steps. L-arabinose is reduced to L-arabitol which is oxidized to L-xylulose. L-xylulose is reduced to xylitol which is oxidized to D-xylulose that is phosphorylated to D-xylulose-5-phosphate, an intermediate of the pentose phosphate pathway. D-xylose is reduced to xylitol and subsequently oxidized to D-xylulose-5-phosphate. All the reduction steps are NADPH-dependent and all the oxidation steps NAD^+-dependent. The equilibrium of the reactions is far in the direction of the polyols, so several unfavourable steps that have to be taken which potentially can obstruct an efficient conversion of L-arabinose to D-xylulose-5-phosphate. The cofactor specificity of the dehydrogenases involved contributes to a higher efficiency since the anabolic reduction charge ([NADPH]/([NADPH]+[$NADP^+$])) is higher than the catabolic reduction charge (([NADH]/([NADH]+[NAD^+])) (Führer *et al.*, 1980). A second mechanism for increasing the efficiency of this pathway was found by studying the two xylitol dehydrogenases of the L-arabinose pathway. This is described in Chapter 7. The NADPH-dependent L-xylulose reductase, catalyzing the reduction of L-xylulose to xylitol, was purified and the NAD^+-dependent xylitol dehydrogenase, catalyzing the oxidation of xylitol to D-xylulose, was partially purified. Comparison of the two enzymes, which catalyze similar reactions leading to the different stereoisomers, made clear that they differ in two major points. 1) When their affinity for xylitol was compared it was found that the NAD^+-dependent enzyme had a much higher affinity for xylitol than the NADPH-dependent enzyme. 2) Near the physiological pH (around 7) the ratio of the rductive relative to the oxidative catalytic activity was higher for the L-xylulose reductase than for the xylitol dehydrogenase. Both characteristics contribute to a more efficient catalysis of the reaction in the *in vivo* direction.

In Chapter 8 an attempt is made to obtain some information on the function of the various polyol pools in *A. niger*. It was found that glycerol was the main polyol involved in osmotic adjustment in the fungus. Furthermore, glycerol accumulation was observed to be related to fast growing hyphae, whereas mannitol and erythritol accumulated in older hyphae. Mannitol also was an

important storage compound in conidiospores. This general scheme of polyol accumulation during different growth phases is modified by environmental parameters like aeration of the culture and the nitrogen source available. Changes in fluxes through metabolic pathways and as a result of that changes in the steady state concentrations of intermediary metabolites from which the polyols derive, presumably play a role in this. It also implies that the function of the polyols in the cell is not completely coupled to specific polyols but can, in part, be taken over by other polyols. It was observed that a considerable part of the accumulated polyols (>50% after 24 h) is found in the medium. Observations made with the glycerol kinase mutant suggested that polyol excretion is a way for the fungus to control the intracellular levels of the polyols, presumably for maintaining the osmotic balance of the cell. Long fermentations (5 days) showed that in late developmental stages the polyol excretion becomes more pronounced. Approximately 45% of the glucose taken up was converted into extracelluar polyols. The type of the polyols excreted was a reflection of the intracellular polyol pool composition.

Chapters 5 and 6 describe glycerol and pentose catabolism which has led to a better understanding of carbon metabolism in *A. niger*. Information on this subject is scarce in this fungus. The isolation of mutants in the degradation of such compounds is quite essential for metabolic studies. It turned out to be very difficult to isolate mutants in carbon metabolism in *A. niger* and still only a few of these mutants are available now. The reason for this is not known but the high intracellular polyol pools and the excretion of the polyols, resulting in cross-feeding during the filtration enrichment techniques, might play a role in this. The analysis of pentose metabolism and the xylitol dehydrogenases involved is also valuable in the light of understanding the mechanisms that play a role in extracellular enzyme production. The knowledge of the L-arabinose catabolic route has already proven useful in the analysis of the araban degrading system of *A. niger*. L-arabitol plays a major role in this (vd Veen *et al.*, 1993). The analysis of polyol accumulation in Chapter 8 indicates that different polyols accumulate in different parts of the hyphae depending on their age. There still remains the question whether specific polyols accumulate in specific compartments of the cell, for example in the vacuoles, which are abundant in older hyphae. No information is available on this. It was shown that polyol excretion is a general phenomenon in *A. niger*. Although it has been observed before (Röhr *et al.*, 1987), it was not considered to be such a common phenomenon in this fungus. The observation of large scale polyol accumulation in the culture fluid of 3-5 days old mycelial cultures grown under low oxygen conditions suggests similarities with organic acid fermentations. These are performed in strongly aerated cultures. An efficient flux to the TCA cycle is apparently not possible under the low aeration conditions used. This results in overflow metabolism in an earlier stage of the catabolic pathway leading to polyol formation. Cofactor regeneration might as well play a role in the polyol accumulation under these conditions.

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Samenvatting

De filamenteuze schimmel *Aspergillus niger* heeft het opmerkelijke vermogen grote hoeveelheden van bepaalde metabolieten te accumuleren. Deze metabolieten zijn in te delen in twee groepen: organische zuren en polyolen. De organische zuren worden vooral gevormd in een latere levensfase van de schimmel en worden uitgescheiden in het medium. De polyolen accumuleren met name in het mycelium en worden in alle levensfasen in grote hoeveelheden teruggevonden. Uitscheiding van de polyolen is met name in latere levensfasen ook aangetoond.

Het in dit proefschrift beschreven onderzoek had als doel het verkrijgen van inzicht in een aantal aspecten van zuurvorming en polyol metabolisme in *Aspergillus niger*. De studie van de vorming van organische zuren heeft zich gericht op gluconzuurvorming. Wat betreft de biosynthetische route gaat het hier om een relatief eenvoudig proces. Bij de vorming is in wezen slechts één enzym betrokken, namelijk glucose oxidase. Dit enzym oxideert een glucose molecuul tot glucono-δ-lacton. Bij deze reactie wordt verder een zuurstof molecuul verbruikt en een waterstofperoxide molecuul gevormd. Deze laatste verbinding is toxisch en maakt daardoor de aanwezigheid van catalase, dat waterstofperoxide afbreekt, noodzakelijk. Het glucono-δ-lacton kan spontaan of gekatalyseerd door lactonase hydrolyseren tot gluconzuur. Gluconzuur wordt commercieel mbv *A. niger* gemaakt. Ook het gezuiverde glucose oxidase kent vele toepassingen.

In hoofdstuk twee is aangetoond dat de drie enzymen die betrokken zijn bij de glucose oxidatie gelocaliseerd zijn aan de buitenkant van de cel in de celwand. Het voordeel voor de schimmel is dat het hierdoor relatief weinig last heeft van het giftige waterstofperoxide.

In hoofdstuk drie en vier is geanalyseerd onder welke omstandigheden glucose oxidase wordt aangemaakt door *A. niger* en hoe dit mechanisme van inductie op moleculair nivo in elkaar zit. Dit probleem is aangepakt door mutanten te isoleren die gestoord waren in dit mechanisme van inductie (hoofdstuk 3). Deze mutanten zijn genetisch geanalyseerd en het bleek dat er mutaties in 9 verschillende genen geïsoleerd waren. Analyse van de inductie omstandigheden voor een wild type stam en een aantal van deze mutanten heeft geleid tot de conclusie dat twee factoren een hoofdrol spelen: zuurstof en glucose. Uit de eigenschappen van de mutanten bleek dat deze factoren deels onafhankelijk waren. Nadere experimenten wezen uit dat waterstofperoxide, gevormd door glucose oxidase, een centrale rol speelt in de inductie van glucose oxidase, lactonase en catalase (hoofdstuk 4). Er zijn sterke aanwijzingen dat het genprodukt van één van de mutanten (*gox*B) betrokken is bij de waterstofperoxide inductie. De specificiteit van glucose oxidase voor glucose bepaalt voor een belangrijk deel ook de glucose-afhankelijkheid van de inductie. De karakteristieken van de *gox*B mutant en de aanwezigheid van andere mutanten klassen geven aan dat ook nog andere factoren invloed hebben op het inductie mechanisme.

Het polyol metabolisme is onderdeel van het koostof metabolisme van *A. niger*. Over dit onderwerp was slechts zeer weinig informatie beschikbaar, zodat

een deel van de tijd werd geïnvesteerd in de analyse van afbraakroutes van een aantal koolstof verbindingen. In hoofdstuk 5 is de afbraakroute van glycerol, één van de belangrijkste polyolen in *A. niger*, geanalyseerd. Hieruit bleek dat de voor schimmels gangbare afbraakroute gevolgd werd. Eerst fosforylering door glycerol-kinase en vervolgens oxidatie door een FAD-afhankelijk glycerol-3-fosfaat dehydrogenase.

In hoofdstuk 6 is de afbraakroute van D-xylose en L-arabinose geanalyseerd. D-xylose wordt verwerkt door reductie tot xylitol dat vervolgens weer geoxideerd wordt tot D-xylulose. D-xylulose wordt gefosforyleerd door D-xylulose kinase tot D-xylulose-5-fosfaat dat een intermediair is van de pentose fosfaat route. L-arabinose wordt via een aantal reductie en oxidatie stappen ook in D-xylulose omgezet. De intermediairen zijn: L-arabitol, L-xylulose en xylitol. Het opvallende in deze twee afbraakroutes zijn de polyolen als intermediairen. Het evenwicht van de redox reacties in de routes ligt ver in de richting van de polyolen. Zeker in het geval van L-arabinose afbraak kan dit een probleem opleveren omdat een aantal thermodynamische barrieres genomen moeten worden hetgeen tot een lage efficientie van de flux door deze route kan leiden. Er zijn een aantal mechanismen die deze efficientie verhogen. In de eerste plaats de cofactor specificiteit van de dehydrogenases. Alle reductie reacties worden gekatalyseerd door NADPH-afhankelijke reductases, terwijl bij alle polyol oxidaties NAD^+-afhankelijke dehydrogenases betrokken zijn. Aangezien de [NADPH]/([NADPH]+[$NADP^+$]) verhouding in de cel hoger is dan de [NADH]/([NADH]+[NAD^+]) verhouding betekent dit een verschuiving van het evenwicht in de richting van D-xylulose vorming. Twee andere efficientie verhogende mechanismen zijn aangetoond in hoofdstuk 7. Daar is de zuivering en karakterisering van L-xylulose reductase en xylitol dehydrogenase beschreven. Deze enzymen katalyseren beiden een bijna identieke reactie, slechts verschillend in het stereoisomeer dat na oxidatie van xylitol gevormd wordt. *In vivo* katalyseren ze twee opeenvolgende stappen in de L-arabinose afbraak en hebben xylitol als gemeenschappelijk product/substraat. De activiteit van de enzymen is aangepast aan hun functie. De affiniteit voor xylitol van het xylitol dehydrogenase blijkt veel hoger te zijn dan van het L-xylulose reductase, waardoor de teruggaande reactie van xylitol naar L-xylulose in de cel nauwelijks plaats zal vinden. De aanpassing van de enzymen aan hun *in vivo* functie blijkt ook in het pH profiel terug te vinden.

In hoofdstuk 8 is de functie van de polyolen in de cel geanalyseerd. Glycerol speelt de belangrijkste rol bij de aanpassing van de osmotische waarde van de cel. Verder is dit kleinste polyol het sterkst vertegenwoordigd in jong mycelium, terwijl in ouder mycelium vooral mannitol en erythritol ophopen. In conidiosporen is naast trehalose vooral een grote hoeveelheid mannitol aanwezig. Uitgaande van een belangrijke rol van de polyolen in het handhaven van de turgor van de cel is het logisch dat in jong, snel groeiend mycelium glycerol wordt opgehoopt omdat dit de meest efficiente manier voor de cel is om zijn osmotische waarde te verhogen. Gezien de ophoping in conidiosporen en vooral in ouder mycelium lijkt opslag een belangrijke functie van mannitol te zijn. Het blijkt dat polyol excretie

onder alle condities aangetoond kon worden. Er zijn aanwijzingen gevonden dat de uitscheiding van polyolen een mechanisme vormt voor de cel om de omvang van de polyol pools te controleren. In latere levensfasen bij een geringe beluchting van het mycelium blijkt dat ongeveer de helft van de verwerkte C-bron wordt omgezet in polyolen welke worden uitgescheiden in het medium. Dit proces vertoont gelijkenis met citroenzuur vorming door *A. niger*.

Curriculum vitae

Cor Witteveen werd op 14 april 1960 geboren te Sneek. In 1978 behaalde hij het VWO diploma aan de rijksscholengemeenschap te Meppel. In datzelfde jaar begon hij met de studie scheikunde aan de Rijksuniversiteit Groningen, waar hij in 1985 cum laude afstudeerde met als hoofdvak biochemie (Prof. B. Witholt) en als bijvak fysische chemie (Prof. R. Kaptein). Van september 1985 tot en met 1990 was hij werkzaam bij de vakgroep Erfelijkheidsleer van de Landbouwuniversiteit Wageningen. De eerste vier jaar en het laatste jaar in dienst van de Stichting Technische Wetenschappen (STW) en in de tussenliggende periode van 4 maanden in dienst van de Landbouwuniversiteit. De STW projecten waren gericht op stamveredeling van *Aspergillus niger* voor gluconzuur produktie. Een rugoperatie in december 1990 heeft zijn laboratorium werkzaamheden voor een periode van twee jaar onderbroken. De eerste 5 maanden van 1993 zijn besteed aan het afronden van dit proefschrift.

Nawoord

Dat dit boekje niet geheel op eigen kracht is gemaakt zal duidelijk zijn. Aan het begin (en eind) van de hoofdstukken staan de mensen genoemd die er een bijdrage aan geleverd hebben. Ik wil hen daarvoor bedanken. Een paar mensen zijn nauw betrokken geweest bij het gehele promotieonderzoek.

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