

## Glycerol catabolism in *Aspergillus nidulans*

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**Glycerol is catabolized in *Aspergillus nidulans* by glycerol kinase and a mitochondrial FAD-dependent *sn*-glycerol-3-phosphate dehydrogenase. The levels of both enzymes are controlled by carbon catabolite repression and by specific induction. Biochemical and genetical analyses show that dihydroxyacetone and D-glyceraldehyde are converted into glycerol and then catabolized by the same pathway. D-Glyceraldehyde can be reduced by NADP<sup>+</sup>-dependent glycerol dehydrogenase or by alcohol dehydrogenase I, while dihydroxyacetone is only reduced by the first enzyme. Three new glycerol non-utilizing mutants have been found. These three mutations define three hitherto unknown loci, *glcE*, *glcF* and *glcG*. The mutation in *glcG* leads to a greatly decreased *sn*-glycerol-3-phosphate dehydrogenase activity.**

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### Introduction

Glycerol is one of the polyols that is formed rapidly by *Aspergillus nidulans* when this fungus is grown on glycolytic carbon sources, especially under strong oxygenation (Dijkema *et al.*, 1985). Upon starvation, glycerol is the first polyol to disappear. Glycerol plays an important role in several physiological processes. Beever & Laracy (1986) showed that in *A. nidulans* glycerol and erythritol are the major osmoregulatory solutes. On hyperosmotic growth media both polyols accumulate, whereas they decrease rapidly in amount after transfer to a hypoosmotic medium. Glycerol formation is also one of the early events during germination of fungal spores (Van Laere & Hulsmans, 1987). In the stage just before citric acid production by *Aspergillus niger* glycerol has been reported to accumulate (Legisa and Matthey, 1986). Glycerol is catabolized again when citric acid production starts. This led Legisa & Matthey (1986) to suggest that glycerol accumulation may even initiate citric acid production in *A. niger*. The catabolism of dihydroxyacetone (DHA) (Schuurink *et al.*, 1990) and in part that of D-galacturonate (Uitzetter *et al.*, 1986) are also likely to proceed through glycerol in *A. nidulans*.

Glycerol catabolism has been studied in many organisms, as reviewed by Lin (1976, 1977). In most

organisms, both prokaryotes and eukaryotes, glycerol utilization is initiated by phosphorylation followed by oxidation of glycerol 3-phosphate (G3P) to dihydroxyacetone phosphate (DHAP) by G3P dehydrogenase. This enzyme is membrane-associated in prokaryotes and located on the inner membrane of mitochondria in eukaryotes. Three alternative pathways have been found. One pathway involves an NAD<sup>+</sup>-dependent glycerol dehydrogenase that converts glycerol into DHA, which is then phosphorylated to DHAP by DHA kinase (May & Sloan, 1981). This pathway can be used by several bacteria and by some yeasts. In the second alternative pathway glycerol is converted to glyceraldehyde by an NADP<sup>+</sup>-dependent glycerol dehydrogenase (Viswanath-Reddy *et al.*, 1978). The third alternative, found amongst others in *Aspergillus japonicus*, involves glycerol oxidase (Uwajima *et al.*, 1980). This enzyme oxidizes glycerol, using molecular oxygen, to D-glyceraldehyde (D-GAD) and hydrogen peroxide without mediation of NAD(P)<sup>+</sup> (Uwajima *et al.*, 1990).

Four glycerol non-utilizing mutant classes have been reported thus far for *A. nidulans*. *glcA* mutations were proposed to lead to glycerol kinase deficiency, *glcB* and *glcD* strains are deficient for the mitochondrial glycerol-3-phosphate dehydrogenase (Payton, 1978; Uitzetter, 1982; Arst *et al.*, 1990) and *glcC* strains are defective in glycerol uptake (Visser *et al.*, 1988). *A. nidulans* is therefore likely to use the phosphorylating pathway.

We present here a more detailed study of glycerol catabolism in *A. nidulans* and describe three new

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*Abbreviations:* ADH, alcohol dehydrogenase; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; D-GAD, D-glyceraldehyde; G3P, glycerol 3-phosphate; MTT, 3-(4,5-dimethylthiazolyl-2)2,5-diphenyltetrazolium bromide.

independent glycerol non-utilizing mutants. The effect of various carbon sources on glycerol catabolism in wild-type and *glc* mutants has also been investigated to initiate studies on regulation.

## Methods

**Materials.** G3P dehydrogenase (rabbit muscle), lactate dehydrogenase (rabbit muscle), triose phosphate isomerase (rabbit muscle), pyruvate kinase (rabbit muscle), horseradish peroxidase, G3P, ATP, glutathione, NAD(P)H and NAD(P)<sup>+</sup> were supplied by Boehringer Mannheim. D-GAD, DHAP, methylglyoxal, MTT and *o*-dianisidine were from Sigma. DHA was from Serva. D-Galacturonic acid was obtained from Fluka. Sephadex G-25 was obtained from Pharmacia LKB. Other reagents were from Merck and were of analytical grade.

**Strains and culture conditions.** *Aspergillus nidulans* strain WG096 (*yA2 pabaA1*) was used as wild-type with respect to carbon source utilization. The glycerol non-utilizing strains WG196 (*yA2 pabaA1 glcA1*), WG197 (*yA2 pabaA1 glcB33*), WG372 (*yA2 pantoB100 glcC2*) and *glcD100* (*pabaA1 pantoA10 glcD100*) were used. Strain WG216 (*pabaA1 creA<sup>d</sup>1*) was used to study the effect of catabolite repression. Linkage group assignment was made using WG202 (*biA1 acrA1 meaB6 pyroA4 pA1 sB3 nicB8 riboB2*). The ethanol non-utilizing strains used were Q8 (*alcR125*), H542-500 (*yA4 pyroA4 sB3 adE20 suadE20 alcA500 alcR500*), H444 (*biA1, alcR125*), H489 (*biA1 nii4 aldA67*), H352/7 (*pabaA1 alX4 alcA527*) and WG379 (*pabaA1 alcA527 aldA67 ureA1*).

For the preparation of conidiospores, *A. nidulans* was grown on complete medium (Pontecorvo *et al.*, 1953) solidified with 1.2% (w/v) agar and containing 25 mM-sucrose as carbon source. Mycelium was grown by inoculating 10<sup>6</sup> spores ml<sup>-1</sup> into minimal medium (Pontecorvo *et al.*, 1953), supplemented with appropriate vitamins, with 50 mM-D-fructose as carbon source, and incubating for 16 h at 37 °C, pH 6.0, in a New Brunswick Scientific orbital shaker (200 r.p.m.) using 1-litre flasks containing 300 ml. The mycelium was harvested by filtration and transferred to fresh minimal medium containing either glycerol (100 mM), DHA (100 mM), D-galacturonate (50 mM), D-glucose/glycerol (25 mM/100 mM), D-glucose (50 mM) or D-fructose (50 mM). After 6 h incubation at 37 °C in the orbital shaker, mycelium was again harvested by filtration, washed with cold saline and squeezed to remove excess fluid. The mycelium was then frozen with liquid nitrogen and stored at -60 °C.

Growth tests were performed at pH 6.0 as described previously (Uitzetter *et al.*, 1986). The final concentrations of the carbon sources were: glycerol 100 mM, DHA 100 mM, D-galacturonate 50 mM, D-glucose/glycerol 25 mM/100 mM, D-glucose 50 mM, D-fructose 50 mM. In other test plates using combinations of carbon sources, glycerol and DHA were used at a concentration of 100 mM, methylglyoxal at 10 mM and 25 mM, D-glucose, sucrose, D-fructose, D-galactose, lactose and D-xylose at 25 mM, acetate and L-glutamate at 100 mM.

**Isolation and genetic mapping of three new glycerol mutants.** A suspension of 10<sup>7</sup> conidiospores from strain WG096 was irradiated for 30 s with a 30 W TUV lamp (Philips) at a dose of 2 μJ mm<sup>-2</sup> s<sup>-1</sup>. Mutants were enriched by filter enrichment, essentially as described by Uitzetter *et al.* (1986). DHA (100 mM) was used as carbon source. Three independent mutants were obtained. The three mutants were tested for complementation to each other and to the *glcA1* and *glcB33* mutations in heterokaryons and in diploids. The linkage groups of the three new mutants were determined by haploidization of diploids formed with strain WG202 (Upshall *et al.*, 1979). The three new mutants were designated *glcE1*, *glcF1* and *glcG1*. The *glcG1* mutant was also tested for complementation to the *glcD100* mutation in heterokaryons and as

diploid. The possible linkage of *glcD100* and *glcG1*, and of *glcE1* and *glcF1*, was investigated by meiotic recombination analysis. Standard genetical techniques were used (Pontecorvo *et al.*, 1953; Clutterbuck, 1974).

**Preparation of cell-free extracts.** Cell-free extracts were prepared according to Arst *et al.* (1990).

**Enzyme assays and protein determination.** The activities of enzymes were measured with an Aminco DW-2 UV-VIS spectrophotometer using the double-beam mode at 340 nm versus 380 nm unless stated otherwise. Reactions were performed at 25 °C. D-GAD reductase [NAD(P)H] and DHA reductase [NAD(P)H] were assayed in 50 mM-sodium phosphate buffer pH 6.5, 0.2 mM-NAD(P)H, 5 mM-D-GAD or DHA. Glycerol dehydrogenase NAD(P)<sup>+</sup> (EC 1.1.1.6 and 1.1.1.72) was assayed in 100 mM-glycine/NaOH pH 9.6, 0.4 mM-NAD(P)<sup>+</sup>, and 100 mM-glycerol. Alcohol dehydrogenase (ADH; EC 1.1.1.1) was assayed according to Creaser *et al.* (1985). DHA kinase activity was determined in 50 mM-Tris/HCl pH 8.0, 5 mM-MgCl<sub>2</sub>, 5 mM-ATP, 0.2 mM-NADH, 10 mM-DHA and 1 unit (U) G3P dehydrogenase. D-GAD kinase was assayed in 50 mM-Tris/HCl pH 8.0, 5 mM-MgCl<sub>2</sub>, 5 mM-ATP, 0.2 mM-NADH, 10 mM D-GAD, 1 U G3P dehydrogenase and 1 U triose phosphate isomerase. NAD-dependent *sn*-G3P dehydrogenase (EC 1.1.1.8) and FAD-dependent *sn*-G3P dehydrogenase (EC 1.1.99.5) were assayed as described previously (Arst *et al.*, 1990). Glycerol oxidase was assayed according to Uwajima *et al.* (1980). Glycerol kinase (EC 2.7.1.30) was assayed in a discontinuous assay according to De Koning *et al.* (1987) and in a continuous assay in 50 mM-HEPES pH 7.0, 5 mM-MgCl<sub>2</sub>, 10 mM-glycerol, 0.1 mM-NADH, 0.2 mM-phosphoenolpyruvate, 1 U lactate dehydrogenase and 0.2 U pyruvate kinase. Glyoxalase I (lactoylglutathione reductase: EC 4.4.1.5) was assayed according to Inoue *et al.* (1987). Glutathione reductase (EC 1.6.4.2) was assayed in 50 mM-sodium phosphate buffer pH 6.5, 0.2 mM-NADPH and 2 mM-glutathione (ox.). Methylglyoxal dehydrogenase was assayed in 100 mM-glycine/NaOH pH 9.6, 0.4 mM-NAD(P)<sup>+</sup>, and 2 mM-methylglyoxal.

Protein content in mycelial extracts was determined by the microbiuret method (Itzhaki & Gill, 1964) using bovine serum albumin as a standard.

## Results

### *Enzyme activities in wild-type A. nidulans after transfer to various carbon sources*

To study the effect of different carbon sources on the level of enzymes involved in glycerol metabolism, wild-type *A. nidulans* and various mutants were grown on fructose and subsequently transferred to media with different carbon sources (see Methods). The transfer methodology enables mycelia at comparable growth stages to be compared. Ideally one would like to test, besides inducing and repressing carbon sources, also a non-repressing, non-inducing carbon source, in order to sort out induction and repression properly. In practice this cannot be realized since glycerol also arises from intermediary metabolism. Table 1 gives the data obtained with the wild-type strain WG096. In Fig. 1 a model for glycerol metabolism in *A. nidulans* is presented which is based on these activity measurements.

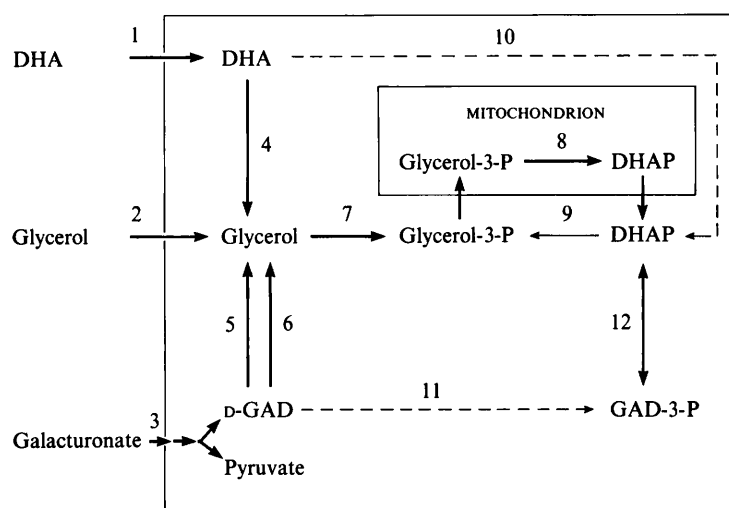


Fig. 1. Glycerol catabolism in *A. nidulans*. The following steps are indicated: 1, DHA uptake; 2, glycerol uptake; 3, D-galacturonate uptake and degradation to pyruvate and D-GAD; 4, NADP<sup>+</sup>-dependent glycerol dehydrogenase; 5, NADP<sup>+</sup>-dependent glycerol dehydrogenase; 6, ADH I; 7, glycerol kinase; 8, FAD-dependent G3P dehydrogenase; 9, NAD<sup>+</sup>-dependent G3P dehydrogenase; 10, DHA kinase; 11, D-GAD kinase; 12, triose phosphate isomerase.

Table 1. Enzyme activities in wild-type WG096 after transfer to medium containing various carbon sources

Mycelia were grown for 16 h in minimal medium with fructose and then transferred for 8 h to fresh minimal medium containing 100 mM-glycerol (Glyc), 100 mM-DHA, 50 mM D-galacturonate (GalA), 25 mM/100 mM-D-glucose/glycerol (Glc/Glyc), 50 mM-D-glucose (Glc) or 50 mM-D-fructose (Fru). The specific activities shown are the means of two determinations, agreeing within 10% in each case. Data for glycerol kinase, G3P dehydrogenase (FAD), DHA reductase (NADPH) and D-GAD reductase (NADPH) were reported previously (Arst *et al.*, 1990).

Enzyme	Activity [nmol min <sup>-1</sup> (mg protein) <sup>-1</sup> ] after transfer to:					
	Glyc	DHA	GalA	Glc/Glyc	Glc	Fru
Glycerol kinase	41	36	40	7	—	—
G3P dehydrogenase (FAD)	53	45	32	8	9	10
G3P dehydrogenase (NAD)	9	2	17	3	6	3
DHA reductase (NADPH)	212	236	166	250	248	202
D-GAD reductase (NADPH)	70	88	56	85	83	31
DHA reductase (NADH)	17	—	—	3	—	—
D-GAD reductase (NADH)	22	—	51	11	—	8
ADH	107	—	278	8	—	20
Glycerol dehydrogenase (NADP)	208	201	156	156	178	143
Glycerol dehydrogenase (NAD)	—	—	—	—	—	—
DHA kinase	—	19	15	—	—	—
D-GAD kinase	—	3	2	—	—	—
Glycerol oxidase	—	—	—	—	—	—

—, Not detectable.

Upon transfer to glycerol, DHA or D-galacturonate, glycerol kinase (step 7 in Fig. 1) and mitochondrial G3P dehydrogenase (step 8) were induced. No glycerol kinase activity could be detected upon transfer to D-glucose or when refreshing the D-fructose medium. A low activity was found after transfer to a mixture of D-glucose and glycerol, indicating that glycerol kinase is subject to glucose catabolite repression. The level of mitochondrial G3P dehydrogenase increased about five-fold on glycerol, DHA or D-galacturonate as compared to cultures

transferred to D-glucose, D-fructose or a mixture of D-glucose and glycerol. These data suggest that the flavoprotein is also subject to glucose catabolite repression. Under the culture conditions used here the catabolic pathway for glycerol is formed by glycerol kinase (step 7) and mitochondrial G3P dehydrogenase (step 8).

DHA reductase (NADPH) (step 4), D-GAD reductase (NADPH) (step 5) and glycerol dehydrogenase (NADP<sup>+</sup>) (reactions 4 and 5 in opposite direction) were



Table 3. *Glycerol kinase and mitochondrial G3P dehydrogenase (FAD) activities in wild-type and mutant strains of A. nidulans*

Mycelia were grown and transferred as described in Table 1. Data for the *glcD100* mutant and for the wild-type were reported previously (Arst *et al.*, 1990) but are included here for completeness and to permit comparisons.

Strain	Activity [nmol min <sup>-1</sup> (mg protein) <sup>-1</sup> ] after transfer to:					
	Glycerol	DHA	Galacturonate	Glucose/ glycerol	Glucose	Fructose
(a) <i>Glycerol kinase</i>						
WG096	41	36	40	7	–	–
<i>glcA</i>	–	–	–	–	–	–
<i>glcB</i>	33	40	31	2	–	–
<i>glcC</i>	10	80	48	–	–	–
<i>glcD</i>	51	70	50	–	–	–
<i>glcE</i>	35	43	38	–	–	–
<i>glcF</i>	29	32	47	3	–	–
<i>glcG</i>	64	31	50	5	–	–
<i>creA</i>	85	48	52	80	8	10
<i>alcA</i>	38	45	27	2	–	–
(b) <i>G3P dehydrogenase (FAD)</i>						
WG096	53	45	32	8	9	10
<i>glcA</i>	70	90	139	31	9	7
<i>glcB</i>	–	–	–	–	–	–
<i>glcC</i>	5	81	21	11	9	6
<i>glcD</i>	–	13	2	–	–	–
<i>glcE</i>	15	59	19	11	5	4
<i>glcF</i>	11	15	42	12	8	6
<i>glcG</i>	5	7	10	11	4	4
<i>creA</i>	18	47	23	30	11	7
<i>alcA</i>	10	19	2	3	3	2

–, Not detectable.

Table 3 presents biochemical evidence which confirms that this mutant lacks glycerol kinase activity. In the *glcB33* mutant no mitochondrial G3P dehydrogenase activity was found. The *glcC2* mutant was impaired in glycerol uptake (Visser *et al.*, 1988). The growth features of the *glcD100* mutant (Arst *et al.*, 1990) and three new glycerol mutants, *glcE1*, *glcF1* and *glcG1*, are also presented. The growth characteristics of the *glcG1* mutant were similar to those of the *glcB33* mutant. The G3P dehydrogenase (FAD) activity measured in this mutant was always very low (Table 3). The *glcB33* mutation, located on chromosome I, and the *glcG1* mutation, located on chromosome VIII, complemented each other both in a heterokaryon and in a diploid. Since all other enzyme levels in this mutant are comparable to those of the wild-type on each of the carbon sources tested (data not shown), it is probable that the *glcG1* mutant, like the *glcB33* mutant, is defective in the mitochondrial G3P dehydrogenase activity. In the *glcD100* mutant FAD-dependent G3P dehydrogenase activities are also greatly decreased (Arst *et al.*, 1990). The *glcD100* mutation was found to be located on chromosome VIII (Arst *et al.*, 1990). Complementation

both in a heterokaryon and in a diploid together with free recombination in meiosis demonstrated that *glcD100* and *glcG1* are non-allelic.

The *glcE1* and *glcF1* mutations have been allocated to chromosome VI. They complement each other, as well as the *glcA1*, *glcB33* and *glcC2* mutations, in both heterokaryons and diploids.

#### *Effects of carbon source on glycerol kinase and mitochondrial G3P dehydrogenase activities in glycerol mutants*

When the glycerol uptake mutant (*glcC2*) was transferred to glycerol no appreciable induction of glycerol kinase and G3P dehydrogenase occurred. Upon transfer to DHA or D-galacturonate, both enzymes were induced to levels comparable to those found in the wild-type WG096. Glycerol uptake or glycerol formation is therefore a likely prerequisite for induction of glycerol kinase and G3P dehydrogenase.

G3P dehydrogenase activity was elevated in the glycerol kinase mutant (*glcA1*) upon transfer of my-

celium to glycerol, DHA, D-galacturonate or D-glucose/glycerol. However, G3P levels remained low in this mutant under these conditions (D. H. A. Hondmann & J. Visser, unpublished results), indicating that in *A. nidulans* G3P is unlikely to be the direct inducer of glycerol catabolism. In *Escherichia coli* G3P has been reported to be the inducer for transcription of the genes involved in glycerol metabolism (Cozarelli *et al.*, 1968).

In both the *glcB33* and *glcG1* mutants glycerol kinase activities were comparable to wild-type levels. G3P accumulated in the *glcB33* mutant when D-fructose-grown mycelium was transferred to glycerol, DHA, D-galacturonate or even to D-glucose/glycerol (J. Visser and others unpublished results). G3P was therefore unlikely to result in feedback inhibition, or to repress transcription, of glycerol kinase.

The *glcE1* and *glcF1* mutations had no pronounced effects on glycerol kinase or G3P dehydrogenase activities. In the *glcE1* mutant G3P dehydrogenase was not induced to the same extent as in the wild-type upon transfer to glycerol or D-galacturonate. G3P dehydrogenase activity remained low in the *glcF1* mutant transferred from a glycolytic carbon source to glycerol or DHA.

The ADH I defective strain (*alcA527*) failed to induce G3P dehydrogenase when it was transferred to D-galacturonate. This can be interpreted as an additional line of evidence that ADH I is involved in D-GAD catabolism. On test plates *alcA* mutants grew as well as the wild-type on D-galacturonate. In this case a transfer of 6 h to a liquid medium was not comparable to growth on a solid medium for 2–3 d.

Catabolite repression affected both glycerol kinase and G3P dehydrogenase, as could be concluded from the enzyme levels found in a *creA<sup>d</sup>-1* mutant. Neither enzyme was repressed in this mutant on a mixed carbon source consisting of D-glucose and glycerol. Carbon catabolite repression cannot be the only regulatory mechanism, since no induction was found in the *creA<sup>d</sup>-1* mutant when transferred to D-glucose or D-fructose. It is apparent that glycerol, or a derivative, must act as an inducer.

The remaining enzyme levels measured in this study indicated no gross differences between the wild-type and the various mutants, with the exception that DHA kinase and D-GAD kinase activities were found to be induced to levels about fivefold higher on DHA and on D-galacturonate in the case of the glycerol kinase mutant (*glcA1*) as compared to the wild-type.

#### *Further characterization of glcE1 and glcF1*

Since the *glcE1* and *glcF1* mutants demonstrated no obvious enzyme lesions, we investigated the possibility that the poor growth of these two mutants on DHA and glycerol could be a result of a toxic effect. In *Saccharo-*

*myces cerevisiae* and *E. coli* mutants have been described that produce methylglyoxal during glycerol catabolism and are therefore subject to glycerol toxicity (Cooper & Anderson, 1970; Penninckx *et al.*, 1983). The non-enzymic formation of methylglyoxal from glyceraldehyde under physiological conditions has also been reported (Riddle & Lorenz, 1968). Several enzymes that were reported to be involved in methylglyoxal metabolism of various micro-organisms (Cooper, 1984; Thornally, 1990) were assayed in wild-type *A. nidulans* and in mycelium of the *glcE1* and *glcF1* mutants which was transferred to DHA or to glycerol. All enzymes tested showed comparable activities in all three strains or were undetectable. Glyoxalase I activities of 15–30 mU (mg protein)<sup>-1</sup> were measured; NADPH-dependent methylglyoxal dehydrogenase activities were in the range 30–50 mU (mg protein)<sup>-1</sup> in all strains. No NADH-dependent methylglyoxal dehydrogenase was detected. Glutathione reductase activities were in the range 20–40 mU (mg protein)<sup>-1</sup>.

On solid media containing D-glucose, sucrose, D-fructose, acetate, D-galactose lactose, L-glutamate or D-xylose, to which DHA, glycerol or methylglyoxal were added, no growth differences between the wild-type and *glcE1* or *glcF1* were observed, suggesting that the growth defects of *glcE1* and *glcF1* mutants are not due to toxicity.

## Discussion

In *Aspergillus nidulans* both biochemical and genetical evidence indicates that the main route for glycerol, DHA and D-GAD catabolism involves glycerol kinase and mitochondrial G3P dehydrogenase (FAD), and therefore depends on respiration. At least two regulatory mechanisms are important for glycerol catabolism. Firstly there is a pathway-specific induction as glycerol kinase and the FAD-dependent G3P dehydrogenase are induced by glycerol (and by DHA and D-galacturonate), whereas other enzymes that could have formed an alternative route were apparently uninduced. Secondly glucose catabolite repression affects the induction of glycerol kinase and FAD-dependent G3P dehydrogenase. This repression was not absolute as low levels of both enzymes are found using mixed substrates, e.g. glucose/glycerol. In the *creA<sup>d</sup>-1* mutant transferred to D-glucose or D-fructose, uninduced levels of glycerol kinase and G3P dehydrogenase activities are found, whereas pathway-specific induction is prominent on a mixture of D-glucose and glycerol. The two mechanisms make it possible for *A. nidulans* to control the amount of glycerol present intracellularly in a flexible way. If glucose repression were too tight this would interfere with the

need to catabolize glycerol in the presence of repressing carbon sources, e.g. under conditions where the osmotic value of the environment drops.

Upon uptake DHA will be largely converted to glycerol by the NADPH-dependent DHA reductase. The properties of the purified enzyme showed that glycerol formation is favoured under physiological conditions (Schuurink *et al.*, 1990). *In vivo* natural abundance  $^{13}\text{C}$  NMR studies show that *A. nidulans* wild-type and glycerol non-utilizing mutants grown on, or transferred to, DHA accumulate glycerol under aerobic conditions. DHA kinase was detected at low levels in the wild-type but was elevated fivefold in the glycerol kinase mutant. An *A. niger* glycerol kinase mutant could grow on DHA (Witteveen *et al.*, 1990), whereas the equivalent *A. nidulans* mutant could not. This is a major difference in glycerol catabolism between these two fungi. In *A. niger* DHA kinase formed an effective bypass which apparently did not function, under the conditions used here, in *A. nidulans*. We believe that the balance between reduction and phosphorylation of DHA is very much in the direction of reduction in *A. nidulans*. DHA kinase and D-GAD activities could be attributed to a single enzyme in *A. niger* (Witteveen *et al.*, 1990). In *A. nidulans* both activities are also likely to be due to a single enzyme. Both activities appear simultaneously on DHA and on D-galacturonate and in the same ratio. DHA kinase may nevertheless be of physiological importance in *A. nidulans* for growth on DHA or D-galacturonate, when the anabolic reduction charge is low or when oxygen levels are low. This is supported by the observation that the *glcA1* mutant does grow under microaerophilic conditions.

D-Galacturonate was reported to be degraded to pyruvate and a triose, probably D-GAD (Uitzetter, 1982). The induction of D-GAD reductase (NADH) upon transfer to D-galacturonate confirmed this prediction. D-GAD formed from D-galacturonate will be almost completely converted to glycerol by ADH I or by NADPH-dependent D-GAD reductase activity catalysed by the well-characterized DHA reductase. In *Schizosaccharomyces pombe* NADH-dependent D-GAD reductase and ADH I were also activities of the same enzyme (Kong *et al.*, 1985). The induction in *A. nidulans* of ADH I upon growth on D-galacturonate indicates an unexpected physiological role for this enzyme. When grown on cell walls of higher plants, *A. nidulans* excretes pectic enzymes, amongst others. One of the major endproducts formed from pectin is D-galacturonate (Pilnik & Rombouts, 1981). Since both D-GAD reductases are found at comparable levels on D-galacturonate, and because both enzymes have comparable  $K_m$  values for D-GAD (Creaser *et al.*, 1985; Schuurink *et al.*, 1990), the balance of fluxes through the two enzymes will be

mainly controlled by the anabolic and catabolic reduction charge. The second major difference with *A. nidulans* is that in *A. niger* an NADPH-dependent D-GAD reductase is induced on D-galacturonate instead of ADH I (Witteveen *et al.*, 1990).

Schuurink *et al.* (1990) proposed the existence of a 'glycerol cycle', composed of DHAP phosphatase, NADPH-dependent DHA reductase, glycerol kinase and FAD-dependent G3P dehydrogenase. An operating glycerol cycle would regenerate  $\text{NADP}^+$  and generate ATP under conditions when excess NADPH is formed. Under the growth conditions used here the glycerol cycle does not seem to be used, since no glycerol kinase activity can be found in mycelium transferred to D-glucose or D-fructose.

The cytosolic  $\text{NAD}^+$ -dependent G3P dehydrogenase is present at basal levels in all mycelia. This enzyme seems to be able to maintain appropriate G3P levels necessary for lipid biosynthesis. This may explain why glycerol non-utilizing mutants do not show an aberrant colonial morphology.

Three independent glycerol non-utilizing mutants, *glcB33*, *glcD100* and *glcG1*, were found to be affected in the mitochondrial G3P dehydrogenase activity. In *E. coli* the FAD-dependent G3P dehydrogenase is composed of three different subunits, a catalytic subunit, a subunit that shuttles reduction equivalents to the electron chain, and an anchor subunit (Cole *et al.*, 1988). If in *A. nidulans* this enzyme has a similar composition, as suggested by the three complementation groups found, the *glcB33* mutation is most likely to affect the catalytic subunit, since no activity can be detected in this mutant. The *glcD100* mutant can grow on mixed carbon sources like D-glucose/glycerol, whereas the *glcG1* mutant cannot. Therefore, inhibition of growth due to G3P accumulation does not seem to occur in the *glcD100* mutant. This can be explained by assuming that the *glcD100* mutation affects the anchor subunit, leading to a partially active enzyme that is not properly bound to the inner mitochondrial membrane, and therefore only found in low activities in the mitochondrial cell-free extract. The *glcG1* mutation will, in this model, disrupt the subunit that shuttles reduction equivalents from the catalytic subunit to the respiratory chain.

On D-glucose/D-galacturonate test plates *glcB33* and *glcG1* strains grow like the wild-type, whereas they do not grow on D-glucose/glycerol or D-glucose/DHA. Glucose catabolite repression is much more restrictive for one or more steps in the uptake and degradation of D-galacturonate to D-GAD and pyruvate than it is for the uptake of DHA and glycerol and for glycerol kinase induction.

The true nature of the *glcE1* and *glcF1* mutations remains unresolved. Involvement of a DHA uptake

system seems unlikely since all enzymes involved in glycerol metabolism were found at normal or slightly diminished levels after transfer to DHA or glycerol. It is also unlikely that these mutants have an impaired detoxification system.

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