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Purification and characterization of an NAD^+-linked formaldehyde dehydrogenase from the facultative RuMP cycle methylotroph *Arthrobacter* P1

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Abstract

When *Arthrobacter* P_1 is grown on choline, betaine, dimethylglycine or sarcosine, an NAD^+-dependent formaldehyde dehydrogenase is induced. This formaldehyde dehydrogenase has been purified using ammonium sulphate fractionation, anion exchange- and hydrophobic interaction chromatography. The molecular mass of the native enzyme was 115 kDa ± 10 kDa. Gel electrophoresis in the presence of sodium dodecyl sulphate indicated that the molecular mass of the subunit was 56 kDa ± 3 kDa, which is consistent with a dimeric enzyme structure. After ammonium sulphate fractionation the partially purified enzyme required the addition of a reducing reagent in the assay mixture for maximum activity. The enzyme was highly specific for its substrates and the K_m values were 0.10 and 0.80 mM for formaldehyde and NAD^+, respectively. The enzyme was heat-stable at 50° C for at least 10 min and showed a broad pH optimum of 8.1 to 8.5. The addition of some metal-binding compounds and thiol reagents inhibited the enzyme activity.

Abbreviation: RuMP – Ribulose monophosphate

Introduction

Arthrobacter P1 is a Gram-positive facultative methylotroph which uses the Ribulose monophosphate (RuMP) cycle for formaldehyde fixation during growth on methylamine. In common with other organisms which use this assimilation pathway, no formaldehyde or formate dehydrogenase was detected but instead the formaldehyde was oxidized to carbon dioxide using RuMP cycle enzymes [Hexulose phosphate synthase and hexulose phosphate isomerase] and pentose phosphate pathway enzymes [glucose 6-phosphate and 6-phosphogluconate dehydrogenases] (Beardsmore et al. 1982; Levering et al. 1981a). Subsequent studies showed that the enzymes associated with the RuMP cycle were synthesized when the organism was grown on choline and the intermediates of choline degradation to sarcosine. The catabolism of choline results in the production of glycine and formaldehyde in the ratio 1:3. One of the formaldehyde molecules reacts with the glycine to produce serine and the remaining molecules were assumed to react with ribulose monophosphate and enter the RuMP cycle (Levering et al. 1981b). Choline metabolism, however, was unimpaired in mutants which lacked the ability to synthesize hexulose phosphate synthase and thereby were unable to grow on methylamine (Levering et al. 1987). Instead the growth on choline was found to be dependent upon the synthesis of an NAD^+-dependent formaldehyde dehydrogenase. The synthesis of this enzyme was not limited to the mutant since it also was detected in wild-type *Arthrobacter* P1 when the organism was grown on choline, betaine, dimethylglycine or sarcosine. Formaldehyde dehydrogenase activity, however, could not be detected in cells grown on methylamine and, more significantly, not in cells from formaldehyde-limited chemostat cultures (Levering et al. 1987). These findings raised several questions, in particular, what are the properties of this NAD^+-dependent formaldehyde dehydrogenase and what is its role? This paper addresses the first question by reporting the purification and characterization of the enzyme and discusses the second question.

Materials and methods

Organism and cultivation

Arthrobacter P1 (NCIB 11625) and its maintenance have been described (Levering et al. 1981a). Batch cultures in conical flasks filled to 25% of the volume with a mineral salts medium (Levering et al. 1981a) containing the appropriate carbon source (0.2%) were grown at 30° C with shaking. Cells in the mid-exponential phase of growth were harvested by centrifugation (6,000 g for 20 min).

Preparation of cell-free extracts

The harvested cells were washed once, resuspended in 50 mM Tris-HCl buffer pH 7.5 containing 5 mM KCl and disrupted using ultrasonication (MSE 150W) 15 × 30s interspersed with 30s cooling periods. Unbroken cells and debris were removed by centrifugation at 38,000 g for 20 min at 0–4° C. The supernatant was used as the cell-free extract for enzyme assays and enzyme purification.

Enzyme assay

Formaldehyde dehydrogenase (NAD^+-dependent) (EC 1.2.1.-) was assayed at 30° C in a reaction mixture (final volume 1 ml) containing 50 mM potassium phosphate buffer pH 8.0, 0.8 mM NAD^+ and extract. The reaction was started by the addition of 10 mM formaldehyde. Dithiothreitol (4 mM) was added to the assay mixture in order to measure maximum activity.

Protein determination

Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Purification of formaldehyde dehydrogenase

All procedures were carried out at 0–4° C. The chromatography columns used were connected to a Pharmacia FPLC system. The cell-free extract was fractionated using ammonium sulphate precipitation. Finely ground solid ammonium sulphate was added with stirring and the protein which precipitated between 40 and 75% saturation was collected and dissolved in the minimum amount of 50 mM Tris-HCl buffer pH 7.5 containing 5 mM KCl (Buffer A). The enzyme was desalted by applying it to a pre-packed Sephadex G25 gel filtration column (Bio-Rad PD 10), equilibrated and eluted with buffer A. The filtrate was added to a Mono-Q HR5/5 anion exchange column washed and equilibrated with 20 mM Tris-HCl buffer pH 7.5 containing 1 mM KCl (Buffer B). Proteins were eluted from the column at a flow rate of $1.0\,ml \cdot min^{-1}$ by applying a 30 ml linear 0–1.0 M KCl gradient in buffer B. Fractions (1 ml) were collected and those containing activity were pooled. Ammonium sulphate was added to a final concentration of 1.0 M. Solid particles were removed by centrifugation for 10 min in an Eppendorf centrifuge (maximum speed) and the supernatant was loaded on a Phenyl-Superose HR5/5 hydrophobic interaction column, equilibrated with buffer B containing 1.0 M ammonium sulphate. Bound protein was eluted with a 30 ml linear 1.0–0 M ammonium sulphate gradient in buffer B at a flow rate of $0.5\,ml \cdot min^{-1}$. Active fractions (0.5 ml) were pooled and stored in small aliquots at − 80° C.

Determination of molecular mass

The molecular mass of the native formaldehyde dehydrogenase was determined by using a calibrated Superose 12 HR10/30 gel filtration column, equilibrated with 100 mM potassium phosphate buffer pH 7.5. A sample of the purified enzyme was applied to the Superose 12 column and eluted at 0.5 ml·min^{-1}. The standard marker components were thyroglobulin (bovine), 670 kDa; gamma globulin (bovine), 150 kDa; ovalbumin (chicken), 44 kDa; myoglobin (horse), 17 kDa and vitamin B12, 1.35 kDa.

Electrophoretic separations

Denaturing gel electrophoresis (SDS-PAGE 12.5% polyacrylamide gel) was used as described by Laemmli and Favre (1973). The proteins used as calibration reference markers were: phosphorylase A, 94 kDa; human transferrin, 80 kDa; bovine serum albumin, 68 kDa; catalase, 58 kDa; fumarase, 50 kDa; citrate synthase, 46 kDa and carbonic anhydrase, 31 kDa. The gels were stained with Coomassie brilliant blue G-250.

Results

Purification of NAD$^+$-formaldehyde dehydrogenase from choline-grown cells

The initial step of the enzyme purification involved protein fractionation using ammonium sulphate precipitation. Thereafter a number of different chromatographic procedures, including DEAE-cellulose ion exchange and hydroxylapatite chromatography, were screened to further purify the enzyme. None of these techniques, however, were successful in retaining significant activity. Finally anion exchange and hydrophobic interaction chromatography columns attached to an FPLC system were used (Table 1). The anion exchange chromatography step yielded a preparation that gave only 4 to 5 protein bands on SDS-PAGE. A subsequent hydrophobic interaction chromatography step for unknown reasons consistently resulted in a considerable loss in both total and specific formaldehyde dehydrogenase activities. This nevertheless allowed further purification of the enzyme, resulting in a homogeneous preparation as judged by SDS-PAGE. During these studies only a single formaldehyde dehydrogenase enzyme species could be detected in choline-grown cells of *Arthrobacter* P1. Since there were no indications that the hydrophobic interaction chromatography step modified the formaldehyde dehydrogenase protein, the properties of the enzyme preparation obtained were studied in more detail.

Molecular mass and structure of the enzyme

The native molecular mass of the enzyme was determined using a Superose 12 gel filtration column. The elution of the enzyme was consistent with a value of 115 kDa ± 10 kDa. SDS-PAGE revealed a subunit molecular mass of 56 kDa ± 3 kDa. This suggested that the NAD$^+$-formaldehyde dehydrogenase is a dimer.

pH specificity

Enzyme activity was detected over the pH range 7.5 to 9.0. Sodium potassium phosphate buffer was

Table 1. Purification of NAD$^+$-dependent formaldehyde dehydrogenase from choline-grown cells of *Arthrobacter* P1.

Step	Total volume (ml)	Total protein (mg)	Total activity (nmol·min^{-1})	Specific activity (nmol·min^{-1}·mg^{-1} protein)	Yield (%)	Purification factor
Crude extract	2.9	57.5	1398	24.3	100	1.00
$(NH_4)_2SO_4$ (40–75%)	1.4	22.1	1492	67.5	107	2.78
Sephadex G25	2.9	21.4	1142	53.4	82	2.19
Mono-Q pool	1.1	1.11	498	449	36	18.5
Phenyl-Superose pool	1.2	0.53	175	330	13	13.6

used over the range pH 7.5 to 8.0 and Tris-HCl from pH 7.8 to 9.0. The Tris-HCl buffer showed 25% inhibition with respect to the activity in sodium potassium phosphate buffer at the same pH value. Subsequent studies revealed that K^+, and to a lesser extent, Mg^{2+} ions restored activity in Tris buffers. Consequently, the buffers used were either potassium phosphate buffer, or Tris-HCl buffer containing 5 mM KCl. The optimum pH for activity was found to occur over a broad peak from pH 8.1 to 8.5.

Stability of the enzyme and the effect of the addition of reducing agents

The enzyme was relatively stable to temperatures above ambient room temperatures. No significant loss of activity was detected for instance when the enzyme was incubated at 50° C for 10 min. When stored at 4° C for two weeks, 50% of the initial activity was retained. When the enzyme was frozen at − 18° C, full activity was retained for at least two weeks. Repeated freezing and thawing did not reduce significantly the activity. The activity in crude cell-free extract was not increased significantly by the addition of a reducing agent, either in the preparation of the extract or in the assay mixture itself. As the purification proceeded beyond the ammonium sulphate fractionation, the requirement for the addition of a reducing agent in the assay mixture became progressively important for the measurement of maximum activity. GSH or DTT (3–5 mM) could be used.

Substrate specificity

The enzyme showed a high specificity for its substrates. Potential aldehyde substrates were tested at concentrations of 1, 5 and 25 mM. No activity was detected when formaldehyde was substituted at the lower concentrations by a range of aldehydes, including propionaldehyde, n-butyraldehyde, isobutyraldehyde, valeraldehyde, glycoaldehyde, benzaldehyde, or methanol. Only at the highest (non-physiological) concentration activity with acetaldehyde was detected, at a level of 14% of the activity measured in the presence of formaldehyde (1 mM). $NADP^+$ could not replace NAD^+. No activity was detected with the artificial electron acceptors phenazine methosulphate (1 mM), phenazine methosulphate (1 mM) plus dichlorophenol indophenol (0.2 mM), ferricyanide (20 mM) and cytochrome c (0.1 mM).

Kinetic properties

The initial rate of enzyme activity (in the absence of dithiothreitol) was studied as a function of the substrate concentration. The enzyme displayed normal Michaelis-Menten type of kinetics and the following kinetic constants were derived from double reciprocal plots: apparent K_m and V_{max} values for formaldehyde, 0.10 mM and 0.70 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ of protein, respectively (at a fixed NAD^+ concentration of 0.80 mM and formaldehyde concentrations varying between 0.05–20 mM); apparent K_m and V_{max} values for NAD^+: 0.80 mM and 0.42 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ of protein, respectively (at a fixed formaldehyde concentration of 10 mM and NAD^+ concentrations varying between 0.075–2.5 mM).

Inhibitor studies

Various metal chelating agents (2.0 mM) were tested as potential inhibitors of enzyme activity. EDTA had no effect but 2, 2′-bipyridine (50%) and 1, 10-phenanthroline, 8-hydroxyquinoline (100%) caused inhibition. Similarly thiol reagents (2.6 mM) were tested. Iodoacetamide had no effect on the enzyme activity but N-ethylmaleimide caused 40% inhibition.

Discussion

Formaldehyde is a toxic compound for all organisms. Its accumulation may be prevented either via fixation by the various pathways of formaldehyde assimilation, or via degradation by the action of formaldehyde dehydrogenases (Attwood & Quayle 1984; Sondossi et al. 1986) or formaldehyde dismutases (Adroer et al. 1990; Kato et al. 1984).

The different formaldehyde dehydrogenases that have been described can be divided into two main groups: the NAD^+-dependent enzymes which are induced by the presence of C_1 compounds and which may or may not require glutathione or other low molecular weight factors for activity (van Dijken et al. 1976; Stirling & Dalton 1978; Eggeling & Sahm 1985), and the dye-linked enzymes which are generally non-specific for the aldehyde substrate and not induced by growth on one-carbon compounds (Mehta 1975; Marison & Attwood 1980).

Since *Arthrobacter* P1 growing on methylamine assimilates the one-carbon compound via the RuMP cycle and oxidizes formaldehyde to carbon dioxide with the cyclic dissimilatory pathway (Levering et al. 1981a), it was unexpected when an NAD^+-formaldehyde dehydrogenase activity was measured in extracts prepared from choline-, betaine-, dimethylglycine- or sarcosine-grown cells (Levering et al. 1987). The purification of the enzyme in *Arthrobacter* P1 allowed a comparison of its properties with the NAD-dependent formaldehyde dehydrogenases from liver (Uotila & Koivusala 1974), pea seeds (Uotila & Koivusala 1979), yeasts (van Dijken et al. 1976; Schütte et al. 1976) and various G^- and G^+ bacteria (Stirling & Dalton 1978; Ando et al. 1979; Betts 1984; Eggeling & Sahm 1985; van Ophem & Duine 1990). To our knowledge, the *Arthrobacter* P1 enzyme is the first NAD-dependent, non-glutathione, non-factor-dependent formaldehyde dehydrogenase characterized from G^+ bacteria. The *Arthrobacter* P1 enzyme most closely resembles the formaldehyde dehydrogenase characterized from betaine-grown cells of *Pseudomonas putida* C-83 (Ando et al. 1979; Ogushi et al. 1984, 1986), although there are significant differences as well (Table 2). Most importantly, these enzymes share a high specificity for formaldehyde, whereas other formaldehyde dehydrogenases studied generally show activity with a small to wide range of aldehydes. Furthermore both these enzymes displayed a higher affinity for formaldehyde than observed for other enzymes.

The structure of most formaldehyde dehydrogenases has been described as dimers with a native molecular mass of 100–120 kDa with identical subunits of 44–47 kDa. The enzyme from *Arthrobacter* P1 also appears to be a dimer with a native molecular mass consistent with that reported for other

Table 2. Comparison of the properties of NAD^+-formaldehyde dehydrogenase from choline-grown *Arthrobacter* P1, betaine-grown *Pseudomonas putida* C-83[1] and methanol-grown *Methylophilus methylotrophus*[2].

Property	*Arthrobacter* P1	*Pseudomonas putida* C-83	*Methylophilus methylotrophus*
*M*r (native)	115,000	150,000	–
*M*r (subunit)	56,000	75,000	–
pH optimum	8.1–8.5	7.8	8.0
Km (mM)			
formaldehyde	0.10	0.067	12
NAD^+	0.80	0.056	0.54
V_{max} ($\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein)			
formaldehyde	0.70	10	0.072
NAD^+	0.42	–	0.049
Substrate specificity	formaldehyde	formaldehyde	wide range of aldehydes
Electron acceptors	NAD^+	NAD^+	NAD^+
Addition of reducing agents	stimulates activity	no effect	stimulates activity

[1] Ando et al. (1979); Ogushi et al. (1984; 1986).
[2] Betts (1984).
–, No data available.

formaldehyde dehydrogenases. The *P. putida* enzyme on the other hand is clearly larger (Table 2). Moreover the enzyme from this latter organism was equally active in phosphate buffer and Tris-HCl buffer (Ogushi et al. 1984). The activity of the enzyme from *Arthrobacter* P1 showed a significant decrease in activity when Tris-HCl was used rather than potassium phosphate buffer. This decrease in activity can be restored, to some extent, by the addition of 5 mM KCl to the Tris-HCl buffer. This requirement for K^+ ions may explain the inhibitory effect observed when 1, 10-phenanthroline or 8-hydroxyquinoline are added to the enzyme from *Arthrobacter* P1. The inability of EDTA to affect enzyme activity is not clear.

The question to be considered is what induces this specific formaldehyde dehydrogenase in *Arthrobacter* P1 and what is its role? Since the enzyme is expressed during growth on choline and betaine and the degradation of these compounds results in the release of formaldehyde the initial thought was that the enzyme was associated with the control of the levels of formaldehyde in the cell. However, during growth on sarcosine it would appear that formaldehyde should be released in a manner similar to the release of formaldehyde during growth on methylamine and yet cells grown on sarcosine synthesize the enzyme whilst methylamine-grown cells do not show activity. Finally it has been shown that formaldehyde induces the synthesis of hexulose phosphate synthase and hexulose phosphate isomerase, the first two enzymes of the RuMP cycle (Levering et al. 1987), but not formaldehyde dehydrogenase. To date the inducer molecule for the synthesis of the NAD^+-formaldehyde dehydrogenase and its specific role in *Arthrobacter* P1 remains to be elucidated.

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