Purification and Properties of Dihydroxyacetone Kinase from *Schizosaccharomyces pombe*

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Dihydroxyacetone kinase (ATP:dihydroxyacetone phosphotransferase) from *Schizosaccharomyces pombe* has been purified and characterized. It has an M_r of 160000 and consists of four polypeptides of M_r 45000. It shows high substrate specificity, dihydroxyacetone being its only phosphoryl group acceptor and ATP its only phosphoryl group donor; the apparent K_m value for dihydroxyacetone was 16 μ M and that for ATP 550 μ M. It has a pH optimum of 6.5, requires Mg^{2+} or Ca^{2+} (the slightly more active ion), and is inhibited by fluoride or ADP but not by dihydroxyacetone phosphate or fructose 1,6-bisphosphate. The isolated enzyme is relatively unstable.

INTRODUCTION

In previous communications, we have shown that the fission yeast *Schizosaccharomyces pombe* can produce both a glycerol dehydrogenase and a dihydroxyacetone kinase (DHAK), and have suggested that these two enzymes may constitute the main pathway for glycerol dissimilation in this organism; we were unable to detect any glycerol kinase activity (May & Sloan, 1981; May *et al.*, 1982). The purification and properties of the first of these enzymes, glycerol:NAD⁺ 2-oxidoreductase, have been described (Marshall *et al.*, 1985). In this paper we report the purification and characterization of the second enzyme, dihydroxyacetone kinase.

METHODS

General methods. Maintenance of the yeast (*S. pombe* 972 h⁻), the general methods used and sources of chemicals have already been described (Kong *et al.*, 1985; Marshall *et al.*, 1985). After disc gel electrophoresis, done as previously described, gels were stained for protein with Coomassie brilliant blue R. Glycerol-3-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, pyruvate kinase and phosphoenolpyruvate (PEP) were obtained from Boehringer-Mannheim.

Growth of cells and preparation of cell extracts. Cells for extraction and purification of DHAK were grown on a modified Edinburgh minimal medium no. 2 (Mitchison, 1970), in which the glucose concentration was reduced to 0.5% (w/v); they were harvested in the stationary phase at a time when all the glucose had been utilized, to ensure full derepression of the enzyme. Cell extracts were prepared as described previously (May & Sloan, 1981) except that the cells were suspended in a Tris/HCl buffer (50 mM, pH 8.0) containing 1 mM-phenylmethylsulphonyl fluoride (PMSF).

Assay of kinase activity. Two methods were used: a method depending on measuring production of a specific phosphorylated product of the substrate (method 1), and a general method in which production of ADP is measured (method 2).

Method 1. Unless otherwise specified, all assays for DHAK were done using this method, with dihydroxyacetone (DHA) as substrate, essentially as described by May *et al.* (1982). The assay mixture (total volume 1.0 ml) contained buffer, the DHAK preparation, 5 mM-ATP, 5 mM-MgCl₂, 3.3 mM-DHA, 0.33 mM-NADH and 8.5 units glycerol-3-phosphate dehydrogenase, the latter being omitted from the blank cuvette. Crude

Abbreviations: DHA, dihydroxyacetone; DHAK, dihydroxyacetone kinase; PEP, phosphoenolpyruvate; PMSF, phenylmethylsulphonyl fluoride.

extracts which contained NADH oxidase activity were assayed at pH 9.5 using 40 mM-glycine/KOH buffer while preparations free of this activity were assayed at pH 6.5 using 50 mM-maleic acid/NaOH buffer. When the substrate was glycerol, glycerol (2 mM) replaced DHA and NAD^+ (0.66 mM) replaced NADH. When the substrate was glyceraldehyde, DL-glyceraldehyde (2 mM) replaced DHA, NAD^+ (0.66 mM) replaced NADH, glyceraldehyde-3-phosphate dehydrogenase (5 units) replaced glycerol-3-phosphate dehydrogenase and 5 mM-sodium arsenate was included (Amelunxen & Carr, 1975).

Method 2. ADP production was measured by the method of Hayashi & Lin (1967) in which it is linked through pyruvate kinase, PEP and lactate dehydrogenase to NADH oxidation.

Purification of DHAK. All steps were done at 0–4 °C.

Step 1. Protamine sulphate precipitation. Protamine sulphate solution (10%, w/v) was added slowly to cell extract to a final concentration of 1 mg protamine sulphate to 10 mg protein. After stirring gently for 30 min, the precipitate was removed by centrifugation at 17000 *g* for 30 min and discarded.

Step 2. Ammonium sulphate fractionation. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant fluid from step 1 to a concentration of 2.6 M. After stirring for 60 min the resulting precipitate was removed by centrifugation at 12000 *g* for 30 min and discarded. More $(\text{NH}_4)_2\text{SO}_4$ was then added to the supernatant fluid to bring its concentration to 3.4 M; this precipitated more material which was again discarded.

Step 3. DEAE-cellulose chromatography. The supernatant fluid from step 2, which contained the DHAK activity, was desalted by passage through a Sephadex G-25 column equilibrated with 50 mM-Tris/HCl, pH 8.0, containing 40 mM-KCl. The protein fraction was concentrated to 10 ml by ultrafiltration through a membrane filter (Diaflo PM10) in a stirred cell (Amicon 8050). The concentrated protein was then applied to a DEAE-cellulose column (Whatman DE 52, 30 × 2.6 cm) which had been equilibrated with the same buffer, and enzyme activity was eluted with a linear KCl gradient (40–150 mM-KCl in 50 mM-Tris/HCl).

Step 4. Gel permeation chromatography. Fractions from step 3 with DHAK activity were pooled and concentrated to 3.0 ml by ultrafiltration. This solution was then applied to a Sephadex G-100 column (82 × 1.6 cm) previously equilibrated with 50 mM-Tris/HCl (pH 8.0) and fractions were eluted from the column with the same buffer; those containing activity were pooled and concentrated.

RESULTS

Purification of enzyme

Initial attempts to purify the enzyme from cell extracts were frustrated by rapid loss of activity during manipulation. Three modifications of the initial procedure reduced, but did not eliminate, these losses: incorporation of PMSF in the buffer used for cell disintegration; use of protamine sulphate in place of ethodin (6,9-diamino-2-ethoxyacridine lactate) for removing nucleic acids; and avoidance of any interruption or delay in the procedure once the cells had been disrupted.

An overall purification of about 50-fold was achieved, but yields were variable and never high. A representative set of results is given in Table 1. The product from step 4 (gel permeation) was used for subsequent characterization. An additional step of gel permeation chromatography using Sephacryl S-300 gave no increase in specific activity and resulted in further loss of enzyme activity.

In the fractionation with $(\text{NH}_4)_2\text{SO}_4$ the two-stage precipitation with first 2.6 M and then 3.4 M- $(\text{NH}_4)_2\text{SO}_4$ proved to be more effective in removing inactive protein than one single precipitation with 3.4 M- $(\text{NH}_4)_2\text{SO}_4$. Increasing the $(\text{NH}_4)_2\text{SO}_4$ concentration of the supernatant fluid to saturation (3.8 M) precipitated some enzyme activity but still left much of it in solution and was not a satisfactory method of purification.

Properties of enzyme

Stability. The purified enzyme was stable when stored at –70 °C for several months but could not be kept for more than a few days at 4 °C; all activity was lost within 5 min at 37 °C. However, glycerol gave some protection; when present at 1.0 M about 15% of the activity was retained after 5 min at 37 °C. Neither EDTA nor dithiothreitol prevented loss of activity.

M_r and subunits. Chromatography on a calibrated column of Sephacryl S-300 gave an M_r value of 160000. Determination of subunit size by electrophoresis under dissociating conditions with SDS gave a single protein band with M_r 45000, indicating that the undissociated protein contains four identical subunits.

Table 1. *Summary of purification procedure*

Activity is expressed as $\mu\text{mol min}^{-1}$, specific activity as $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. The crude extract contained 4.3 g protein. For details see Methods.

Fraction	Volume (ml)	Total activity	Specific activity	Purification factor	Yield (%)
Crude extract	180	34	0.008	1.0	100
Protamine sulphate treatment	175	19	0.009	1.12	56
$(\text{NH}_4)_2\text{SO}_4$ fractionation	10	10	0.12	15	29
DEAE-cellulose chromatography	3	3	0.19	24	9
Sephadex G-100 chromatography	0.7	0.5	0.41	51	1.5

pH optimum. The optimum pH for enzyme activity was 6.5. Measurement of activity between pH 5.5 and 7.5 was made with a mixed buffer (40 mM-Tris/40 mM-HEPES/40 mM-MES/HCl or NaOH) and at pH 8.6 and 9.5 with 50 mM-glycine/KOH. There was a sharp decrease in activity on the acid side of pH 6.5, and a more gradual decrease on the alkaline side. Essentially the same results were obtained over a pH range of 5.5 to 8.5 when another mixed buffer (50 mM-maleic acid/50 mM-triethanolamine/HCl or NaOH) was used.

Effect of divalent cations. When Mg^{2+} in the assay mixture was replaced by 5 mM- Ca^{2+} , a 20% increase in activity was observed; replacement by Mn^{2+} or Co^{2+} decreased activity by 85%.

Substrate specificity. The only substrate whose phosphorylation was catalysed by the enzyme was DHA. No activity was detected with glycerol or DL-glyceraldehyde by either method, or with sodium DL-glycerate, hydroxyacetone, propan-1-ol, 1,2-propanediol, 1,3-propanediol or D-xylulose (each at 2 mM) by method 2. The requirement for ATP was equally specific, no activity being detectable when ATP was replaced by 5 mM-GTP or 5 mM-UTP.

Effect of inhibitors. Several compounds were tested for their ability to inhibit DHAK by measuring their effect on its activity using assay method 1. Those which were inhibitory were then tested separately for any inhibitory effect on glycerol-3-phosphate dehydrogenase; only cyanide inhibited this enzyme and thus its effect on DHAK was not measurable by this method. Fluoride produced 50% inhibition at 10 mM and complete inhibition at 100 mM, while ADP produced 40% inhibition at 5 mM. Addition of dihydroxyacetone phosphate (5 mM), fructose 1,6-bisphosphate (5 mM) or glycerol (up to 1 M) caused no inhibition.

Michaelis constants. At pH 6.5 in the presence of 5 mM-ATP the apparent K_m and V_{max} for DHA were 16 μM and 1.0 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ respectively. In the presence of 3.3 mM-DHA the apparent K_m and V_{max} for ATP were 550 μM and 1.0 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ respectively.

DISCUSSION

The results show that the DHAK of *S. pombe*, as suggested previously (May *et al.*, 1982), is a highly specific enzyme whose only phosphoryl group acceptor is DHA, and whose only phosphoryl group donor is ATP. The high specificity and several other properties such as lack of inhibition by fructose 1,6-bisphosphate distinguish it from the various glycerol kinases (ATP:glycerol phosphotransferases; EC 2.7.1.30) whose primary function appears to be in glycerol metabolism but which nevertheless are also capable, to varying degrees, of catalysing phosphorylation of DHA or glyceraldehyde (Thorner & Paulus, 1973). It is also different from the triokinases (ATP:D-glyceraldehyde-3-phosphotransferases; EC 2.7.1.28) which react with glyceraldehyde or DHA but not with glycerol (Hers, 1962; Beutler & Guinto, 1973).

An enzyme whose primary function appears to be the phosphorylation of DHA (i.e. an ATP:dihydroxyacetone phosphotransferase) has been reported in the methanol-utilizing yeasts *Hansenula polymorpha* and *Candida boidinii* (van Dijken *et al.*, 1978) and *Candida methylca* (Hofmann & Babel, 1981). It resembles the *S. pombe* enzyme in being readily inactivated, so much so that only partial purification was achieved, but differs in showing considerable activity

with DL-glyceraldehyde (25% of the activity shown with DHA). The main metabolic function of this enzyme would seem to be as an essential component of the pathway for methanol utilization.

A closely similar enzyme was obtained from the halophilic green algae *Dunaliella parva* and *Dunaliella salina* (Lerner & Avron, 1977; Lerner *et al.*, 1980). It also was relatively unstable; while its rate of inactivation was reduced by the presence of 2 M-glycerol, only partial purification was achieved. This enzyme, like the *S. pombe* enzyme, appeared to be highly specific for DHA and without activity on glyceraldehyde; its M_r was lower than that of the *S. pombe* enzyme (between 65000 and 100000) and its pH optimum higher (7.5).

A DHAK from *Klebsiella pneumoniae* has also been purified and partly characterized; it appears to resemble the *S. pombe* enzyme in several respects, but differs in being a dimer instead of a tetramer and has an M_r of 95000 as opposed to 160000. Though it reacted with DHA and not with glycerol, glyceraldehyde does not appear to have been tested (Johnson *et al.*, 1984).

An unusual feature of the DHAK of *S. pombe* is the relatively high activity, as a phosphoryl group donor, of CaATP, which was 20% more active than MgATP under the conditions used. For other kinase enzymes the essential divalent cation is normally Mg^{2+} . However, the DHAK of *K. pneumoniae* was also reported to be at least as active with Ca^{2+} as with Mg^{2+} (Johnson *et al.*, 1984) though that from *C. methylica* was only 30% as active with Ca^{2+} as with Mg^{2+} (Hofmann & Babel, 1981).

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