Purification and Properties of Glycerol:NADP⁺ 2-Oxidoreductase from *Schizosaccharomyces pombe*

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Glycerol:NADP⁺ 2-oxidoreductase (EC 1.1.1.156) was isolated from *Schizosaccharomyces pombe*, purified and characterized. It had an M_r of 57000, and SDS-PAGE revealed two polypeptides, of M_r 25000 and 30000. Its coenzyme requirement was satisfied exclusively by NADP. The pH optimum for glycerol oxidation was 9.5, for dihydroxyacetone reduction 6.0. Rates of oxidation with some structurally related diols were three- to six-fold lower than for glycerol, while glyceraldehyde and other carbonyl compounds showed negligible rates of reduction. Neither monovalent nor divalent cations activated the enzyme. Apparent K_m and V_{max} values were determined. The enzyme is similar to glycerol dehydrogenases isolated from *Mucor javanicus* and from *Dunaliella parva* but differs considerably from the glycerol:NAD⁺ 2-oxidoreductase of *S. pombe*.

INTRODUCTION

The fission yeast *Schizosaccharomyces pombe* can produce four enzymes capable of catalysing pyridine-nucleotide-linked oxidation of glycerol (Kong *et al.*, 1985). These enzymes differ in coenzyme specificity and in the mode of glycerol oxidation, whether at C-1 or C-2. One of these activities, glycerol:NAD⁺ 1-oxidoreductase, was shown to be a property of alcohol dehydrogenase (ethanol:NAD⁺ oxidoreductase, EC 1.1.1.1). The purification and properties of another of these enzymes, glycerol:NAD⁺ 2-oxidoreductase (GDH2; EC 1.1.1.6) has already been described (Marshall *et al.*, 1985). In this paper we describe the purification and characterization of a third enzyme, glycerol:NADP⁺ 2-oxidoreductase (GDH4; EC 1.1.1.156).

METHODS

General methods. The organism used was *S. pombe* strain 972 h⁻. Its maintenance and method of growth, the preparation of cell extracts, and methods for enzyme assay, protein estimation, enzyme purification, and disc gel electrophoresis (including the location of protein-containing and enzymically active bands) were as described by Kong *et al.* (1985). Methods for determination of M_r and subunit size, pH optima and kinetic data for the enzyme were as described by Marshall *et al.* (1985).

Growth of cells and preparation of cell extracts. Batches of cells for extraction and purification of GDH4 were grown on Edinburgh minimal medium no. 2 (Mitchison, 1970), modified to contain 2% (w/v) glucose, and harvested towards the end of the exponential growth phase; these conditions were optimal for production of this enzyme while ensuring repression of GDH2. Cells were suspended in 20 mM-Tris/HCl, pH 7.6, containing 0.5 mM-phenylmethylsulphonyl fluoride and cooled to 4 °C before disruption in a French pressure cell.

Purification of enzyme. Following disruption of the cells, all subsequent stages in the purification were done at 0–4 °C. Protamine sulphate was added (1 mg per 10 mg protein) to the clear cell-free extract obtained by

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Abbreviations: DHA, dihydroxyacetone; GDH2, glycerol:NAD⁺ 2-oxidoreductase; GDH4, glycerol:NADP⁺ 2-oxidoreductase.

centrifugation. The mixture was stirred for 15 min, centrifuged at 27000 *g* for 30 min and the precipitate discarded. $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant fluid to a final concentration of 2.5 M; the mixture was allowed to stand for 90 min and then centrifuged. The precipitate was dissolved in a small volume of 20 mM-phosphate buffer ($\text{KH}_2\text{PO}_4/\text{KOH}$, pH 6.5) and desalted by passage through a column of Sephadex G-25. This solution was then added to a column (2.5 $\times$ 24 cm) of Matrex Gel Red A which had been equilibrated with 20 mM-phosphate. After standing for 30 min the column was washed with 50 ml 20 mM-phosphate containing 0.8 M-KCl and 0.2 mM-NADH, which eluted most of the protein, followed by 20 mM-phosphate containing 1.0 M-KCl and 0.2 mM-NADPH, which eluted GDH4. The enzyme was precipitated from the eluate with 2.5 M- $(\text{NH}_4)_2\text{SO}_4$, dissolved in a small volume of 20 mM-Tris/HCl (pH 8.0) and desalted by passage through Sephadex G-25. Material prepared in this way was stored at -70°C and was used in subsequent characterization procedures.

Assay of GDH4. GDH4 activity was assayed by measurement of the rate of NADPH oxidation with dihydroxyacetone (DHA) as substrate. The reaction mixture contained enzyme, 50 mM-potassium hydrogen phthalate/KOH (pH 6.0), 0.3 mM-NADPH and 5 mM-DHA (Kong *et al.*, 1985). When measuring activity over a range of pH values to determine pH optima, the phthalate buffer was replaced by one of the following mixtures at a concentration of 40 mM and adjusted to the required pH value: sodium acetate/acetic acid (pH 3.5–5.5); $\text{KH}_2\text{PO}_4/\text{KOH}$ (pH 6.0–8.0); Tris/HCl (pH 7.5–9.0); glycine/KOH (pH 9.0–11.0).

Electrophoresis. In addition to PAGE in an anionic system using 7.5% (w/v) polyacrylamide gel at pH 8.3 (Kong *et al.*, 1985), a cationic system with the same gel strength at pH 4.6 was also used [modified from Williams & Reisfeld (1964)].

Chemicals. Sources of chemicals have been listed previously (Marshall *et al.*, 1985). The dye matrix gel, Matrex Gel Red A, was obtained from Amicon Corporation. It consists of the dye Procion HE-3B linked to agarose and shows high affinity for NADP-dependent enzymes (Watson *et al.*, 1978).

RESULTS

Purification of the enzyme

Table 1 shows the results obtained in a typical experiment in which the enzyme was purified 130-fold with 35% recovery. Attempts at further purification by gel permeation chromatography on Sephacryl S-300 gave no increase in specific activity.

Properties of the enzyme

Stability. The purified enzyme showed no loss of activity when stored for several months at -70°C , nor had it lost activity after 2 weeks at 4°C ; by contrast crude extracts lost up to 90% of their activity within a week at 4°C . After 5 min at pH 7 about half the activity was lost at 50°C ; all of it was lost at 65°C . The enzyme appeared to be as stable at pH 11 as it was at pH 7, but much less stable at pH 4. The presence of 1 M-glycerol gave some protection against loss of activity both at high temperature and at low pH.

Electrophoretic mobility. The enzyme did not migrate in an anionic gel at pH 8.3. In a cationic gel at pH 4.6 it migrated as a single protein band with an *R* value of 0.57, the position coinciding with the band of enzyme activity. To stain the protein band on the cationic gel it was found that initial fixing with 12.5% trichloroacetic acid for 60 min was necessary; the standard mixture (methanol/acetic acid/water) was not adequate.

M_r and subunits. Chromatography on a calibrated column of Sephacryl S-300 gave an M_r value of 57000. Determination of subunit size by electrophoresis under dissociating conditions with SDS gave two protein bands, with M_r values of 25000 and 30000. The enzyme thus appears to be a dimer of two unequal subunits.

pH optimum. Glycerol oxidation was most rapid under alkaline conditions, decreasing from a maximum at pH 9.5 to zero at pH 6.0. DHA reduction was most rapid at pH 6.0, decreasing to about one-tenth of its maximum value at pH 3.8 and pH 8.0.

Inorganic ion effects. No significant variation in activity was observed for either the forward or reverse reactions when the reaction system contained 100 mM monovalent cation supplied as NH_4^+ , Li^+ , Na^+ , K^+ or $(\text{CH}_3)_4\text{N}^+$. No activation or inhibition of the enzyme was observed when it was incubated with any of the divalent cations Ca^{2+} , Cu^{2+} , Fe^{2+} , Mg^{2+} , Mn^{2+} or Zn^{2+} at 0.1 mM.

Table 1. *Summary of a typical GDH4 purification*

Activity is expressed as $\mu\text{mol min}^{-1}$, specific activity as $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$, measuring NADPH-dependent DHA reduction. Each activity value is the mean of three determinations. The crude extract contained 9.6 g protein.

Fraction	Volume (ml)	Total activity	Specific activity	Purification factor	Yield (%)
Crude extract	400	1120	0.12	1	100
Protamine sulphate treatment	400	1060	0.11	0.92	94
1st $(\text{NH}_4)_2\text{SO}_4$ precipitation	43	920	0.36	3.0	82
Matrex Gel Red A chromatography	150	390	13.1	110	35
2nd $(\text{NH}_4)_2\text{SO}_4$ precipitation	42	390	15.7	130	35

Table 2. *Substrate specificity of GDH4*

Oxidation reactions were measured in 40 mM-glycine/KOH, pH 9.5, with 0.8 mM-NADP⁺, 100 mM-substrate and 20 μg enzyme ml^{-1} ; the specific activity with glycerol was $1.8 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. Reduction reactions were measured in 40 mM-potassium hydrogen phthalate/KOH, pH 6.0, with 0.4 mM-NADPH, 20 mM-substrate and 8 μg enzyme ml^{-1} ; the specific activity with DHA was $12.4 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. Each value is the mean of three determinations from one typical experiment.

Oxidation reaction		Reduction reaction	
Reduced substrate	Relative activity	Corresponding oxidized substrate	Relative activity
Glycerol	100	DHA	100
Ethanediol	0	DL-Glyceraldehyde	1
1,2-Propanediol	30	Glycolaldehyde	1
1,2-Butanediol	19	Hydroxyacetone	2
2,3-Butanediol	25	(1-Hydroxybutan-2-one)	NT
1,2,3-Butanetriol	18	3-Hydroxybutan-2-one	1
(Lactaldehyde) [~]	NT	(1,3-Dihydroxybutan-2-one)	NT
Propan-2-ol	3	Pyrvaldehyde	6
Other alcohols*	0	Acetone	NT
		Other carbonyl compounds†	0

NT, Not tested.

* Includes methanol, ethanol, propan-1-ol, butan-1-ol, 1,3-propanediol.

† Includes formaldehyde, acetaldehyde, butyraldehyde.

Substrate and coenzyme specificity. Table 2 gives a comparison of the relative rates of oxidation or reduction of some compounds structurally related to glycerol or DHA, in the presence of NADP⁺ or NADPH as coenzyme. Glycerol was the substrate most rapidly oxidized, lower activity (20–30%) being found with some diols possessing hydroxyl groups on adjacent carbon atoms, and slight activity with propan-2-ol. DHA was the substrate most rapidly reduced, activity with pyruvaldehyde being about 6% of maximum value, and only slight activity being found with other substrates. There was no measurable activity when NAD⁺ replaced NADP⁺ or NADH replaced NADPH.

Effect of thiol and chelating compounds. Of three thiol compounds tested, L-cysteine and dithiothreitol showed some inhibitory effect (40% and 25% respectively at 10 mM) while 2-mercaptoethanol was more inhibitory (80% at 1 mM). The thiol antagonists iodoacetamide and *N*-ethylmaleimide had little or no effect on activity but *p*-chloromercuribenzoate inhibited strongly. 8-Hydroxyquinoline was also strongly inhibitory (complete inhibition at 10 mM) but 10 mM-EDTA had little effect.

Kinetic properties. Values for apparent K_m and V_{max} measured at optimal pH values for the four principal substrates are given in Table 3.

Table 3. *Kinetic data for GDH4*

K_m and V_{max} values were calculated from Lineweaver-Burk plots in which each point representing enzyme activity was the mean of three determinations.

Substrate tested	Other reactant (mM)	pH of assay	K_m (mM)	V_{max} [$\mu\text{mol min}^{-1}$ ($\mu\text{g protein}^{-1}$)]
Glycerol	NADP ⁺ (0.8)	9.5	51	1.6
NADP ⁺	Glycerol (100)	9.5	0.1	3.4
DHA	NADPH (0.4)	6.0	0.67	2.9
NADPH	DHA (17)	6.0	0.34	10

DISCUSSION

In an earlier paper on glycerol oxidation and triose reduction by *S. pombe* (Kong *et al.*, 1985) we presented evidence that the enzyme we designated GDH4 is a glycerol:NADP⁺ 2-oxidoreductase (EC 1.1.1.156). The present study with the purified enzyme confirms this classification; its activity with glyceraldehyde is no more than 1% of that with DHA and it has an absolute requirement for NADP⁺ or NADPH as coenzyme, NAD⁺ and NADH being inactive. It is thus clearly distinguished from the other types of glycerol:NADP⁺ oxidoreductases which have been described, namely EC 1.1.1.72, which is relatively specific for glycerol and trioses (Viswanath-Reddy *et al.*, 1978), and EC 1.1.1.21, a broad-specificity aldehyde or aldose reductase (Sheys *et al.*, 1971). Both these enzymes, although possessing activity as NADP-dependent DHA reductases, are more active as glyceraldehyde reductases. Only the enzymes from *Aspergillus niger* (Baliga *et al.*, 1962), *Mucor javanicus* (Dutler *et al.*, 1977; Hochuli *et al.*, 1977) and the halophilic alga *Dunaliella parva* (Ben-Amotz & Avron, 1974) appear to be specific DHA reductases similar to GDH4 from *S. pombe*. The enzymes from *S. pombe*, *M. javanicus* and *D. parva* are also similar in size, substrate specificity and lack of activation by monovalent or divalent cations. Little information is available about the *A. niger* enzyme.

Comparing the two glycerol 2-dehydrogenases from *S. pombe*, GDH4 is a dimer, M_r 57000, containing two polypeptides of different sizes, while GDH2 is an octamer, M_r 400000, containing eight polypeptides of the same size. GDH4 differs from GDH2 in not being activated by either monovalent or divalent cations, in showing higher specificity for glycerol or DHA as substrates but lower affinity for them (10-fold less for DHA, 100-fold less for glycerol), and in oxidizing 1,2-propanediol less rapidly than glycerol (activity ratio 0.3; for GDH2, 1.6). Except for propan-2-ol, which was oxidized very slowly by GDH4, the only other compounds oxidized were closely related structurally to glycerol and possessed hydroxyl groups on adjacent carbon atoms; little activity was found with any carbonyl compound other than DHA in the reverse reaction.

Our initial proposal that glycerol is utilized in *S. pombe*, first by oxidation involving GDH2, then by phosphorylation of DHA by a kinase (May & Sloan, 1981; May *et al.*, 1982) has received support from studies with mutants which lack these enzymes (Gancedo *et al.*, 1986; Kong *et al.*, 1987). The metabolic function of GDH2 seems clear, but that of GDH4 is uncertain. It is produced not only in cells growing on glycerol or in glucose-grown cells following exhaustion of glucose, but also in cells growing exponentially on glucose. Since its affinity for glycerol is 100-fold less than that of GDH2, it would seem unlikely that it would have any role in glycerol oxidation when both enzymes are present; only in the absence of GDH2 and in relatively high glycerol concentration would it be likely to function in glycerol oxidation. On the other hand it could operate efficiently in the reverse direction as a DHA reductase synthesizing glycerol. This would agree with the commonly accepted distinction between NAD-dependent enzymes as oxidizing enzymes and NADP-dependent enzymes as reducing enzymes.

The halophile *D. parva*, when subjected to osmotic stress, counteracts the decreased water activity by production of glycerol as a compatible solute (Ben-Amotz & Avron, 1973), and it has been proposed that in this organism GDH4 may be involved in synthesis or removal of glycerol to counteract changes in water activity (Ben-Amotz *et al.*, 1982). When *S. pombe* grows in high

concentrations of glucose or other sugars it is subject to similar osmotic stress, which leads to increased production of glycerol (unpublished results). A possible role of GDH4 in *S. pombe* may therefore be in synthesis of glycerol to counteract osmotic stress, or in removal of excess glycerol when such stress is removed.

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