Purification and properties of NADP⁺-dependent glycerol dehydrogenases from *Aspergillus nidulans* and *A. niger*

R. SCHUURINK,† R. BUSINK, D. H. A. HONDMANN, C. F. B. WITTEVEEN and J. VISSER*

Department of Genetics, Agricultural University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands

(Received 14 November 1989; revised 2 February 1990; accepted 9 February 1990)

Glycerol dehydrogenase, NADP⁺-specific (EC 1.1.1.72), was purified from mycelium of *Aspergillus nidulans* and *Aspergillus niger* using different purification procedures. Both enzymes had an M_r of approximately 38000 and were immunologically cross-reactive, but had different amino acid compositions and isoelectric points. For both enzymes, the substrate specificity was limited to glycerol and erythritol for the oxidative reaction and to dihydroxyacetone (DHA), diacetyl, methylglyoxal, erythrose and D-glyceraldehyde for the reductive reaction. The *A. nidulans* enzyme had a turnover number twice that of the *A. niger* enzyme at pH 6.0, whereas inhibition by NADP⁺ was less ($K_i = 45 \mu\text{M}$ vs $13 \mu\text{M}$). It is proposed that both enzymes catalyse *in vivo* the reduction of DHA to glycerol and that they are regulated by the anabolic reduction charge.

Introduction

In *Aspergillus nidulans*, three classes of mutations involved in glycerol metabolism have been assigned functions recently (Visser *et al.*, 1988; J. Visser and others, unpublished results): these are glycerol uptake, glycerol kinase and glycerol-3-phosphate dehydrogenase (mitochondrial membrane-bound flavoprotein). Strains carrying the latter two mutations did not grow on glycerol or DHA and accumulated glycerol and glycerol 3-phosphate respectively, within 2 h of transfer from glucose to DHA, as indicated by ¹³C NMR spectroscopy. The glycerol uptake mutant accumulated glycerol upon transfer to DHA while it depleted its other polyol pools completely. DHA was never observed in the mycelium and it is proposed that this toxic compound is rapidly converted into glycerol. Under these conditions the other polyol pools seem to accommodate the cellular requirement for NADPH by increased catabolism. We therefore became interested in the properties of the enzyme catalysing the conversion of DHA into glycerol.

The amount of glycerol formed in wild-type *A. nidulans*

under different growth conditions has been studied previously by *in vivo* NMR spectroscopy (Dijkema *et al.*, 1985, 1986). Glycerol formation was stimulated by nitrate, high oxygen levels and by lowering the growth temperature. These conditions all lead to activation of the pentose phosphate pathway and therefore increased generation of NADPH.

Glycerol dehydrogenase has been found in different filamentous fungi (Jennings, 1984). The enzyme has been purified from *Mucor javanicus* (Dutler *et al.*, 1977; Hochuli *et al.*, 1977), *Neurospora crassa* (Viswanath-Reddy *et al.*, 1978) and a *Rhodotorula* sp. (Watson *et al.*, 1969). It has also been partially purified from *A. niger* (Baliga *et al.*, 1962). The present paper however, describes the first complete purification of glycerol dehydrogenase of *Aspergillus*.

Studies in our laboratory indicated that glycerol metabolism in *A. niger* differs somewhat from that in *A. nidulans*. A glycerol kinase mutant of *A. niger*, unlike a similar *A. nidulans* mutant, was found to grow on DHA (C. F. B. Witteveen and others, unpublished results). Röhr *et al.* (1983) and Legisa & Matthey (1986) observed the appearance of glycerol in the growth medium of *A. niger* in the initial stages of citric acid fermentation. Although the enzyme was not purified, its kinetics was studied to some extent and activation by bicarbonate was found in the non-physiologically important reaction (Inayat & Matthey, 1988). In order to assess the role of glycerol in *A. niger*, we also purified and characterized the glycerol dehydrogenase of this fungus.

† Present address: Centre of Phytotechnology RUL-TNO, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands.

Abbreviations: BCA, bicinechonic acid; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PMS, phenazine methosulphate.

Methods

Materials. DEAE-Sepharose Fast Flow, FPLC equipment and the Mono P column were from Pharmacia. Ampholytes were from Serva; the TSK 3000 gel permeation column was from LKB. Procion Red HE3B agarose (Matrex Red A) was obtained from Amicon. NAD⁺, NADH, NADP⁺ and NADPH were from Boehringer and DHA was from Serva. All other chemicals were from Merck and were of analytical grade.

Strains and culture conditions. *Aspergillus nidulans* WG096 (*yA2 paba1*) and *A. niger* N400 (CBS 120-49) were used to obtain fungal biomass for enzyme purification purposes. The strains were maintained on agar plates using complete medium with 25 mM-sucrose as the carbon source (Pontecorvo *et al.*, 1953). They were batch cultured at room temperature for 40 h under vigorous aeration in liquid minimal medium with sucrose as the carbon source and NO₃⁻ as the nitrogen source (Dijkema *et al.*, 1986). The mycelium was harvested by filtration, washed with cold saline (0.85% NaCl), squeezed to remove excess liquid and frozen in liquid nitrogen to be stored at -60 °C until further use.

Preparation of cell-free extract. Frozen mycelium (50–100 g) was ground in a stainless steel Waring blender with liquid nitrogen for 8 min. After evaporation of the nitrogen, 3 ml of extraction buffer (20 mM-potassium phosphate, pH 6.0, 1 mM- β -mercaptoethanol, 1 mM-MgCl₂, 0.1 mM-EDTA) was added per gram of mycelium. This mixture was stirred for 1 h at 4 °C before centrifugation for 30 min at 20000 *g*. In the case of *A. niger*, the extraction buffer contained 5 mM-EDTA instead of 0.1 mM.

Enzyme assays and protein determination. Glycerol dehydrogenase activity was determined by measuring the formation of NADPH at 340 nm. The enzyme assay consisted of 50 mM-glycine/NaOH, pH 9.6, 50 mM-glycerol (*A. nidulans*) or 100 mM-glycerol (*A. niger*) and 0.2 mM-NADP⁺. A unit of enzyme activity (U) is defined as the amount of enzyme required to form 1 μ mol NADP⁺ min⁻¹. In some experiments the formation of NADPH was coupled to PMS (250 μ M) and MTT (335 μ M). This reaction was monitored at 578 nm using a value of 15 mM⁻¹ cm⁻¹ for the millimolar absorption coefficient. The reduction of DHA was determined by measuring the disappearance of NADPH at 340 nm. The enzyme assay mixture contained 50 mM-potassium phosphate, 5 mM-DHA and 0.2 mM-NADPH.

The kinetic experiments were done at 25 °C and measurements made using an Aminco double beam DW-2UV/VIS spectrophotometer. Protein in crude cell-free extracts was determined by the micro-biuret method (Itzhaki & Gill, 1964; Jerneck *et al.*, 1986). In the various steps of the purification procedures the protein was determined by the BCA method according to the instructions of the supplier (Pierce). For both methods, BSA was used as a standard.

Electrophoresis and isoelectric focusing. Electrophoresis in 10% (w/v) polyacrylamide gels containing 0.1% SDS was done according to Laemmli (1970). The *M_r* of glycerol dehydrogenase was determined by comparison with the Serva marker kit protein standards; carbonic anhydrase (*M_r* 29000), ovalbumin (45000), BSA (67000) and phosphorylase B (92500). Analytical thin-layer gel 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.

Antibodies and Western blotting. Antibodies against the purified glycerol dehydrogenase of *A. nidulans* were raised in a male New Zealand White rabbit. The antiserum obtained was stored at -60 °C. SDS-polyacrylamide gels were blotted on nitrocellulose with the LKB-Novablot apparatus under the conditions recommended in the manual. The blots were developed by the procedure described by Uitzetter

(1982). Staining was done in 100 ml 50 mM-Tris/HCl, pH 7.5, 30 μ l 30% (w/v) H₂O₂ and 40 mg 4-chloro-1-naphthol.

Amino acid analysis. This was done according to the method described by Bidlingmeyer *et al.* (1984), using a Supelcosyl LC18 DB column on a Spectra-Physics SP 8000 HPLC. The proteins were gas-phase hydrolysed with 6 M-HCl for 24 and 96 h at 110 °C. The results appeared to correspond well to those obtained with the LKB Amino Acid Analyzer α -plus 4101, using a sulphonated resin column. The presence of ornithine was double-checked with the methane sulphonic acid method and the performic acid method.

Enzyme purification

(a) *Aspergillus nidulans* glycerol dehydrogenase. Solid ammonium sulphate was added to the crude extract to 65% saturation at 4 °C. The precipitate was removed by centrifugation at 20000 *g* for 30 min and an additional amount of ammonium sulphate was added to 95% saturation. The precipitate, collected by an identical centrifugation step, was dissolved in a minimal amount of extraction buffer and dialysed extensively. The dialysed protein solution was applied to a DEAE-Sepharose Fast Flow column equilibrated with extraction buffer. The adsorbed proteins were eluted with a linear gradient of NaCl in the same buffer (0–0.5 M). The active fractions were pooled and dialysed extensively against the Mono P buffer used to equilibrate the Mono-P column (25 mM-bis-Tris/HCl, pH 6.3, 1 mM- β -mercaptoethanol, 1 mM-MgCl₂, 1 mM-EDTA). The dialysed active enzyme pool was applied at room temperature to a chromatofocusing column (Mono P) on the FPLC system. The adsorbed proteins were eluted through a pH gradient (pH 6.0–4.0), using Pharmacia Polybuffer 74 (pH 4.0). The active fraction eluted at pH 5.1 and the ampholytes were removed on a TSK 3000 gel-permeation column in Mono P buffer containing 50 mM-NaCl. The native *M_r* of the protein was determined by comparison with the following standards: chymotrypsin (*M_r* 25000), egg albumin (45000) and BSA (67000).

(b) *Aspergillus niger* glycerol dehydrogenase. Crude extract was applied directly to a Red-A column equilibrated with extraction buffer. The ratio between the crude extract applied and the amount of column material used was critical to obtain the correct adsorption and elution conditions. Column loading was continued up to the point where glycerol dehydrogenase did not bind further. The column was washed with extraction buffer before applying a linear NADP⁺ gradient (0–3 mM) in the same buffer. The active fractions were pooled, dialysed extensively against 20 mM-Tris/HCl, pH 7.5, containing 1 mM- β -mercaptoethanol, 1 mM-MgCl₂ and 0.1 mM-EDTA. The enzyme solution was then applied to a DEAE-Sepharose Fast Flow column (60 ml) equilibrated with the same buffer. The adsorbed proteins were eluted with a linear NaCl gradient (0–0.2 M); the enzyme eluted at about 50 mM-NaCl. The NADP⁺ still present was removed by extensive dialysis against extraction buffer containing 100 mM-NaCl followed by another dialysis step with extraction buffer to remove the NaCl.

Enzyme storage. Both enzymes were usually stored at -20 °C in 20 mM-potassium phosphate buffer, pH 6.0, 5 mM-EDTA, 1 mM-MgCl₂, 1 mM- β -mercaptoethanol. They remained active at 4 °C for at least 1 month.

Results

Glycerol dehydrogenase activity under different growth conditions

In mycelial extracts a NADP⁺-dependent glycerol dehydrogenase activity was readily detected spectrophotometrically. The enzyme was constitutive both in *A.*

Table 1. Purification of glycerol dehydrogenase from *A. nidulans* and *A. niger*

	Vol. (ml)	Total protein (mg)	Total activity (U)	Specific activity* [U (mg protein) ⁻¹]	Yield (%)
<i>A. nidulans</i>					
Crude extract	500	1500	75	0.05	100
(NH ₄) ₂ SO ₄ precipitation	50	34	52	0.76	69
DEAE chromatography	30	8.6	40	4.70	53
Mono-P chromatography	5.5	3.2	36	24.1	48
<i>A. niger</i>					
Crude extract	175	814	95	0.116	100
Red A chromatography	55	22	71	3.25	75
DEAE chromatography	20	2.0	65	32.5	68

* In the glycerol dehydrogenase assay 50 mM-glycerol was used in the case of *A. nidulans* and 100 mM in the case of *A. niger*. Due to the low affinity for glycerol these values are quite distinct from V (maximal velocity) values.

nidulans and in *A. niger* and was present at a level of 0.05–0.1 U (mg protein)⁻¹. Activities in crude cell-free extracts of *A. nidulans* grown on different carbon sources (glucose, DHA, glycerol) with urea or nitrate as the nitrogen source were all in this range [0.05–0.08 U (mg protein)⁻¹]. The specific activity in *A. niger* extracts was approximately twice that observed in *A. nidulans* (Table 1).

Enzyme purification

In Table 1 the results of the *A. nidulans* and *A. niger* glycerol dehydrogenase purifications are summarized. Purifications of 480-fold and 280-fold were obtained with recoveries of approximately 50% and 70% respectively. In the case of the *A. nidulans* enzyme, the ammonium sulphate fractionation (0–65%) effectively removed the bulk of the protein. The enzyme was found to be pure after subsequent anion exchange chromatography and a chromatofocusing step, giving a single band on SDS-PAGE (Fig. 1). After raising the pH from 5.1 to 6.0, the ampholytes were removed by gel-permeation chromatography. The pure enzyme was used to raise polyclonal antibodies which also cross-reacted with the *A. niger* enzyme (Fig. 1b).

The purification procedure used for *A. nidulans* glycerol dehydrogenase could not be used for the *A. niger* enzyme. Ammonium sulphate precipitation still resulted in a high recovery (80%), but, the enzyme did not bind to DEAE-Sepharose at pH 6.0, using the *A. nidulans* extraction buffer, although the pI of the *A. niger* enzyme is only slightly higher than that of the *A. nidulans* enzyme. Only at pH 7.5, when already in an almost purified state, did the enzyme show weak binding. In the crude extract however, other proteins seem to displace the glycerol dehydrogenase from the column.

Several other approaches were considered. Hydrophobic interaction chromatography using butyl- and phenyl-Sepharose was unsuccessful. Cation exchange chromatography was also not feasible as it caused enzyme inactivation, although the enzyme did bind to CM-Sepharose in acetate buffer at pH 4.7. Western blotting showed that in crude extracts both enzymes were sensitive to proteolytic degradation (Fig. 1b). Addition of 5 mM-EDTA prevented this but at the concentration required EDTA addition could not be combined with an anion exchange chromatography step. Finally, a number of different affinity matrices based on reactive dyes were tested. In crude extracts the *A. niger* enzyme did bind in the presence of 5 mM-EDTA to Procion Red HE3B-agarose (Red A) from which it could be specifically eluted by NADP⁺. However, the crude extract had to be loaded until the point where glycerol dehydrogenase activity appeared in the eluate (approximately 8 ml of crude extract per ml of gel). Otherwise, the enzyme bound so strongly that it could only be removed under less specific elution conditions using high salt concentrations.

Enzyme activity was eluted from the Red A column at the higher concentrations of the NADP⁺ gradient (0–3 mM). After dialysis to lower the EDTA concentration, the enzyme could be applied to a DEAE-Sepharose column. The enzyme preparation obtained in this step still contained NADP⁺ but the cofactor could be dissociated from the enzyme by increasing the NaCl concentration and was then removed by dialysis.

Physico-chemical characterization

The *A. nidulans* enzyme had an isoelectric point of 5.1 whereas the *A. niger* enzyme had a pI value of 5.3. The M_r

Fig. 1 (a). SDS-PAGE of glycerol dehydrogenase from *A. nidulans* at different stages of purification. Lane 1, crude extract; 2, active fraction after DEAE-Sephrose Fast Flow chromatography; 3, active fraction after Mono-P chromatography; (b) Western blot incubated with antiserum raised against glycerol dehydrogenase of *A. nidulans*. Lane 1, 30 µg of *A. nidulans* cell-free extract; 2, 1 µg of purified glycerol dehydrogenase of *A. nidulans*; 3, 30 µg of *A. niger* cell-free extract; 4, 2 µg of purified glycerol dehydrogenase of *A. niger*.

of both enzymes was 38000 as determined by SDS-PAGE and by gel-permeation chromatography. Therefore the active enzyme is a monomer. The amino acid composition of both enzymes is shown in Table 2. As the number of basic amino acid residues in the *A. nidulans* enzyme exceeds the number in the *A. niger* enzyme, whereas the pI value is lower, the *A. nidulans* enzyme must contain more acidic residues to account for its lower pI.

A remarkable finding was the presence in the *A. nidulans* glycerol dehydrogenase of ornithine residues.

Table 2. Amino acid composition of the glycerol dehydrogenases from *A. nidulans* and *A. niger*

Amino acid	No. of residues*	
	<i>A. nidulans</i>	<i>A. niger</i>
Asx†	35	38
Thr	16	17
Ser	19	14
Glx†	49	52
Pro	21	24
Gly	24	35
Ala	24	28
Val	24	26
Met	2	2
Ile	19	18
Leu	29	28
Tyr	12	10
Phe	13	12
His	8	7
Lys	31	26
Arg	15	11
Cys	1	1
Trp	0	0
Orn	1-5	1

* Based on an M_r of 38000.

† Sum of acid and amide forms.

The number of residues found varied with the enzyme batch prepared from between one to five residues per mole. In the purified *A. niger* enzyme one ornithine residue was found. The presence of ornithine was confirmed independently by the methanesulphonic acid and performic acid hydrolysis methods. The modification must be due to the action of an arginase but we did not investigate whether this occurred *in vivo* or during the purification procedure itself. Conversion of arginine to ornithine is known to occur in some structural proteins but has not to our knowledge been reported for an enzyme.

Enzyme stability and pH activity profile

The influence of pH on the activity of both enzymes was studied over the range 4.2-10.6 in different buffers with the same ionic strength ($I=0.056$). For the pH range 4.2-7.0 10 mM-citrate buffer was used, for the pH range 6.8-8.6 25 mM-triethanolamine buffer and for pH 8.0-10.6 50 mM-glycine/NaOH buffer. The ionic strength in all cases was equalized with NaCl, which does not affect the activity of either enzyme. The pH optima for DHA reduction were 4.8 and 5.4 for the *A. nidulans* and *A. niger* enzymes respectively, and for glycerol oxidation pH 10.0 for both enzymes.

Table 3. Substrate specificity of the glycerol dehydrogenases from *A. nidulans* and *A. niger*

Substrate	Relative activity (%)	
	<i>A. nidulans</i>	<i>A. niger</i>
(a) Reductive activity*		
Dihydroxyacetone	100	100
Erythrose	4	4
Acetone	0	0
Acetol	0	0
Diacetyl	72	62
Acetylacetone	0	0
Glyoxal	0	0
Methylglyoxal	18	18
Acetaldehyde	0	0
Acetoin	0	0
D-Glyceraldehyde	10	9
DL-Glyceraldehyde	0	0
Pyruvate	0	0
(b) Oxidative activity†		
Glycerol	100	100
Erythritol	17	17
DL-Threitol	0	0
Xylitol	0	0
Ethylene glycol	0	0
Arabitol	0	0
Mannitol	0	0
Galactitol	0	0

* Assay conditions: 0.5 mM-substrate, 0.2 mM-NADPH, 10 mM-citrate buffer, pH 6.0; $I = 0.056$.

† Assay conditions: 50 mM-substrate, 0.2 mM-NADP⁺, 50 mM-glycine/NaOH buffer, pH 10.0; $I = 0.056$.

The *A. nidulans* enzyme was partially inactivated by freezing (−20 °C) and thawing whereas the *A. niger* enzyme remained stable.

Substrate specificity

The purified glycerol dehydrogenases were NADP⁺ dependent; no activity was observed with NAD⁺ as electron acceptor. Both enzymes had the same substrate specificity (Table 3). It is interesting that besides glycerol, erythritol was also a substrate. This C₄-polyol is also observed in natural abundance ¹³C NMR spectra under the same growth conditions that lead to glycerol accumulation (Dijkema *et al.*, 1985, 1986). For the reduction reaction, D-glyceraldehyde gave only 10% of the activity observed for DHA. L-Glyceraldehyde seemed to inhibit the reaction since no activity was observed when DL-glyceraldehyde was used. The active sites of both enzymes seem to be able to accommodate C₃ and C₄ compounds which contain adjacent carbonyl functions (diacetyl and methylglyoxal) or one carbonyl

Table 4. Kinetic parameters of the glycerol dehydrogenases of *A. nidulans* and *A. niger*

Parameter	<i>A. nidulans</i>		<i>A. niger</i>	
	pH 4.8	pH 6.0	pH 5.4	pH 6.0
K_m (NADPH)	50 μ M	19 μ M	30 μ M	17 μ M
K_m (DHA)	0.45 mM	0.29 mM	0.41 mM	0.39 mM
v^*	200	150	100	80
$K_i = K_{ii}$ (NADP ⁺)†	ND	45 μ M	ND	13 μ M
Parameter	<i>A. nidulans</i>		<i>A. niger</i>	
	pH 10.0		pH 10.0	
K_m (NADP ⁺)	15 μ M		17 μ M	
K_m (glycerol)	1 M		0.7 M	
v^*	400		120	

ND, Not determined.

* μ mol min^{−1} (mg protein)^{−1}.

† $K_i = [E][I]/[EI]$; $K_{ii} = [EDHA][I]/[EDHA][I]$.

and two adjacent hydroxyl groups like DHA or also at one side of the molecule like D-glyceraldehyde and erythrose.

Kinetic parameters

Initial velocities were measured for NADPH oxidation and DHA reduction at the pH optimum for each particular enzyme and also at pH 6.0 for both enzymes. Glycerol oxidation was measured at pH 10.0, the pH optimum of this reaction for both enzymes. The sequential nature of the kinetic mechanism is shown by the intersecting pattern of lines in Fig. 2 (a, b). Both enzymes have a random bi-bi mechanism, since the intersection of lines in the forward direction is on the abscissa, whereas in the reverse direction it is below the abscissa (Fromm, 1975). The kinetic constants differed significantly with respect to NADP⁺ inhibition (Table 4): the K_i for the *A. niger* enzyme was smaller than the K_m for NADPH but for the *A. nidulans* enzyme it was larger (Fig. 2 c, d). Apparently, the *A. niger* enzyme is more sensitive to NADP⁺ inhibition. The secondary plot derived from Fig. 2(c) which can be obtained by plotting intercepts and slopes against the reciprocal NADP⁺ concentration, revealed that the $K_{ii} = K_i$. Thus NADP⁺ does not discriminate between free enzyme or enzyme that has already bound DHA. The turnover of the *A. nidulans* enzyme is approximately twice that of the *A. niger* enzyme. This is also true for the reverse reaction where the difference is even larger.

Fig. 2. Lineweaver-Burk plots of *A. nidulans* and *A. niger* glycerol dehydrogenases. (a) Determination of the K_m values for NADPH of glycerol dehydrogenase of *A. nidulans* at pH 4.8 at different DHA concentrations. (b) Determination of the K_m for NADP⁺ of glycerol dehydrogenase of *A. nidulans* at pH 10.0 at different glycerol concentrations. (c) Inhibition by NADP⁺ of DHA reduction by glycerol dehydrogenase of *A. niger* at pH 6.0; effect of varying the DHA concentration. (d) Inhibition by NADP⁺ of DHA reduction by glycerol dehydrogenase of *A. niger* at pH 6.0; effect of varying the NADPH concentration.

Both Michaelis-Menten constants for glycerol were extremely high; the constants were measured with glycerol concentrations below the K_m and extrapolated in a secondary plot. There was hardly any product inhibition by glycerol: the rate of DHA reduction by NADPH remained almost the same in the presence of 500 mM-glycerol. There was no activation by NaHCO₃ of the *A. niger* enzyme, in contrast to the results reported by Legisa & Matthey (1986). The effects of glycerol 3-phosphate and DHAP on the activities of both enzymes were negligible.

Discussion

The accumulation by aspergilli of large amounts of different polyols including glycerol (Dijkema *et al.*, 1985, 1986) emphasizes the importance of these compounds in

fungal physiology. Moreover, in citric acid fermentation glycerol is initially excreted and accumulates in the medium (Legisa & Matthey, 1986). Beever & Laracy (1986), analysing the xerotolerant behaviour of *A. nidulans* concluded that glycerol and erythritol were the major osmoregulatory solutes of *A. nidulans*. Under our experimental conditions no osmotic stress is imposed, yet the intracellular glycerol pool changes rapidly in comparison to other polyol pools in response to changes in physiological conditions such as aeration. Here the role of glycerol may be in maintaining a proper anabolic reduction charge. We therefore investigated the properties of various enzymes involved in glycerol metabolism in *Aspergillus*, and in this particular study we purified and characterized NADP⁺-dependent glycerol dehydrogenase.

The *A. nidulans* and *A. niger* glycerol dehydrogenases both have an M_r of 38000, are active as monomers and

are immunologically cross-reactive. The M_r is by far the lowest found amongst the glycerol dehydrogenases studied. In other fungi, the native enzyme is either a dimer or a tetramer (Sheys & Doughty, 1971; Dutler *et al.*, 1977; Viswanath-Reddy *et al.*, 1978).

The substrate specificities of the glycerol dehydrogenases of *Rhodotorula* (Watson *et al.*, 1969) and *Neurospora crassa* (Viswanath-Reddy *et al.*, 1978) for the NADPH oxidation were 100% for D-glyceraldehyde as compared to 5% and 43%, respectively, for DHA. In contrast, the enzyme from *Mucor javanicus* (Dutler *et al.*, 1977; Hochuli *et al.*, 1977) had DHA as the preferred substrate and only 0.3% of activity was found with D-glyceraldehyde. The *Aspergillus* enzymes also have a preference for DHA but differ in that the activity with D-glyceraldehyde is inhibited by L-glyceraldehyde (Table 3). The K_m for DHA of the *Mucor* enzyme is 8 mM, whereas the K_m for both *Aspergillus* enzymes is below 0.5 mM. The concentration of DHA found in *Mucor* mycelium, grown on glucose, was 0.5 mM. This is much higher than the level in mycelium of *Aspergillus*.

Inoue *et al.* (1987) reported the purification and characterization of glyoxalase from *A. niger*. This enzyme catalyses the conversion of the toxic compound methylglyoxal into S-lactoylglutathione in the presence of glutathione. It must be noted that the *Aspergillus* glycerol dehydrogenases are also able to inactivate methylglyoxal.

The kinetics of the glycerol dehydrogenases of *A. nidulans* and *A. niger* are very similar. Both enzymes have a random bi-bi mechanism although they differ with respect to turnover and inhibition by NADP⁺. The K_i (and K_{ii}) for the *A. niger* enzyme is approximately 13 μ M whereas that for the *A. nidulans* enzyme is 45 μ M.

Glycerol dehydrogenase must be a physiologically relevant enzyme as it is constitutively present in high concentrations under a variety of growth conditions. However, the reaction product of the enzyme is found only in high concentrations when *A. nidulans* is strongly aerated and grown at moderate pH values (Dijkema *et*

al., 1986). These conditions also stimulate the pentose phosphate pathway, thus generating NADPH. Under such conditions a high anabolic reduction charge will occur (Führer *et al.*, 1980), which evidently favours the flux from DHA to glycerol, as can be clearly understood from the kinetic parameters. The K_i value of NADP⁺, however, is such that an increase in NADP⁺ will efficiently inhibit this reaction; the enzyme is therefore likely to be part of a scheme to control the anabolic reduction charge. Another important control point which will be required to realize this has to occur at the step preceding the reduction of DHA. This concerns the dephosphorylation of DHAP, since this represents an essentially irreversible step. The glycerol thus formed can be recycled to DHAP by glycerol kinase and the mitochondrially located glycerol-3-phosphate dehydrogenase. Both enzymes are present (J. Visser and others, unpublished results). We therefore propose the existence of a glycerol cycle (shown below) that regenerates NADP⁺ when NADPH is generated in excess. At the same time a net synthesis of ATP occurs due to the involvement of the mitochondrial flavoprotein.

Kiser & Niehaus (1981) purified and characterized a mannitol-1-phosphate dehydrogenase (EC 1.1.1.17) from *A. niger*. This enzyme is NAD⁺-dependent and is not influenced by NADP(H). A mannitol cycle has been proposed that involves subsequent action of mannitol-1-phosphate phosphatase and mannitol dehydrogenase which would then lead to NADPH formation (Hult *et al.*, 1980). At the expense of ATP and NADH mannitol 1-phosphate can be regenerated. Although this cycle is also supposed to be regulated by the anabolic reduction charge (Niehaus & Dilts, 1982), this may be relevant only under extreme conditions where the pentose phosphate pathway does not generate sufficient NADPH and yet sufficient ATP and NADH are synthesized. The glycerol cycle, however, would balance the anabolic reduction charge under physiologically different conditions that result in an active pentose phosphate pathway.

This study is part of the Agricultural University Biotechnology Programme on Biocatalysts. We thank Mr Rutger van Rooijen for his contribution to this work, and Drs van Rooijen and Visser (NIZO-Ede) who performed some amino acid determinations.

References

- BALIGA, B. S., BHATNAGAR, G. M. & JAGANNATHAN, V. (1962). Triphosphopyridine nucleotide-specific glycerol dehydrogenase from *Aspergillus niger*. *Biochimica et Biophysica Acta* **58**, 384–385.
- BEEVER, R. E. & LARACY, E. P. (1986). Osmotic adjustment in the filamentous fungus *Aspergillus nidulans*. *Journal of Bacteriology* **168**, 1358–1365.
- BIDLINGMEYER, B. A., COHEN, S. A. & TARVIN, T. L. (1984). Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography* **336**, 93–104.
- DIKEMA, C., KESTER, H. C. M. & VISSER, J. (1985). ¹³C NMR studies of carbon metabolism in the hyphal fungus *Aspergillus nidulans*. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 14–18.
- DIKEMA, C., RIJCKEN, R. P., KESTER, H. C. M. & VISSER, J. (1986). ¹³C-NMR studies on the influence of pH and nitrogen source on polyol pool formation in *Aspergillus nidulans*. *FEMS Microbiology Letters* **33**, 125–131.
- DUTLER, H., VAN DER BAAN, J. L., HOCHULI, E., KIS, Z., TAYLOR, K. E. & PRELOG, V. (1977). Dihydroxyacetone reductase from *Mucor javanicus*. *European Journal of Biochemistry* **75**, 423–432.
- FROMM, H. J. (1975). Initial rate enzyme kinetics. *Molecular Biology, Biochemistry and Biophysics* **22**, 75.
- FÜHRER, L., KUBICEK, C. P. & RÖHR, M. (1980). Pyridine nucleotide levels and ratios in *Aspergillus niger*. *Canadian Journal of Microbiology* **26**, 405–408.
- HOCHULI, E., TAYLOR, K. E. & DUTLER, H. (1977). Dihydroxyacetone reductase from *Mucor javanicus*. *European Journal of Biochemistry* **75**, 433–439.
- HULT, K., VEIDE, A. & GATENBECK, S. (1980). The distribution of the NADPH regenerating mannitol cycle among fungal species. *Archives of Microbiology* **128**, 253–255.
- INAYAT, M. J. & MATTEY, M. (1988). Regulation of NADP⁺-specific glycerol dehydrogenase in *Aspergillus niger*. *Biochemical Society Transactions* **16**, 976.
- INOUE, Y., GHEE, H., WATANABE, K., MURATA, K. & KIMURA, A. (1987). Metabolism of 2-ketoaldehydes in mold: purification and characterization for glyoxalase I from *Aspergillus niger*. *Journal of Biochemistry* **102**, 583–589.
- ITZHAKI, R. F. & GILL, D. M. (1964). A micro-biuret method for estimating proteins. *Analytical Biochemistry* **9**, 401–410.
- JENNINGS, D. H. (1984). Polyol metabolism in fungi. *Advances in Microbial Physiology* **25**, 149–193.
- JERNECJ, K., CIMERMAN, A. & PERDIH, A. (1986). Comparison of different methods for protein determination in *A. niger* mycelium. *Applied Microbiology and Biotechnology* **23**, 445–448.
- KISER, R. C. & NIEHAUS, W. G. (1981). Purification and kinetic characterization of mannitol-1-phosphate dehydrogenase from *Aspergillus niger*. *Archives of Biochemistry and Biophysics* **211**, 613–621.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LEGISA, M. & MATTEY, M. (1986). Glycerol synthesis by *Aspergillus niger* under citric acid accumulating conditions. *Enzyme and Microbial Technology* **8**, 607–609.
- NIEHAUS, W. G. & DILTS, G. P. (1982). Purification and characterization of mannitol dehydrogenase from *Aspergillus parasiticus*. *Journal of Bacteriology* **151**, 243–250.
- PONTECORVO, G., ROPER, J. A., HEMMONS, L. J., MACDONALD, K. D. & BUFTON, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Advances in Genetics* **5**, 141–238.
- RÖHR, M., KUBICEK, C. P., ZEHENTGRUBER, O. & ORTHOFER, R. (1983). Balance of carbon and oxygen conversion rates during pilot plant citric acid fermentation by *Aspergillus niger*: identification of polyols as major byproducts. *International Journal of Microbiology* **1**, 19–25.
- SHEYS, G. H. & DOUGHTY, C. C. (1971). Subunits of aldose reductase from *Rhodotorula*. *Biochimica et Biophysica Acta* **235**, 414–417.
- UITZETTER, J. H. A. A. (1982). *Studies on carbon metabolism in wild type and mutants of Aspergillus nidulans*. PhD thesis, Agricultural University Wageningen, The Netherlands.
- VISSER, J., VAN ROOIJEN, R., DIKEMA, C., SWART, K. & SEALY-LEWIS, H. M. (1988). Glycerol uptake mutants of the hyphal fungus *Aspergillus nidulans*. *Journal of General Microbiology* **134**, 655–659.
- VISWANATH-REDDY, M., PYLE, J. E. & HOWE, H. B. (1978). Purification and properties of NADP-linked glycerol dehydrogenase from *Neurospora crassa*. *Journal of General Microbiology* **107**, 289–296.
- WATSON, J. A., HAYASHI, J. A., SCHUYTEMA, E. & DOUGHTY, C. C. (1969). Identification of reduced nicotinamide adenine dinucleotide phosphate dependent aldehyde reductase in a *Rhodotorula* strain. *Journal of Bacteriology* **100**, 110–116.