Purification and Properties of NADP⁺-linked Glycerol Dehydrogenase from *Neurospora crassa*

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The properties of NADP⁺-linked glycerol dehydrogenase (EC 1.1.1.72) from *Neurospora crassa* were studied following 505-fold purification, with 54 % yield, by gel filtration, ion exchange chromatography and affinity chromatography. Specific staining for the purified enzyme after disc gel electrophoresis revealed a single band and after isoelectric focusing, five bands. No enzymically inactive bands were detected by general protein staining in control gels. Total molecular weight was estimated to be about 160000, with a subunit molecular weight of about 43000. The forward reaction from glycerol to D-glyceraldehyde had a pH optimum of 9.5 and was specific for glycerol as substrate, since no activity was detected with other polyols tested. The reverse reaction had a pH optimum of 6.5 and was maximal with D-glyceraldehyde as substrate. K_m values for glycerol and D-glyceraldehyde were 1.43×10^{-1} M and 1.15×10^{-2} M, respectively. Dihydroxyacetone also served as substrate in the reverse reaction. The enzyme was NADP⁺-specific.

INTRODUCTION

Two oxidative pathways are known to be involved in the dissimilation of glycerol in various organisms. In one pathway, glycerol is oxidized to dihydroxyacetone by an NAD⁺-linked glycerol dehydrogenase. In the other pathway, glycerol is oxidized to glyceraldehyde by an NADP⁺-linked glycerol dehydrogenase. During a previous study, we detected NADP⁺-linked glycerol dehydrogenase in wild-type strains of *Neurospora*, whereas the NAD⁺-linked enzyme was apparently absent (Viswanath-Reddy *et al.*, 1977). NAD⁺-linked glycerol dehydrogenase has been reported mostly in prokaryotes (Lin, 1976).

Two types of NADP⁺-linked glycerol dehydrogenase have been described. First, a relatively substrate-specific type which reversibly catalyses the conversion of glycerol to glyceraldehyde (Toews, 1966, 1967; Sheys *et al.*, 1971; Kormann *et al.*, 1972) or to dihydroxyacetone (Ben-Amotz & Avron, 1973; Dutler *et al.*, 1977; Hochuli *et al.*, 1977), and secondly, a non-specific type called aldehyde reductase which not only catalyses the reduction of aldoses such as glyceraldehyde but also oxidizes several sugar alcohols (polyols) such as D-sorbitol, L-arabitol and glycerol (Bosron & Prairie, 1972). We have detected high levels of NADP⁺-linked polyol dehydrogenase activity with sorbitol, mannitol, xylitol and glycerol in crude extracts of *Neurospora crassa*. The purpose of the present study was to determine the nature of NADP⁺-linked glycerol dehydrogenase activity in *N. crassa*. We report here a purification procedure for NADP⁺-linked glycerol dehydrogenase and describe some of the properties of this enzyme; we found it to be a relatively substrate-specific type and distinct from polyol dehydrogenase.

METHODS

Organism. *Neurospora crassa* wild-type strain 74-OR23-1A, from the Fungal Genetics Stock Centre, Arcata, California, U.S.A., was used.

Chemicals. Sodium dodecyl sulphate (SDS) was obtained from Matheson Coleman and Bell, East Rutherford, New Jersey, U.S.A.; DEAE Bio-Gel A, Bio-Lyte 3/10 ampholytes and acrylamide from Bio-Rad Laboratories, Richmond, California, U.S.A.; Good's buffers from U.S. Biochemical Corp., Cleveland, Ohio, U.S.A.; chymotrypsinogen, ovalbumin and ribonuclease A from Isolab, Akron, Ohio, U.S.A.; enzyme grade ammonium sulphate and riboflavin-5'-phosphate from ICN Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; Sephadex G-100 and G-200 from Pharmacia; and Cibacron Blue F3GA from Ciba-Geigy. Other biochemical reagents, of the highest purity available, were obtained from Sigma. Inorganic chemicals were obtained from Fisher Scientific Co., Fairlawn, New Jersey, U.S.A.

Culture conditions and preparation of crude enzyme extract. The fungus was grown at 25 °C for 36 h in 10 l Westergaard & Mitchell medium (1947) in 14 l carboys. Carboys were inoculated at a final concentration of 10^6 conidia ml⁻¹ and sparged with sterile air. Mycelia were harvested by filtration through cheesecloth on a Buchner funnel, washed thoroughly with cold distilled water, dried between paper towels and weighed. Mycelial weights averaged 85 g per carboy.

The temperature was maintained at 0 to 4 °C throughout the purification procedure. Mycelia (100 g) were cut into small pieces and suspended in 2 vol. cold 0.1 M-Tris/HCl buffer (pH 7.4), containing 0.5 mM-2-mercaptoethanol and 100 g glass beads (0.5 mm diameter). The mixture was homogenized intermittently in a Virtis homogenizer up to a setting of 90 for 30 min. The homogenate was centrifuged at 1500 g for 15 min in a Sorvall refrigerated centrifuge to remove glass beads and debris, and the supernatant was centrifuged at 20000 g for 30 min. The supernatant from the second centrifugation constituted the crude enzyme extract.

Enzyme assay. Glycerol dehydrogenase (glycerol:NADP⁺ oxidoreductase; EC 1.1.1.72) activity was assayed by following the change in absorbance of pyridine nucleotide at 340 nm in cuvettes of 1 cm light path in an Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer equipped with a Sargent model SRLG recorder.

The forward reaction i.e. glycerol oxidation to D-glyceraldehyde, was used to determine enzyme activity throughout the purification procedure.

The forward reaction mixture contained 50 mM-sodium bicarbonate buffer (pH 10), 0.25 mM-NADP⁺, 100 mM-glycerol, 5 to 10 µl enzyme extract (as required) and distilled water to 2 ml final volume. The reaction was initiated by the addition of NADP⁺ and was linear up to 5 min.

The reverse reaction mixture contained 50 mM-2-(N-morpholino)ethanesulphonic acid (MES) buffer (pH 6.5), 0.25 mM-NADPH, 50 mM-D-glyceraldehyde, 5 to 10 µl enzyme extract (as required) and distilled water to 2 ml final volume. The reaction was initiated by the addition of NADPH and was linear up to 3 min.

One unit of enzyme activity was defined as that amount of activity which catalysed the formation of 1 µmol NADP⁺ or NADPH per min at 25 °C. An absorption coefficient of 6.22×10^3 l mol⁻¹ cm⁻¹ at 340 nm was used to calculate the amount of coenzyme. The specific activity was expressed as enzyme units (mg protein)⁻¹. Biuret and Lowry protein determinations were performed according to procedures outlined by Bergmeyer (1974).

Michaelis-Menten constants were determined from Lineweaver-Burk plots, with limiting substrate or cofactor added in appropriate amounts. The pH optima of the enzyme were determined by substitution of the appropriate Good's buffer (Good *et al.*, 1966) in the reaction mixture. The buffers used were 0.1 M-CAPS (pH 10 to 11), 0.1 M-CHES (pH 9 to 9.5) 0.1 M-HEPPS (pH 8.5), 0.1 M-HEPES (pH 7 to 8), 0.1 M-MOPS (pH 6.5 to 7) and 0.1 M-MES (pH 5.5 to 6.5).

Preparation of Cibacron Blue-Sepharose and calcium phosphate gel. Cibacron Blue was attached to Sepharose 4B-200 by a slightly modified method of Böhme *et al.* (1972). Sepharose 4B-200 (200 ml) was suspended in 200 ml distilled water in a 60 °C water bath, and the dye solution (2 g Cibacron Blue in 60 ml distilled water) was added dropwise with gentle stirring. After addition of the dye, stirring was continued for 15 min, 45 g NaCl was added and the mixture was stirred for a further 30 min. The mixture was then heated to 70 °C, treated with 4 g Na₂CO₃ and stirring was continued for another 1.5 h. The mixture was allowed to cool to room temperature and the unreacted dye was removed by filtration, with repeated washing of the gel with distilled water until the filtrate became colourless. Calcium phosphate gel was prepared according to the method of Keilin & Hartree (1938). The gel was stored at 4 °C at least 1 month before use.

Polyacrylamide disc gel electrophoresis. Disc gel electrophoresis was performed in 7.5 % (w/v) polyacrylamide gels with 0.1 M-Tris/glycine buffer (pH 8.9) in 5 × 75 mm glass tubes, according to the procedure of Davis (1964). The samples were run at 2.5 mA per gel at 4 °C. Gels were stained for protein in three steps:

Table 1. Purification of NADP⁺-linked glycerol dehydrogenase from *Neurospora crassa*

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Specific activity	Yield (%)	Purification factor
Crude extract	952	9900	405	0.041	100	
Ethodin	983	3846	406	0.106	100	2.6
Ammonium sulphate	100	1357	328	0.242	81	5.9
Calcium phosphate	20	1021	297	0.291	73	7.1
Sephadex G-100	20	564	292	0.518	72	12.6
DEAE Bio-Gel A	20	147	266	1.81	66	44
Cibacron Blue	14	10.5	218	20.7	54	505

(i) fixation overnight in a solution containing 0.5 g CuSO₄, 0.05 g Coomassie Brilliant Blue R, 10 ml acetic acid, 27 ml absolute ethanol and distilled water to 100 ml; (ii) fixation for 6 h in a solution containing 0.01 g Coomassie Brilliant Blue R, 10 ml acetic acid, 25 ml absolute ethanol and distilled water to 100 ml; and (iii) destaining in a solution containing 10 ml acetic acid, 10 ml absolute ethanol and distilled water to 100 ml. Gels were stained specifically for glycerol dehydrogenase as follows. The staining mixture consisted of 10 ml 0.1 M-sodium bicarbonate buffer (pH 10), 4 mg 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl monotetrazolium bromide (MTT), 400 µg phenazine methosulphate, 6 mg NADP⁺, 2 ml 1 M-glycerol and 8 ml distilled water. The gels were incubated in the staining solution at 37 °C until adequately stained, usually 20 to 30 min. Control gels stained with this mixture without glycerol showed no bands.

Isoelectric focusing in polyacrylamide gels. Isoelectric focusing was performed in 7.5 % polyacrylamide gels containing Bio-Lyte ampholytes (1 %, w/v; pH range 3 to 10) in 5 × 75 mm glass tubes. The gels were photopolymerized with riboflavin (4 µg ml⁻¹). Protein samples (50 µg in 50 µl) were applied to the gels as 25 % (w/v) sucrose solutions and overlaid, successively, with 50 µl 20 % sucrose and 50 µl 10 % sucrose solutions. The electrolyte solution in the upper chamber was 0.03 M-H₂SO₄; in the lower chamber, it was 0.01 M-Ca(OH)₂ in 0.04 M-NaOH. The lower chamber was maintained at 4 °C by cold water circulation. With the anode in the upper chamber, an electric potential of 20 V cm⁻¹ of gel was applied for 4 h. Several gels were cut in 5 mm sections, crushed in 1 ml distilled water and the pH gradient was determined. The other gels were washed with cold 0.1 M-sodium bicarbonate buffer (pH 10) and stained for proteins by the procedure of Otavsky & Drysdale (1975) or specifically for glycerol dehydrogenase, as described above for polyacrylamide disc gel electrophoresis.

SDS-polyacrylamide gel electrophoresis. The enzyme was denatured with SDS according to the procedure of Fairbanks *et al.* (1971). The enzyme sample (500 µg) was prepared in 1 ml 0.04 M-Tris/acetate buffer (pH 6.4) containing 0.001 M-EDTA, 0.14 M-2-mercaptoethanol and 1 % recrystallized SDS. The sample was then incubated for 12 h at 37 °C. The marker proteins ribonuclease A, chymotrypsinogen, ovalbumin and bovine serum albumin were treated in a similar way. Tracking dye concentrate containing 0.5 % (w/v) bromophenol blue and 30 % sucrose was added to the sample to make the protein samples 6 % with respect to sucrose. The 7.5 % gels were prepared in 0.1-M Tris/acetate buffer (pH 6.4) containing 1 % SDS in 5 × 75 mm glass tubes. The same buffer was used for running the electrophoresis. Approximately 25 µg protein was applied to each gel and subjected to electrophoresis at 2.5 mA per tube for 4.5 h. The gels were fixed in isopropyl alcohol/acetic acid/water (4:1:5, by vol.) for 4 h and stained for protein in 0.25 % Coomassie Brilliant Blue R in 7 % acetic acid for 4 h. The gels were then destained in a solution containing 7.5 % acetic acid and 5 % methanol.

RESULTS

Enzyme purification

The results of the purification scheme used for NADP⁺-linked glycerol dehydrogenase are presented in Table 1. The crude extract was markedly pigmented and turbid and normally had a specific activity of about 0.041.

Step 1 – ethodin treatment. Nucleic acids were precipitated from the crude extract by the dropwise addition of a 2 % aqueous solution of ethodin (6,9-diamino-2-ethoxyacridine lactate) over 30 min, with constant stirring, to a final concentration of 200 mg ethodin (g protein)⁻¹. After 4 h, the yellow precipitate was removed by centrifuging at 20000 g for 15 min. This step resulted in no loss of enzyme activity and in 2.6-fold purification.

Step 2 – ammonium sulphate fractionation. Finely powdered ammonium sulphate was added to the supernatant from the previous step over 30 min to 55 % saturation. The

precipitate was removed by centrifugation (20000 *g* for 20 min) and discarded, and the supernatant was brought to 75% saturation by further addition of finely powdered ammonium sulphate. After 2 h the precipitate was collected by centrifuging at 20000 *g* for 20 min. The precipitate was resuspended in 100 ml 0.1 M-Tris/HCl buffer (pH 7.4). This step resulted in the loss of 19% of enzyme activity but in an overall 5.9-fold purification.

Step 3 – calcium phosphate gel negative absorption. Calcium phosphate gel was added to the enzyme fraction from the previous step at a gel:protein ratio of 3:1, and gently stirred for 1 h. The suspension was then centrifuged at 15000 *g* for 15 min, and the supernatant was collected. The enzyme was precipitated by the addition of ammonium sulphate to 75% saturation and collected by centrifugation (15000 *g* for 15 min), dissolved in 20 ml 0.1 M-Tris/HCl buffer (pH 7.4) and centrifuged at 35000 *g* for 30 min to remove any remaining gel.

Step 4 – Sephadex G-100 gel filtration. A Sephadex G-100 column (Pharmacia K 26/70) with a bed volume of 300 ml was equilibrated by washing with 0.1 M-Tris/HCl buffer (pH 7.4). The enzyme fraction from the previous step was applied to the column and eluted with 300 ml 0.1 M-Tris/HCl buffer (pH 7.4) at a flow rate of 40 ml h⁻¹. The enzyme fractions of about 60 ml were pooled, diafiltered with 100 ml 0.01 M-Tris/HCl buffer (pH 8.6) in an Amicon ultrafiltration cell with PM-10 filter, and adjusted to 20 ml with similar buffer. This step removed about 45% of extraneous protein without any significant loss of enzyme activity.

Step 5 – DEAE Bio-Gel A ion exchange chromatography. A DEAE Bio-Gel A column (Pharmacia K 26/70) with a bed volume of 250 ml was equilibrated by washing with 0.01 M-Tris/HCl buffer (pH 8.6). The enzyme fraction from the previous step was applied to the column and washed with 200 ml of similar buffer. The enzyme was found to be bound to the gel at this ionic concentration and was eluted in two steps. First, 200 ml 0.01 M-Tris/HCl buffer containing 0.025 M-KCl (pH 8.6) was passed through the column. Secondly, the enzyme was eluted with 200 ml 0.01 M-Tris/HCl buffer containing 0.05 M-KCl (pH 8.6). The enzyme fractions of about 50 ml were pooled, diafiltered with 100 ml 0.1 M-Tris/HCl buffer (pH 7.4) in the Amicon ultrafiltration cell, and adjusted to 20 ml with similar buffer. This step resulted in 9% loss of enzyme activity and in an overall 44-fold purification.

Step 6 – Cibacron Blue affinity chromatography. A Cibacron Blue–Sephacrose column (Pharmacia K 26/40) with a bed volume of 150 ml was washed and equilibrated with 0.1 M-Tris/HCl buffer (pH 7.4). The enzyme fraction from the previous step was applied to the column and washed with 100 ml of similar buffer. The enzyme was found to be bound to the gel and was eluted in two steps. First, 100 ml 0.1 M-Tris/HCl buffer containing 0.1 M-KCl (pH 7.4) was passed through the column. Secondly, the enzyme was eluted with 100 ml 0.1 M-Tris/HCl buffer containing 0.2 M-KCl (pH 7.4). The enzyme fractions of about 50 ml were pooled, diafiltered with 100 ml 0.1 M-Tris/HCl buffer (pH 7.4) in the Amicon ultrafiltration cell, and adjusted to 14 ml with similar buffer. This step resulted in a specific activity of 20.7 with 54% yield and an overall 505-fold purification. This preparation was used for further studies. No significant differences were observed in the specific activities of three repetitive purifications of the enzyme.

Purity of the enzyme

Polyacrylamide disc gel electrophoresis of purified glycerol dehydrogenase showed a single band after staining for specific enzyme activity (Fig. 1, left gel). No enzymically inactive bands were detected by Coomassie Brilliant Blue R staining of proteins in parallel gels.

Isoelectric focusing of purified glycerol dehydrogenase in polyacrylamide gels showed five bands after staining for specific enzyme activity (Fig. 1, right gel). No enzymically inactive bands were detected by Coomassie Brilliant Blue R staining of proteins in parallel gels.

Fig. 1. Specific staining for NADP⁺-linked glycerol dehydrogenase in polyacrylamide gels. Left gel: single band following disc gel electrophoresis. Right gel: five bands following isoelectric focusing, with pI values (top to bottom) of 3.8, 4.0, 4.5, 5.0 and 5.1.

Fig. 2

Fig. 2. Subunit molecular weight determination of NADP⁺-linked glycerol dehydrogenase (arrow) following SDS-polyacrylamide gel electrophoresis. Reference proteins and their molecular weights are: 1, ribonuclease A, 13700; 2, chymotrypsinogen, 25000; 3, ovalbumin, 45000; 4, bovine serum albumin, 68000.

Fig. 3

Fig. 3. Effect of pH on the specific activity of NADP⁺-linked glycerol dehydrogenase in the forward (glycerol → glyceraldehyde, ●) and reverse (glyceraldehyde → glycerol, ○) reactions. Determinations were made with substrates at 50 mM, cofactors at 0.25 mM and 10 μl purified enzyme.

Molecular weight

Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis. A single protein band was found, and a molecular weight of about 43000 was estimated from the standard curve (Fig. 2). A total molecular weight was estimated by gel filtration with Sephadex G-100 and G-200 to be about 160000.

Effect of pH

The specific activity of glycerol dehydrogenase was determined between pH 5.5 and 11. The oxidation of glycerol to glyceraldehyde was optimal at pH 9.5, whereas the reduction of glyceraldehyde to glycerol was optimal at pH 6.5 (Fig. 3).

Table 2. *Substrate specificities for forward and reverse reactions of NADP⁺-linked glycerol dehydrogenase at optimum pH values*

Determinations were made with 0.25 mM-NADP⁺ and NADPH and 10 μ l purified enzyme.

Forward reaction (pH 9.5)		Reverse reaction (pH 6.5)	
Substrate (100 mM)	Activity (%)	Substrate (25 mM)	Activity (%)
Glycerol	100	D-Glyceraldehyde	100
D-Mannitol	0	L-Glyceraldehyde	36
D-Sorbitol	0	Dihydroxyacetone	43
Xylitol	0	D-Fructose	0
		D-Ribose	0
		D-Xylose	0

Table 3. *Kinetic data for NADP⁺-linked glycerol dehydrogenase*

Kinetic parameters were obtained from Lineweaver-Burk plots at near-saturating concentrations of substrates. Determinations were made with 50 mM-CHES buffer (pH 9.5) or 50 mM-MES buffer (pH 6.5) and 10 μ l purified enzyme.

Reaction pH	Substrate	K_m (M)	V_{max}
9.5	Glycerol	1.43×10^{-1}	2.20×10^4
9.5	NADP ⁺	5.60×10^{-5}	1.65×10^4
6.5	D-Glyceraldehyde	1.15×10^{-2}	2.63×10^4
6.5	NADPH	2.40×10^{-5}	3.10×10^4

Substrate and cofactor specificity

The forward reaction was specific for glycerol, as no activity was detected with sugar alcohols such as D-mannitol, D-sorbitol or xylitol with NADP⁺ or NAD⁺ as cofactors. The reverse reaction was maximal with D-glyceraldehyde as substrate; with L-glyceraldehyde, a decrease to 36% of the activity with D-glyceraldehyde was observed, suggesting stereospecificity of the enzyme. Dihydroxyacetone also served as substrate for the reverse reaction, with 43% of the activity with D-glyceraldehyde. No activity was detected with D-fructose, D-ribose or D-xylose (Table 2). The enzyme reaction had a specific requirement for NADP⁺ or NADPH as cofactors, since no activity was detected with NAD⁺ or NADH.

Kinetic data

Kinetic parameters for glycerol dehydrogenase in the oxidative and reductive reactions were determined at the optimum pH values. Lineweaver-Burk plots were used to calculate the apparent K_m and V_{max} of each substrate (Table 3). The K_m values obtained for glycerol and D-glyceraldehyde were 1.43×10^{-1} M and 1.15×10^{-2} M, respectively. The K_m values for NADP⁺ and NADPH were 5.60×10^{-5} M and 2.40×10^{-5} M, respectively.

Stability of the enzyme

Purified enzyme preparations did not lose activity for at least 3 months when stored at -20 or 4 °C. Enzyme preparations obtained after ammonium sulphate fractionation were stable in the absence of 2-mercaptoethanol. Heat treatments of the enzyme were done with 50 μ g protein in 1 ml 0.1 M-Tris/HCl buffer (pH 7.4). The enzyme lost 98% activity at 50 °C after 5 min. At 45 °C, the half-life of the enzyme was approximately 25 min, whereas addition of 400 mM-glycerol increased the half-life to approximately 45 min.

Miscellaneous properties

Ammonium ion was reported to activate NAD⁺-linked glycerol dehydrogenase from bacteria, as well as some NADP⁺-linked glycerol dehydrogenases (aldose reductases) from

mammalian tissues (Kormann *et al.*, 1972). Another group of NADP⁺-linked glycerol dehydrogenases from yeast and lens was activated by sulphate ions (Sheys & Doughty, 1971*b*). In the present study the effect of NH₄Cl and Na₂SO₄ on enzyme activity was determined at various concentrations. Ammonium ion had no effect on enzyme activity at 5 to 20 mM. Similarly, sulphate ion had little effect on enzyme activity at 10 mM and reduced activity to 83% at 20 mM. No metal ion requirements for enzyme activity were detected. In the presence of 5 and 10 mM-EDTA, respectively, 90 and 80% of the enzyme activity remained. *p*-Hydroxymercuribenzoate at 1 mM inhibited 50% of the enzyme activity and at 2 mM, 100%.

DISCUSSION

The molecular weights of NADP⁺-linked glycerol dehydrogenase from different sources vary considerably. The total molecular weight of the enzyme from rabbit skeletal muscle was estimated to be 34000 (Kormann *et al.*, 1972) and from the alga *Dunaliella*, 65000 (Ben-Amotz & Avron, 1974). The enzyme from *Rhodotorula* was reported to have a total molecular weight of 61000 with two subunits of 38000 and 23000; moreover, the 38000 molecular weight species had enzyme activity, whereas the smaller species was inactive (Sheys & Doughty, 1971*a*). The enzyme from *Mucor* was reported to have a total molecular weight of about 100000 with a subunit molecular weight of 28000 (Dutler *et al.*, 1977). The enzyme from *Neurospora* in the present study was estimated by gel filtration to have a total molecular weight of about 160000 and, by SDS-polyacrylamide gel electrophoresis, to have a subunit molecular weight of about 43000.

The purified glycerol dehydrogenase from *Neurospora*, upon polyacrylamide disc gel electrophoresis, migrated as a single protein band. However, when the enzyme preparation was subjected to isoelectric focusing in polyacrylamide gels with ampholytes in the pH range 3 to 10, five protein bands were revealed, all of which showed specific enzyme activity. The existence of several active species of enzyme with pI values ranging from 3.8 to 5.1 suggested the presence of multimeric forms, possibly isozymes.

The optimum pH for the forward reaction was 9.5 and for the reverse reaction, 6.5. Similar pH profiles were reported for glycerol dehydrogenase from rat skeletal muscle (Toews, 1966), *Dunaliella* (Ben-Amotz & Avron, 1973, 1974) and *Mucor* (Dutler *et al.*, 1977).

Our studies on substrate specificities of glycerol dehydrogenase from *Neurospora* showed that this enzyme had an absolute requirement for NADP⁺ or NADPH as cofactors, since NAD⁺ or NADH could not be substituted. It was also apparent from the results that D-glyceraldehyde was the preferred substrate for the enzyme, since the activity with L-glyceraldehyde was reduced to 36% of that with D-glyceraldehyde. Similar properties were reported for the enzyme from rat skeletal muscle (Kormann *et al.*, 1972). The enzyme from *Neurospora* had activity with dihydroxyacetone reduced to 43% of that with D-glyceraldehyde, whereas Kormann *et al.* (1972) reported reduction to 4% for the enzyme from rabbit skeletal muscle. In *Dunaliella* (Ben-Amotz & Avron, 1973), dihydroxyacetone was the preferred substrate, and less than 5% relative activity was found with DL-glyceraldehyde. In *Mucor* (Hochuli *et al.*, 1977) dihydroxyacetone was the preferred substrate, and 0.3 and 6.1% relative activity, respectively, was found with D- and L-glyceraldehyde. In contrast, NAD⁺-linked glycerol dehydrogenase from *Aerobacter* had 5.4 and 52.8% activity, respectively, with D- and L-glyceraldehyde compared to that with dihydroxyacetone (Hochuli *et al.*, 1977).

At substrate concentrations of less than 100 mM, glyceraldehyde reduction proceeded at twice the rate of glycerol oxidation in the present study. This was also evident from the Michaelis constants for glycerol (1.43×10^{-1} M) and D-glyceraldehyde (1.15×10^{-2} M) determined at the optimum pH values of the forward and reverse reactions, respectively. For a similar enzyme from *Rhodotorula*, K_m values of 4.5×10^{-3} M for glycerol and 9×10^{-4} M

for D-glyceraldehyde were reported (Sheys *et al.*, 1971; Sheys & Doughty, 1971*b*). Kormann *et al.* (1972) reported a K_m value of 1.5×10^{-4} M for D-glyceraldehyde, using glycerol dehydrogenase from rabbit skeletal muscle. The K_m values for NADP⁺ and NADPH were 5.60×10^{-5} M and 2.40×10^{-5} M, respectively, with the enzyme from *Neurospora*. Although the observed K_m values for glycerol dehydrogenase from different organisms vary, the enzyme was found to have higher affinity for D-glyceraldehyde and NADPH than for glycerol and NADP⁺.

Purified glycerol dehydrogenase from *Neurospora* showed no detectable activity with the following sugar alcohols (polyols) and sugars: D-mannitol, D-sorbitol, xylitol, D-fructose, D-ribose or D-xylose. We detected high levels of NADP⁺-linked dehydrogenase activity for each of these substrates with crude enzyme preparations from *Neurospora*. Our results suggest that the NADP⁺-linked glycerol dehydrogenase was distinct from polyol dehydrogenase.

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