Purification and Properties of Glycerol Dehydrogenase from *Candida valida*

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Glycerol:NAD⁺ 2-oxidoreductase (EC 1.1.1.6) was purified to homogeneity from the non-methylotrophic yeast *Candida valida* H 122. Results of electrophoresis in polyacrylamide gels, gel filtration and ultracentrifugation were compatible with the enzyme's consisting of two subunits with a molecular weight 38000. No heterogeneity was observed by isoelectric focusing. The pH optima were 10.0 for glycerol oxidation and 7.5 for dihydroxyacetone reduction. The K_m values for glycerol, dihydroxyacetone, NAD⁺ and NADH were 5.8×10^{-2} M, 7.7×10^{-4} M, 1.4×10^{-4} M and 4.8×10^{-5} M, respectively. 1,2-Propanediol also served as substrate in the forward and DL-glyceraldehyde in the reverse reaction. The oxidation product of glycerol was identified as dihydroxyacetone. An ordered bi-bi mechanism was deduced from product-inhibition studies. Of several anions and cations tested, pyrophosphate and ammonium ions stimulated the dehydrogenating activity the most. 2-Mercaptoethanol, ethylene glycol and Tris inhibited activity; an inhibition was also observed by phosphorylated coenzymes and substrates. The purified enzyme, which is labile at low concentrations, was stabilized by the addition of neutralized filtrate from the culture liquid.

INTRODUCTION

Glycerol can be utilized as carbon source by diverse eukaryotic micro-organisms. In yeast the utilization of glycerol is initiated by phosphorylation (Gancedo *et al.*, 1968; Sprague & Cronan, 1977). Recently, an alternative route of glycerol metabolism and its regulation involving glycerol dehydrogenase as primary enzyme have been studied in *Candida valida* (Hofmann & Babel, 1979) and *Schizosaccharomyces pombe* (May & Sloan, 1981). In both species, dihydroxyacetone kinase activities were also demonstrated (Babel & Hofmann, 1982; May *et al.*, 1982), which apparently co-operate with the dehydrogenases. However, the dehydrogenases are not sufficiently characterized to allow one to understand their physiological function. The presence of glycerol dehydrogenase (EC 1.1.1.6) in *Geotrichum candidum* has also been shown recently, and two isoenzymes were isolated and characterized (Itoh, 1982). In this paper, the purification and properties of a NAD⁺-linked glycerol dehydrogenase from the non-methylotrophic strain *Candida valida* H 122 are described.

METHODS

Organisms and growth conditions. *Candida valida* strain H 122 was kindly provided by Professor H. A. Koch, Medical Academy, Erfurt, GDR. Cells were cultured at 30 °C in Erlenmeyer flasks (100 ml in 500 ml flasks) on a shaker rotating at about 200 r.p.m. A medium (Reader, 1927) containing 0.1% yeast extract and 2% (w/v) glycerol was used. The initial pH was adjusted to 5.5 with 2 M-NaOH. Cells were harvested during the early stationary phase of growth by centrifugation.

Enzyme extraction and purification. All manipulations were carried out at 4 °C. Cells (30 g wet wt) were washed three times by resuspension and centrifugation with buffer A (50 mM-potassium phosphate buffer, pH 7.5, 10 mM-glycerol, 3 mM-2-mercaptoethanol), then resuspended to about 0.6 g wet wt ml⁻¹ and passed twice through a chilled French pressure cell at 9.9 kN m⁻². Cell debris was removed by centrifugation at 20000 g for 30 min. The supernatant was supplemented with solid (NH₄)₂SO₄ up to 65% saturation, stirred for 1 h and then centrifuged at

20000 g for 30 min. The precipitate was resuspended in an appropriate volume of buffer A supplemented with 1.2 M-(NH₄)₂SO₄ to give a final saturation of 40%. The solution was stirred for 30 min and centrifuged at 25000 g for 30 min. The supernatant was desalted on a Sephadex G-25 column equilibrated with buffer A containing 50 mM-KCl and then applied to a column (2.5 × 20 cm) of Servacel DEAE 23 SS equilibrated with the same buffer. The enzyme was eluted at a flow rate of 30 ml h⁻¹ by a linear increase of KCl concentration from 50 to 250 mM in a total buffer volume of 400 ml, 9 ml fractions being collected. Active fractions were pooled and concentrated to less than 10 ml by ultrafiltration using stirred Amicon cells equipped with a Diaflo PM30 membrane. The concentrated enzyme was then chromatographed on a Sephadex G-150 column (2.5 × 100 cm). Buffer B (20 mM-Tris/HCl buffer, pH 7.0 at 25 °C, 10 mM-glycerol, 3 mM-2-mercaptoethanol) was pumped through the column at a rate of 10 ml h⁻¹ and 2.5 ml fractions were collected. The pooled active fractions were applied to a column (2 × 10 cm) of calcium phosphate gel and the enzyme was eluted with a linear gradient of 0 to 50 mM-potassium phosphate in buffer B (total volume of 50 ml). The combined active fractions were concentrated by vacuum dialysis (Collodion bags SM 13200; Sartorius) to more than 1 mg protein ml⁻¹. Enzyme was stored at -15 °C in buffer B.

Enzyme assays and kinetic studies. The activity of glycerol dehydrogenase was measured at 25 °C by following the change in absorbance of pyridine nucleotide at 340 nm with a Reaction Rate Analyzer (LKB) or a Unicam SP 1750. The reaction mixture of the routine assays contained: 100 mM-sodium pyrophosphate (pH 9.5), 400 mM-glycerol, 50 mM-(NH₄)₂SO₄, and 1 mM-NAD⁺. For measuring the reduction of dihydroxyacetone the assay mixture contained: 100 mM-potassium phosphate (pH 7.5), 100 mM-dihydroxyacetone, and 0.25 mM-NADH. The assay systems were tested for enzyme inactivation according to Selwyn (1965). One unit of enzyme activity was defined as the amount of enzyme which catalysed the conversion of 1 μmol of substrate per minute at 25 °C. Specific activity was expressed as units (mg protein)⁻¹. The apparent kinetic parameters were determined from initial-velocity measurements in the absence of products by using the direct linear plot as described by Eisenthal & Cornish-Bowden (1974). The graphical representation of the kinetic data was performed according to Hanes (1932). The equilibrium constant, as defined by the equation $K_{eq} = (NADH_{eq} \times \text{dihydroxyacetone}_{eq} \times H^+) / (\text{glycerol}_{eq} \times NAD^+_{eq})$, was determined in 0.1 M-sodium pyrophosphate buffer at proton concentrations ranging from 3.16×10^{-8} M to 3.16×10^{-9} M with 50 mM-glycerol, 0.2 mM-dihydroxyacetone, 0.5 mM-NAD⁺ and 0.05 mM-NADH. Concentrations of reactants and products were calculated from the change in absorbance at 340 nm after equilibration, i.e. after incubation for 2 h at 25 °C.

Analytical methods. Polyacrylamide gel electrophoresis with a linear gradient of acrylamide (3 to 15%, w/v) was performed as described by Kopperschlager *et al.* (1977). Enzyme activity in gels was localized by incubating the gel rods in the reaction mixture used for the determination of glycerol oxidation rate supplemented with 0.15 mM-8-dimethylamino-2,3-benzophenoxazine (Meldola Blue) and 1 mM-2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT). Gel rods were developed by keeping in the dark at 25 °C for 15 to 20 min.

SDS-gel electrophoresis was carried out according to Weber & Osborn (1969) using 10% (w/v) acrylamide. The molecular weight standards were cytochrome *c* (12400) and its cross-linked oligomers (25000 to 75000).

For isoelectric focusing, glycerol dehydrogenase (10 μg protein per rod) was resolved in 5% (w/v) polyacrylamide gel containing Pharmalyte (pH 4 to 6.5) according to Laas & Fast-Johansson (1979). For determination of the pH gradient the gels were sliced into 3 mm pieces and crushed in 0.6 ml distilled water (freshly boiled) per gel slice. After 30 min the pH and enzyme activity were measured. Control gels were stained with Coomassie Brilliant Blue G-250.

The molecular weight of glycerol dehydrogenase was estimated by gel filtration on Sephadex G-150 as described by Andrews (1964). For calibration the following marker proteins were used: aldolase (rabbit muscle), mol. wt 158000; bovine serum albumin, 68000; ovalbumin, 45000; chymotrypsinogen A, 25000; cytochrome *c*, 12400.

Sedimentation velocity experiments were carried out in 5 mM-Tris/HCl buffer (pH 7.5), containing 10 mM-glycerol and 3 mM-2-mercaptoethanol, at 50000 r.p.m. and 20 °C with a Phywe Model U60L analytical ultracentrifuge (Phywe AG, Göttingen, FRG) using the UV optics. The apparent sedimentation coefficient was determined at 0.6 mg protein ml⁻¹. The molecular weight was roughly estimated from the equation, mol. wt = 6500 ($s_{20,w}$)^{3.2}, according to Scrimgeour (1977).

Protein was measured by the method of Bradford (1976) using bovine serum albumin as the standard.

For reaction product analysis the glycerol oxidation was coupled to a NAD⁺-regenerating system. The reaction mixture contained in 2 ml: 200 μmol potassium phosphate buffer (pH 8.0), 1 mmol glycerol, 1 mmol sodium pyruvate, 240 μmol NAD⁺, 550 units lactate dehydrogenase, 450 units purified glycerol dehydrogenase. After 1 h incubation at 30 °C the reaction mixture was passed through a Sephadex G-10 column. The fractions containing product were applied to an Amberlite MB-2 column and eluted with distilled water. The break-through fraction was subjected to TLC on silica gel plates (Silufol, Kavalier, Czechoslovakia) using butanol/acetic acid/water (14:3:3, by vol.). Chromatograms were stained by vaporization of crystalline iodine.

Chemicals and prepared substances. Ethylene glycol, dihydroxyacetone, DL-glyceraldehyde, glycerol, DL-1,2-propanediol, sugars and sugar alcohols were obtained from Merck. Other substrates, coenzymes, enzymes, Tris and Meldola Blue were from Boehringer; 2-mercaptoethanol, dithiothreitol, cysteine, reagents for electrophoresis,

ion exchangers and human serum albumin were from Serva Feinbiochemica (Heidelberg, FRG); Pharmalyte (pH 4 to 6.5), Sephadex and Sepharose gels were from Pharmacia; Cibacron Blue F3G-A was from Ciba-Geigy. All other chemicals were of analytical grade. Tetraethylammonium hydroxide was prepared by passing the chloride through a Dowex SAR anion exchanger. Cibacron Blue F3G-A-Sephadex G-100 was prepared according to Böhme *et al.* (1972), calcium phosphate gel according to Koike & Hamada (1971), and cytochrome *c*-oligomers as described by Dunker & Kenyon (1976) for lysozyme. Boiled crude cell extract was obtained by boiling the extract for 5 min in a water bath. Denatured protein was removed by centrifuging at 25000 *g* for 30 min.

Filtrate of culture liquid was prepared with a UM05 membrane (Amicon) and neutralized.

RESULTS

Purification and stability of glycerol dehydrogenase

A summary of a representative enzyme purification is presented in Table 1. The re-extraction of the ammonium sulphate precipitate with a solution of lower saturation (see Methods) gave higher purification than the usual salt fractionation. From the DEAE-cellulose column, glycerol dehydrogenase activity was eluted as a single peak at 0.13 M-KCl in buffer A. Gel filtration on Sephadex G-150 mostly resulted in an enzyme fraction consisting of one major band and a faint band of higher molecular weight, as indicated by polyacrylamide gel electrophoresis under non-denaturing conditions. Only the main band stained for enzyme activity. Finally the chromatography on calcium phosphate gel yielded a homogeneous preparation (Fig. 1), but the recovery from this step was only about 40% of the enzyme activity.

In solutions of purified enzyme with concentrations of 0.5 mg protein ml⁻¹ or less, the activity completely disappeared within 2 to 3 weeks after storage at 4 °C or -15 °C. However, in the presence of 5% (v/v) culture filtrate or boiled crude cell extract, more than 80% of enzyme activity remained. The fact that the culture filtrate and boiled crude extract were able to stabilize the purified enzyme was consistent with decreasing enzyme stability with increasing purity. Divalent cations added as chlorides were not required for stability (Mg²⁺, Ca²⁺, Mn²⁺, Co²⁺, Zn²⁺, Cu²⁺, Fe²⁺). The freshly prepared medium for cultivation was not capable of stabilizing the enzyme. Apparently a hitherto unknown factor produced during fermentation was responsible for enzyme stabilization. The enzyme activity was also stable for 1 month at -15 °C in the presence of 10% (w/v) Ficoll.

Molecular weight and isoelectric point

SDS-polyacrylamide gels showed one band with an apparent molecular weight of 38000. The molecular weight of the native enzyme as determined by filtration on Sephadex G-150 was 76000. From comparing relative mobilities of glycerol dehydrogenase with those of human serum albumin and its oligomers in a gradient of polyacrylamide (3 to 15%, w/v) under non-denaturing conditions a molecular weight of 80000 was calculated. In the analytical ultracentrifuge the isolated enzyme sedimented with an apparent sedimentation coefficient of 5.2 ± 0.3 S. This value corresponded to an approximate molecular weight of 77000 when calculated by the equation according to Scrimgeour (1977). The enzyme therefore behaves as a dimer consisting of two equal subunits. Isoelectric focusing revealed one single band with a pI of 4.8.

Table 1. *Purification of NAD⁺-linked glycerol dehydrogenase from Candida valida H 122*

Purification step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units mg ⁻¹)	Yield (%)	Purification factor
Crude cell extract (from 30 g wet cells)	65	1150	2290	1.98	100	
Ammonium sulphate	73	427	1920	4.50	84	2.3
Servacel DEAE 23 SS	82	30.0	1110	37.0	49	18.7
Sephadex G-150	15	7.19	708	98.5	31	49.8
Calcium phosphate	24	1.05	297	282	13	142.7

Fig. 1. Polyacrylamide gel electrophoresis of purified glycerol dehydrogenase from *Candida valida* H 122 after calcium phosphate gel chromatography. (a) 30 µg Protein on a 3 to 15% gradient gel. Left gel: protein staining. Right gel: active staining. (b) 20 µg Protein on an SDS gel.

Fig. 2. Effect of temperature on the stability of glycerol dehydrogenase. Samples of 25 µg enzyme in 0.5 ml 0.02 M-potassium phosphate buffer (pH 7.5) containing 10 mM-glycerol and 3 mM-2-mercaptoethanol were incubated at 40 °C (○), 50 °C (●), 55 °C (□), 60 °C (■) and at various times samples were assayed for remaining activity. 100% activity corresponds to 14 units ml⁻¹.

Effect of pH and temperature on enzyme activity and stability

Oxidation reaction was maximum between pH 9.5 and 10.5 in 0.1 M-pyrophosphate buffer, reduction reaction with dihydroxyacetone between pH 7.5 and 8.5 in 0.1 M-phosphate buffer. Rates decreased more rapidly at the alkaline pH. Alkaline buffers with pH values above 10 gave rise to other non-enzymic reactions and made the measurement of initial velocities difficult. In 0.1 M-tetraethyl ammonium chloride/tetraethylammonium hydroxide buffer the pH profile for glycerol oxidation was shifted to a lower pH range with an optimum around pH 8.0. Glycerol dehydrogenase was most stable at pH 7.5.

The enzyme had a temperature optimum at 36 °C. Thermostability of the purified enzyme is demonstrated in Fig. 2. 2-Mercaptoethanol had little protective effect against thermal inactivation. At 40 °C after 2 h the remaining activities in the presence and absence of 2-mercaptoethanol were 75% and 65%, respectively.

Table 2. *Kinetic parameters for glycerol dehydrogenase*

Values were obtained from appropriate replots of primary plots with varied concentration of the first substrate at various fixed concentrations of the second substrate. Maximum velocities are expressed as maximum specific activities.

Substrate	K_m (M)	K_i (M)	V_{max} [units (mg protein) ⁻¹]
Glycerol	5.80×10^{-2}		448
NAD ⁺	1.44×10^{-4}	1.09×10^{-3}	442
Dihydroxyacetone	7.70×10^{-4}		202
NADH	4.80×10^{-5}	2.50×10^{-5}	206

Influence of ions

The effects of several monovalent cations on glycerol oxidation were assayed at pH 9.5. NH₄⁺ gave rise to the highest extent of activity. At concentrations of 50 mM or higher, the activation effects of several chloride salts were as follows: NH₄⁺ > K⁺ > Na⁺ > N(C₂H₅)₄⁺. No detectable effect was produced by a series of divalent cations (Mn²⁺, Zn²⁺, Mg²⁺, Ca²⁺, Co²⁺, Fe²⁺) when added as chlorides at concentrations of 0.1 and 1 mM in pyrophosphate buffer. The influence of various anions on the enzyme activity was estimated using sodium salts. At pH 9.5 maximum activity was obtained in pyrophosphate buffer. Half-maximum velocity was already observed at 0.3 mM-sodium pyrophosphate. Generally, the enzyme was more active in the presence of multivalent anions (citrate, phosphate, sulphate) in comparison with monovalent anions (acetate, chloride). Optimal conditions for activity measurement were found at 30 to 60 mM-ammonium sulphate in 0.1 M-pyrophosphate buffer.

Kinetic studies

Initial-velocity measurements revealed typical sets of primary plots for an enzyme that obeyed the equation:

$$v = \frac{V_{max} [A][B]}{K_{ia}K_b + K_a[B] + K_b[A] + [A][B]}$$

where V_{max} is the maximum initial velocity, K_a and K_b are Michaelis constants for the substrates A and B, and K_{ia} is the dissociation constant for the E-A complex (according to the nomenclature of Cleland, 1963). From these initial-velocity measurements, kinetic parameters for glycerol oxidation and dihydroxyacetone reduction were determined (Table 2).

Product inhibition by NADH was competitive with respect to NAD⁺ (Fig. 3*a*). Dihydroxyacetone at subsaturating glycerol concentration (0.2 M) with NAD⁺ as variable substrate gave rise to mixed inhibition (Fig. 3*b*). When glycerol was varied at 1 mM-NAD⁺, dihydroxyacetone revealed a mixed type of inhibition (Fig. 3*c*). Such an inhibition pattern was consistent with the compulsory-order ternary-complex mechanism. The observed substrate inhibition (not shown) which occurred at more than 0.5 M-glycerol was in accord with this type of reaction.

The average equilibrium constant value (K_{eq}) obtained from five individual determinations was $6.3 \times 10^{-12} \pm 2.2 \times 10^{-12}$ M. This value was similar to that of 10.4×10^{-12} M, as calculated from the kinetic constants by using the Haldane relationship.

Substrate and coenzyme specificity

In Table 3 the combinations of compounds tested as substrates and the pyridine nucleotides are summarized. The enzyme was found to exhibit a rather narrow substrate specificity. 1,2-Propanediol was more rapidly dehydrogenated than glycerol due to the higher affinity ($K_m^{1,2\text{-Propanediol}} 3 \times 10^{-3}$ M). In the reverse reaction, dihydroxyacetone was more active than DL-glyceraldehyde. The apparent K_m value for DL-glyceraldehyde (6.2×10^{-3} M) was eightfold higher. The oxidation product of glycerol was identified as dihydroxyacetone by TLC (see

Fig. 3. Product inhibition of glycerol dehydrogenase at pH 8.5. (a) Inhibition by NADH with NAD^+ as variable substrate at constant glycerol (202 mM). Fixed NADH levels were 0.034 mM (●), 0.068 mM (□), 0.136 mM (■), 0.272 mM (△) and none (○). (b) Inhibition by dihydroxyacetone under conditions as indicated in (a). Fixed dihydroxyacetone levels were 0.76 mM (●), 1.89 mM (□), 3.79 mM (■) and none (○). (c) Inhibition by dihydroxyacetone with glycerol as variable substrate at constant NAD^+ concentration (1 mM). Fixed dihydroxyacetone levels were 0.53 mM (●), 1.07 mM (□), 4.27 mM (■) and none (○).

Methods). Phosphorylated substrates were inactive, as also were $NADP^+$ and $NADPH$. On the other hand, glycerol 3-phosphate, dihydroxyacetone phosphate, $NADP^+$ and $NADPH$ were effective inhibitors. Inhibition data yielded values of $I_{0.5}$ for these compounds of 130, 1.9, 5 and 0.19 mM, respectively.

Table 3. *Substrate specificity of glycerol dehydrogenase for dehydrogenation and reduction*

Determinations were made with 1 mM- NAD^+ or $NADP^+$ and 0.25 mM- $NADH$ or $NADPH$ under standard assay conditions (see Methods). No activity in the forward reaction was found with the following compounds: methanol, ethanol, 1-propanol, ethylene glycol, *meso*-erythritol, ribitol, xylitol, D-sorbitol, D-mannitol, D-glucose, L-lactate, L-glycerol 3-phosphate, D-glucose 6-phosphate, D-fructose 6-phosphate, D-glyceraldehyde 3-phosphate. No activity in the reverse reaction was found with the following compounds: D-ribose, D-xylose, D-xylulose, D-fructose, D-mannose, D-galactose, acetone, acetaldehyde, pyruvate, D-glycerate 1,3-diphosphate and dihydroxyacetone phosphate.

Substrate	Concn (mM)	Relative activity (%) with:			
		NAD^+	$NADP^+$	$NADH$	$NADPH$
Glycerol	200	100	0		
1,2-Propanediol	100	130	0		
Dihydroxyacetone	100			100	0
DL-Glyceraldehyde	100			33	0

Effect of various reagents on enzyme activity

2-Mercaptoethanol, Tris and ethylene glycol showed strong inhibition of the enzyme activity ($I_{0.5}$ 0.3, 2 and 4 mM, respectively). Furthermore, the enzyme was slightly inhibited by cysteine and dithiothreitol but not affected by glutathione. *N*-Ethylmaleimide inactivated the enzyme rapidly ($I_{0.5}$ 0.2 mM), indicating the presence of essential thiol groups.

DISCUSSION

The non-methylotrophic strain *Candida valida* H 122 proved to be a suitable source for the purification of glycerol dehydrogenase. The enzyme represented approximately 0.7% of the extracted cell protein when the cells were grown on glycerol.

Several glycerol dehydrogenating enzymes from animal and microbiological sources have been purified by affinity chromatography using immobilized Cibacron Blue F3G-A (Flynn & Cromlish, 1982; Ruch *et al.*, 1980; Kawagishi *et al.*, 1980; Viswanath-Reddy *et al.*, 1978). However, the enzyme from *C. valida* was not inhibited by Blue Dextran 2000 and showed 85% of residual activity even in the presence of 0.1 mM-Cibacron Blue F3G-A. Accordingly, attempts to bind the enzyme to Cibacron Blue-Sephadex G-100 failed. Similar behaviour was reported by Jervis & Schmidt (1977) for potato lactate dehydrogenase.

Glycerol dehydrogenase from *C. valida* was shown to be a dimer consisting of subunits having an equal molecular weight. The enzyme was not capable of forming higher oligomers. In the course of the purification procedure the native enzyme always exhibited the same electrophoretic mobility in polyacrylamide gels. The enzyme did not appear heterogeneous when analysed by isoelectric focusing. The NAD⁺-linked isoenzymes of glycerol dehydrogenase from *Geotrichum candidum* were reported to be tetramers (Itoh, 1982). The aldose reductase from *Rhodotorula* was found to consist of two subunits being different in size and function (Sheys & Doughty, 1971*a*). Compared with the isoenzymes from *G. candidum*, which were completely inactive after heating at 50 °C for 20 min, the glycerol dehydrogenase from *C. valida* retained about 80% of its initial activity under these conditions.

The specific activity of the enzyme from *C. valida* for glycerol oxidation [448 units (mg protein)⁻¹] was significantly higher than reported in the literature for other glycerol dehydrogenases. The different conditions used to assay activity cannot account for these differences.

The higher affinity for 1,2-propanediol is not unusual and has also been observed for prokaryotic glycerol dehydrogenases (Lin, 1976; Tang *et al.*, 1979; Yamada *et al.*, 1982). Accordingly, substrate inhibition by 1,2-propanediol occurred at lower concentrations (above 100 mM) than with glycerol. No inhibition by dihydroxyacetone was found in the reverse reaction, as was described for the glycerol dehydrogenase from *Aerobacter aerogenes* (Strickland & Miller, 1968) and *Cellulomonas* (Yamada *et al.*, 1982). Furthermore, some similarity to the glycerol dehydrogenases from bacteria is apparent in the degree of activation of the enzymes by monovalent cations (Lin & Magasanik, 1960; McGregor *et al.*, 1974). A requirement for divalent cations as recently shown for the glycerol dehydrogenase from *Bacillus megaterium* (Scharschmidt *et al.*, 1983) could not be established. Concerning the influence of anions, the enzyme from *C. valida* appeared different from the isoenzymes in *G. candidum*. Itoh (1982) reported an activation by chloride in the forward reaction and an inhibition by borate and phosphate. In contrast, chloride salts were less effective on the *Candida* enzyme, but multivalent anions, in particular pyrophosphate, showed the highest stimulating effect on the activity of the enzyme. For aldose reductase from *Rhodotorula* it was demonstrated that pyrophosphate affected competitively the association between the cofactor and the enzyme (Sheys & Doughty, 1971*b*). This may also be true for the glycerol dehydrogenase from *C. valida*, where dissociation of NADH from the enzyme seems to be the rate-limiting step ($K_{\text{NADH}}^{\text{NADH}} 2.5 \times 10^{-5}$ M).

Glycerol dehydrogenase from *C. valida* was inhibited by Tris, as is a similar enzyme from *Microcyclops eburneus* (Nishio *et al.*, 1978); however, the isoenzymes from *G. candidum* apparently behaved differently (Itoh, 1982).

Studies on substrate specificity of the *Candida* enzyme revealed an absolute requirement for NAD⁺ and NADH. The identification of the oxidation product as dihydroxyacetone

demonstrated that the enzyme attacked the internal carbon (C2) and was therefore a glycerol : NAD⁺ 2-oxido-reductase (EC 1.1.1.6). The enzyme showed higher specificity than the glycerol dehydrogenases from bacteria. Prokaryotic glycerol dehydrogenases exerted detectable activities on ethylene glycol and glycerol 3-phosphate (Burton, 1955; Hochuli *et al.*, 1977; Yamada *et al.*, 1982) and accepted NADPH as the dehydrogenase from *Escherichia coli* (Tang *et al.*, 1979).

The inhibiting effect of 2-mercaptoethanol on glycerol dehydrogenase from *C. valida* could be due to a structural analogy to ethylene glycol. The fact that only slight inhibition was produced by dithiothreitol and cysteine suggests that the reduction of disulphide bridges is unlikely.

The significance of glycerol dehydrogenase for glycerol utilization in *C. valida* H 122 becomes evident from the observation that it was subject to catabolite repression (Babel & Hofmann, 1982). The relatively high K_m value for glycerol (58 mM) found for this enzyme matches the requirements of the cell because of the extremely high affinity of the subsequent kinase for dihydroxyacetone (Babel & Hofmann, 1982). Moreover, a methylglyoxal synthase has been found in *C. valida* H 122 (Babel & Hofmann, 1981). In the presence of this enzyme the increased levels of dihydroxyacetone phosphate, which might occur with a glycerol dehydrogenase of lower K_m , would give rise to a lethal concentration of methylglyoxal within the cell (Hofmann & Babel, 1983). Therefore, the low-affinity pathway for glycerol utilization apparently prevents the formation of an excessive level of dihydroxyacetone phosphate which could lead to a toxic production of methylglyoxal.

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