Characterization of a glycerol kinase mutant of *Aspergillus niger*

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A glycerol-kinase-deficient mutant of *Aspergillus niger* 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 *A. niger* 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 responsible 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 were formed under different physiological conditions: the NAD+-dependent enzyme described above, a constitutive NADP+-dependent enzyme and a D-glyceraldehyde-specific enzyme 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 cells is generally recognized (Brown, 1976, 1978). The function of glycerol as an osmoticum has also been demonstrated in filamentous fungi (Hocking & Norton, 1983; Beever & Laracy, 1986). Furthermore, glycerol is involved in metabolic processes occurring during germination of fungal spores (Van Laere & Hulsms, 1987) and is also an intermediate in the breakdown of some compounds, for example galacturonic acid (Uitzetter et al., 1986). Glycerol formation plays a role during citric acid production by *Aspergillus niger* (Röhrt el al., 1987). Legisa & Mattey (1986) suggested that glycerol accumulation initiates this process.

The catabolic pathways of glycerol utilization have been investigated in many organisms (Lin, 1976, 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 & 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, 1975a, b; Holm et al., 1976). Three classes of glycerol non-utilizing mutants have been isolated in *Aspergillus nidulans*: glcA, glcB (Payton, 1978; Uitzetter et al., 1986) and glcC (Visser et al., 1988). glcA and glcB are deficient in glycerol kinase and FAD-dependent glycerol-3-phosphate dehydrogenase respectively (J. Visser and co-workers, unpublished results), and glcC 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 has an important role in osmotic regulation; and (3) it may be involved 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 methosulphate (PMS) and dihydroxyacetone (DHA) were from Serva. Other chemicals were from Merck. Sephadex G-25 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 (cspAZ) and nicotinamide deficiency (niaC1) (Bos et al., 1988).

Mutations were induced by UV treatment. A suspension of conidia (8 x 10^6 ml^-1) in a Petri dish was irradiated for 2-5 min with 2 µJ 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 β-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 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 selected for further analyses.

The linkage group of the mutation was determined by somatic recombination with a tester strain (N655) carrying a colour marker and (II), (1988). Isolation of diploids and haploidization was done according to (I), hisD4 (II), lysA7, bioA1 (III), leuA1 (IV), metB1 (V) and pabA1 (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 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 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)_2MoO_4, 24H_2O, 0.29 mg CaCl_2, 2H_2O and 0.2 mg FeSO_4, 7H_2O. In all experiments minimal medium was supplemented with 1 ml 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 of medium, inoculated with approximately lo^6 conidia/ml and incubated in a New Brunswick rotary shaker for 20-24 h at 280 r.p.m.

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 done on Petri dishes containing minimal medium, 1% (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 (wt wt) frozen mycelium was crushed in a mortar and added in small portions to 3 ml ice-cold 10% (v/v) HClO_4 in a Potter-Elvehjem 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 10000 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. 13C 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 g for 30 s to remove cell debris. The supernatant was centrifuged at 10000 g for 10 min. The pellet from this high-speed centrifugation was resuspended in extraction buffer and used for the measurement of FAD-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.1.5). The supernatant was used for the other enzyme measurements.

Fractionation of cell-free extract. Cell-free extract (1 ml) was fractionated on a 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-β-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 using an Amino DW-2 spectrophotometer in the dual wavelength mode, measuring A_M - A_M. 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 DHA kinase except that 10 mM-GAD was used. FAD-independent membrane-bound glycerol-3-phosphate dehydrogenase (EC 1.1.1.95) was measured in the resuspended pellet from the high-speed centrifugation step (see above) using a modification of the method of 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 and 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 a mixture containing 100 mM-glycine/NaOH buffer, pH 9-6, 0.4 mM-NAD(P)+ and 100 mM-glycerol. DHA reductase and GAD reductase were measured in a mixture containing 50 mM-sodium phosphate buffer, pH 6.5, 0.2 mM-NAD(P)+ and 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 & Weinstein, 1976), by the microbiuret method (Itzhaki & Gill, 1964) using BSA as standard.
Results

Isolation of the mutant and genetic mapping of the mutation

After the filtration enrichment 1.3 × 10⁶ 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 this glycerol-negative mutant was denoted glcA1.

Upon haploidization of a diploid obtained from a heterokaryon of NW201 and the tester strain N655, 105 segregants were analysed. The frequency of recombination between glcA1 and pabA1 was 8-6%, whereas the recombination frequency with all the other markers varied between 44% and 53%. Therefore the glcA1 mutation is located on linkage group VI. The recombination between markers known to be located in one linkage group was 1.9% for nicA1 and metB1 (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 greater 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-negative mutant to sporulate.

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 mutant NW201, while the other enzymes were present. It was thus concluded that the glcA1 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, seemed to cause greater induction of a number of activities in the mutant: DHA kinase, GAD kinase, 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 other glycerol dehydrogenases, both NADP+-dependent. One is induced on D-galacturonate and is 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

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

<table>
<thead>
<tr>
<th>Enzyme activity [nmol min⁻¹ (mg protein)⁻¹]</th>
<th>A. niger NW201 grown on:</th>
<th>A. niger N423 grown on:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>Glucose</td>
<td>DHA (transfer to)</td>
</tr>
<tr>
<td>DHAP reductase</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>DHAP kinase</td>
<td>0</td>
<td>208</td>
</tr>
<tr>
<td>d-Glyceraldehyde kinase</td>
<td>0</td>
<td>136</td>
</tr>
<tr>
<td>Glycerol-3-phosphate dehydrogenase</td>
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<td>55</td>
</tr>
<tr>
<td>Glycerol dehydrogenase (NADP⁺)</td>
<td>145</td>
<td>19</td>
</tr>
<tr>
<td>Glycerol dehydrogenase (NAD⁺)</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>DHA reductase (NADPH)</td>
<td>925</td>
<td>70</td>
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<tr>
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<td>64</td>
</tr>
<tr>
<td>GAD reductase (NADPH)</td>
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</tr>
<tr>
<td>GAD reductase (NADH)</td>
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<td>35</td>
</tr>
<tr>
<td>Glycerol kinase</td>
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</table>
Fig. 1. $^{13}$C NMR spectra of extracts of mycelia (A, B) and of mycelia (C, D, E, 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.

Enzyme is relatively specific for DHA and is probably the same enzyme as the one partly purified by Baliga et al. (1962). It has been purified and characterized in our laboratory (Schuurink et al., 1990) and is relatively specific for DHA, GAD giving only 10% of the DHA reductase activity.

Metabolite accumulation in the wild-type and in 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 wild-type 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 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 A. 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 (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 take up glycerol. The mannitol and erythritol pools were depleted by the mutant under these conditions, since no other substrates were 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 are catalysed by a single enzyme. Since it was 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
kinase mutant NW201. The mutant was grown for 24 h on glucose and subsequently transferred for 15 h to minimal medium with D-galacturonic acid. An FPLC Mono-Q 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 A280 (—). DHA kinase (O) and GAD kinase (Δ) activities were measured in all fractions; only the levels in fractions containing detectable kinase activities are indicated.

activities. The two activities eluted simultaneously (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 & 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 both in the glycerol kinase and in the FAD-dependent glycerol-3-phosphate dehydrogenase genes, and thus unable 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 in the mutant as compared to the parental strain grown on DHA. Therefore, it may 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 unable to grow on D-galacturonic acid, 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-galacturonic acid, the NMR spectra of mycelia transferred to D-galacturonic acid and the induction of a number of enzymes from glycerol metabolism by D-galacturonic acid confirm that D-galacturonic acid is catabolized, in part, via glycerol. High levels of GAD kinase are present in the mutant. With the situation for DHA in mind, it must be considered why GAD formed from D-galacturonic acid is not directly phosphorylated thus allowing the mutant to grow on D-galacturonic acid. 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 catalysing 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 that is relatively specific for DHA are found in glucose-grown mycelium. This enzyme might function in the synthesis of glycerol which accumulates in A. niger 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 the catabolism of these substrates. The second NADPH-dependent enzyme is induced by D-galacturonate and is involved in a step in the degradation of this substrate. Considering the relative levels of NADPH-dependent GAD reductase and DHA reductase, this particular enzyme is also very likely to be present when A. niger is grown on one of the other substrates. This enzyme might be comparable to the glycerol dehydrogenase isolated from N. 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 to 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 is similar to the situation in the yeast Schizosaccharomyces pombe, in which Kong et al. (1985) found four different glycerol dehydrogenases, with characteristics comparable to the enzymes described here.

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References


