Purification and Properties of an NAD(P)⁺-linked Formaldehyde Dehydrogenase from *Methylococcus capsulatus* (Bath)

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Crude soluble extracts of *Methylococcus capsulatus* strain Bath, grown on methane, were found to contain NAD(P)⁺-linked formaldehyde dehydrogenase activity. Activity in the extract was lost on dialysis against phosphate buffer, but could be restored by supplementing with inactive, heat-treated extract (70 °C for 12 min). The non-dialysable, heat-sensitive component was isolated and purified, and has a molecular weight of about 115000. Sodium dodecyl sulphate gel electrophoresis of the protein suggested there were two equal subunits with molecular weights of 57000. The heat-stable fraction, which was necessary for activity of the heat-sensitive protein, was trypsin-sensitive and presumed to be a low molecular weight protein or peptide. A number of thiol compounds and other common cofactors could not replace the component present in the heat-treated soluble extract. The purified formaldehyde dehydrogenase oxidized three other aldehydes with the following K_m values: 0.68 mM (formaldehyde); 0.075 mM (glyoxal); 7.0 mM (glycolaldehyde); and 2.0 mM (DL-glyceraldehyde). NAD⁺ or NADP⁺ was required for activity, with K_m values of 0.063 and 0.155 mM respectively, and could not be replaced by any of the artificial electron acceptors tested. The enzyme was heat-stable at 45 °C for at least 10 min and had temperature and pH optima of 45 °C and pH 7.2 respectively. A number of metal-binding agents and substrate analogues were not inhibitory. Thiol reagents gave varying degrees of inhibition, the most potent being *p*-hydroxymercuribenzoate which at 1 mM gave 100% inhibition. The importance of possessing an NAD(P)⁺-linked formaldehyde dehydrogenase, with respect to *M. capsulatus*, is discussed.

INTRODUCTION

Seven different types of formaldehyde dehydrogenase activity have been reported in C₁-utilizing bacteria (Table 1). These various types can be roughly divided into two groups: (1) NAD(P)⁺-linked enzymes, and (2) NAD(P)⁺-independent enzymes. The enzymes in the first group include both formaldehyde-specific and non-specific aldehyde dehydrogenases, and many require glutathione for activity. The second group of enzymes all require artificial electron acceptors to assay their activity *in vitro*, and again some can utilize various other aldehydes as substrates. One prominent enzyme in group 2 is type 7, the ubiquitous methanol dehydrogenase, first described by Anthony & Zatman (1964), found in all methylotrophs capable of growing on methanol and methane. The ability of this enzyme to oxidize formaldehyde and, in some cases, acetaldehyde (Sperl, Forrest & Gibson, 1974) may be due to the substrate being predominantly in the hydrated form in solution.

Formaldehyde can also be oxidized to CO₂ and water by a cyclic series of reactions involving hexulose phosphate synthase (Strøm, Ferenci & Quayle, 1974; Colby & Zatman, 1975). Theoretically, this pathway could generate two molecules of NAD(P)H per molecule of formaldehyde oxidized.

Table 1. *Different types of formaldehyde dehydrogenase enzymes found in C₁-utilizing bacteria*

Type	NAD(P) ⁺ -linked		Artificial electron acceptor*	Substrate specificity			Activator	Organism	Ref.†
	Gluta-thione dependent	Gluta-thione independent		Form-aldehyde	Other aldehydes	Other compounds			
Group 1									
1	+	—	—	+	—	—	—	<i>Methylomonas methanica</i> (Texas)	1
								<i>Methylomonas methanica</i> (Iowa)	2
								<i>Pseudomonas extorquens</i>	1
								Bacterium 5H2	3
								<i>Protaminobacter ruber</i>	1
								<i>Pseudomonas aminovorans</i>	4
2	—	+	—	+	—	—	—	<i>Pseudomonas</i> MS	5
3	—	+	—	+	+	—	—	Bacterium 4B6	6
4	—	+	—	+	—	—	Tetrahydrofolate	<i>Pseudomonas</i> AM1	7
								<i>Methylobacterium organophilum</i>	8
Group 2									
5	—	—	DCPIP or K ₃ Fe(CN) ₆	+	—	—	—	<i>Pseudomonas</i> RJ ₁	9
6	—	—	DCPIP or PMS	+	+	—	—	<i>Pseudomonas</i> AM1	1
								<i>Pseudomonas aminovorans</i>	4
7	—	—	PMS	+	± (CH ₃ CHO)	+	NH ₃ or CH ₃ NH ₂	<i>Methylococcus capsulatus</i> (Texas)	10
								<i>Pseudomonas</i> M27	11
								<i>Hyphomicrobium</i> WC	12
								<i>Pseudomonas</i> TPI	12
								<i>Pseudomonas</i> W	12
								<i>Methylosinus sporium</i>	13
								<i>Pseudomonas</i> RJ ₁	14
								<i>Methylobacterium organophilum</i>	8

* DCPIP, 2,6-Dichlorophenolindophenol; PMS, phenazine methosulphate.

† (1) Johnson & Quayle, 1964; (2) Harrington & Kallio, 1960; (3) Hampton & Zatman, 1973; (4) Boulton & Large, 1977; (5) Kung & Wagner, 1970; (6) Colby & Zatman, 1973; (7) Large & Quayle, 1963; (8) O'Connor & Hanson, 1977; (9) Mehta, 1975; (10) Patel & Hoare, 1971; (11) Anthony & Zatman, 1964; (12) Sperl *et al.*, 1974; (13) Patel & Felix, 1976; (14) Mehta, 1973.

To date, no formaldehyde dehydrogenase has been found in *Methylococcus capsulatus* (Texas) (Patel & Hoare, 1971) or *M. capsulatus* (Bath) other than the non-specific primary alcohol dehydrogenase, present in both. This paper describes the isolation of an NAD(P)⁺-linked formaldehyde dehydrogenase from *M. capsulatus* (Bath) which consists of at least two components. The purification of the heat-sensitive, non-dialysable component of the enzyme and some of its properties are also described.

METHODS

Growth of organism and preparation of soluble extracts. *Methylococcus capsulatus* (Bath) (Whittenbury, Phillips & Wilkinson, 1970) was grown at 45 °C as a batch culture on ammonium mineral salts (AMS) medium (Dalton & Whittenbury, 1976) in a 100 l fermenter (L. H. Engineering, Stoke Poges, Buckinghamshire). Methane (25%, v/v, in air) was supplied as the carbon source. Cells were harvested and soluble extracts were prepared as described previously (Colby, Stirling & Dalton, 1977).

Enzyme assay and units. Formaldehyde dehydrogenase activity was assayed spectrophotometrically by following the reduction of NAD⁺ at 340 nm. All assays, except where specified, were carried out in 1.5 ml cuvettes at 45 °C. The reaction mixture contained (in 1.5 ml): potassium phosphate buffer, pH 7.2, 30 μmol; NAD⁺, 1 μmol; KCN, 3.75 μmol; and various amounts of test protein. After 3 min preincubation, the

reaction was started by adding formaldehyde (3.75 μmol). After step 1 in the purification of the heat-sensitive component (HSC) of the formaldehyde dehydrogenase, the partially purified or purified HSC required the presence of heat-treated soluble extract (HTSE) to regain activity. One unit of activity was defined as the amount of enzyme required to reduce 1 $\mu\text{mol NAD}^+$ min^{-1} .

Preparation of heat-treated soluble extract. The HTSE was prepared by incubating crude soluble extract at 70 °C in a water bath for 12 min, then centrifuging the extract for 3 min to remove the precipitated protein. The protein concentration of the HTSE was 16 mg ml^{-1} .

Trypsin treatment of the heat-treated soluble extract. Samples of HTSE (0.75 ml, 12 mg protein) were dispensed into four micro-centrifuge tubes (1.5 ml capacity). Tube 1 was kept in ice for 30 min and tubes 2, 3 and 4 were incubated at 37 °C in a shaking water bath. Before incubation, 2 mg trypsin (280 BAEE units) was added to tube 3, and 2 mg trypsin plus 4 mg trypsin inhibitor were added to tube 4. After the 30 min incubation period, 4 mg trypsin inhibitor was added to tube 3, and then all four tubes were stored in ice.

Purification of heat-sensitive component of formaldehyde dehydrogenase. Step 1: ammonium sulphate treatment. Ammonium sulphate fractionation of 15 ml crude soluble extract was carried out at 4 °C in the usual manner, with the fraction precipitating between 30 and 55 % saturation being collected. The pellet was redissolved in the minimum volume (2.8 ml) of 20 mM-potassium phosphate buffer, pH 7.2, at 4 °C.

Step 2: Sephadex G-75 column chromatography. The redissolved pellet was applied at 4 °C to a column (3.8 $\times$ 90 cm) of Sephadex G-75, previously equilibrated with 20 mM-potassium phosphate buffer, pH 7.2, and eluted with similar buffer at 4 °C. Fractions (3 ml) were collected, and those with formaldehyde dehydrogenase activity were pooled (28 ml) and concentrated at 4 °C in an Amicon ultrafiltration unit (model 12) fitted with a PM10 Diaflo membrane (Amicon, High Wycombe, Buckinghamshire) to give a final volume of 6 ml.

Step 3: Sephadex G-200 column chromatography. The material from step 2 was applied at 4 °C to a column (3 $\times$ 90 cm) of Sephadex G-200, previously equilibrated with 20 mM-potassium phosphate buffer, pH 7.2, and eluted with similar buffer at 4 °C. Fractions (3 ml) were collected as for step 2 and pooled.

Step 4: DEAE-cellulose treatment. DEAE-cellulose slurry (25 ml, containing approximately 2 g DEAE-cellulose) was slowly mixed with the pooled fractions from step 3 (44 ml) at 4 °C. The DEAE-cellulose had previously been extensively washed with 20 mM-potassium phosphate buffer, pH 7.2, and adjusted to pH 7.2. The mixture was slowly stirred and allowed to equilibrate for 20 min at 4 °C, then centrifuged (4 °C) at 38000g for 10 min. The pellet was discarded and the supernatant was frozen by dropwise addition to liquid nitrogen, the resulting pellets being stored at -80°C .

Analytical procedures. Formaldehyde solutions were prepared by heating aqueous solutions of para-formaldehyde at 100 °C for 2 to 3 h. Formaldehyde was determined by the formaldemethone method of Frisell & MacKenzie (1963). Routinely, formaldehyde disappearance was monitored by the acetylacetone method of Nash (1953).

Formate was determined by the method of Lang & Lang (1972).

Protein was measured by the method of Lowry *et al.* (1951) using dried, crystalline bovine plasma albumin as the standard.

Polyacrylamide gel electrophoresis. The procedure was based on that of Gabriel (1971). Separating gels of 7.5 % (w/v) acrylamide were used at pH 8.8, to which 0.2 ml of stacking gel (1.25 %, w/v, acrylamide) was added. Bromophenol blue tracking dye (1 ml; 0.01 %, w/v) was added to the reservoir (electrode) buffer (50 mM-Tris/glycine, pH 8.3). Electrophoresis was carried out at 2.5 mA per tube until the tracking dye had almost reached the base of the gel. The gels were fixed and stained with 0.1 % (w/v) Coomassie brilliant blue R in 7.5 % (v/v) acetic acid/46 % (v/v) methanol for 2 to 4 h and destained by soaking overnight in similar solvent.

The molecular weights of the HSC subunits of formaldehyde dehydrogenase were estimated by sodium dodecyl sulphate (SDS) gel electrophoresis in a 13 % (w/v) gel system. The gel buffer was 300 mM-Tris/HCl, pH 8.8, and the electrode buffer was 50 mM-Tris/glycine, pH 8.3, both containing 0.1 % (w/v) SDS. The samples were prepared by mixing 0.4 ml of sample protein (0.18 mg) with 0.5 ml 10 % (w/v) SDS in 50 mM-Tris/HCl buffer, pH 7.8, containing 0.1 % (v/v) 2-mercaptoethanol and 0.1 ml 60 % (w/v) sucrose solution. The mixture was heated at 100 °C in a boiling bath for 10 min and then cooled to 4 °C. Electrophoresis and staining were done as before and the relative mobilities of the sample protein and the standard marker proteins were compared.

Activity stain. Formaldehyde dehydrogenase activity was located in unstained (7.5 %, w/v) gels by incubating them in the dark with a mixture containing: 0.08 g nitrobluetetrazolium; 0.03 g NAD^+ ; 1.4 mg phenazine methosulphate; 20 ml 200 mM-formaldehyde; 160 mg HTSE (protein); 70 ml 20 mM-Tris/HCl buffer, pH 7.2. Two gels were incubated at 45 °C for 30 min, one being a control gel without formaldehyde, and a third gel was incubated at 30 °C for 90 min. Purified formaldehyde dehydrogenase HSC (20 μg) was applied to each gel.

Molecular weight determination. The molecular weight of the purified HSC of the formaldehyde dehydrogenase was determined by Sephadex G-200 gel filtration as described by Andrews (1964). The elution volumes for the purified protein and various proteins of known molecular weight were determined and their K_{av} values were calculated: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume, V_0 is the void volume as determined by blue dextran, and V_t is the bed volume.

Chemicals. Biochemicals and enzymes were the best grade available and were mainly from Sigma; chemicals were supplied by BDH and Kodak. Acrylamide (pure) was obtained from Fluka, Buchs, Switzerland. DL-Glyceraldehyde 3-phosphate diethyl acetal, barium salt (obtained from Sigma) was converted to DL-glyceraldehyde 3-phosphate in accordance with the supplier's instructions.

RESULTS

Presence of NAD⁺-linked formaldehyde dehydrogenase activity in crude soluble extracts. Soluble extracts of *M. capsulatus* strain Bath catalysed the disappearance of formaldehyde with the concomitant production of NADH when supplied with exogenous formaldehyde and NAD⁺. The reaction rates were unaffected by the presence of high concentrations of KCN which completely inhibited both the methanol oxidase activity (0.5 mM-KCN) (Colby & Dalton, 1976) and the NAD⁺-linked formate dehydrogenase (oxidase) activity (0.05 mM-KCN) in soluble extracts of *M. capsulatus*. The possibility that a cyclic pathway of formaldehyde oxidation to CO₂ and water was generating NAD(P)H, as reported in a number of facultative and obligate methylotrophs (Strøm *et al.*, 1974; Colby & Zatman, 1975), was discounted since negligible CO₂ was produced from formaldehyde in the presence of exogenous NAD⁺ and KCN. However, this does not discount the possibility that such a pathway operates *in vivo* as the enzymic requirements for such a system have been found in extracts of *M. capsulatus* (unpublished observations).

Purification of heat-sensitive component of the formaldehyde dehydrogenase. When soluble extracts of *M. capsulatus* were dialysed (24 h, 4 °C) against 20 mM-potassium phosphate buffer, pH 7.0, formaldehyde dehydrogenase activity was lost. However, activity was restored when the dialysed extract was supplemented with heat-treated extract (HTSE, prepared as described in Methods), which alone had no formaldehyde dehydrogenase activity. Therefore, as neither the dialysed extract nor the HTSE had formaldehyde dehydrogenase activity when assayed separately and yet regained activity when assayed together, the formaldehyde dehydrogenase must be composed of at least two components, one of which is heat-stable at 70 °C. The purification (Table 2) of the non-dialysable, heat-sensitive component of the enzyme was monitored by polyacrylamide gel electrophoresis, as shown in Fig. 1.

Products and stoichiometry. Formate and NAD(P)H were the only products of formaldehyde oxidation detected by the colorimetric and spectrophotometric methods described. Quantitatively, 3.90 µmol formaldehyde gave rise to 3.91 µmol formate and 3.36 µmol NADH, suggesting a 1:1:1 molar ratio.

Substrate specificity. The following potential substrates were tested for oxidation in the standard spectrophotometric assay at 0.25, 1, 2.4 and 20 mM: methanol, ethanol, 1-propanol, acetaldehyde, propionaldehyde, butyraldehyde, ethylene glycol, glycolaldehyde, glyoxal, glyoxylic acid, DL-glyceraldehyde and DL-glyceraldehyde 3-phosphate. Three of the compounds tested, glycolaldehyde, glyoxal and DL-glyceraldehyde, were oxidized suggesting that the enzyme is not formaldehyde-specific. There was no evidence for the occurrence of the reverse reaction on incubating 2.5 mM-potassium formate with 0.5 mM-NADH.

Apparent K_m values, with NAD⁺ at 0.67 mM, were 0.68, 7.0, 0.075 and 2.0 mM for formaldehyde, glycolaldehyde, glyoxal and DL-glyceraldehyde, respectively, as determined by the method of Eisenthal & Cornish-Bowden (1974). Similarly, V_{max} values for the four enzyme substrates, determined with 11 µg purified HSC, were: formaldehyde (570 m-units), glycolaldehyde (73 m-units), glyoxal (281 m-units) and DL-glyceraldehyde (15 m-units).

Fig. 1. Polyacrylamide gel electrophoresis of various preparations obtained during purification of the formaldehyde dehydrogenase heat-sensitive component: (1) crude soluble extract (200 μ g protein); (2) after ammonium sulphate treatment (step 1) (200 μ g protein); (3) after Sephadex G-75 chromatography (step 2) (200 μ g protein); (4) after Sephadex G-200 chromatography (step 3) (100 μ g protein); (5) after DEAE-cellulose treatment (step 4) (20 μ g protein); (6) after DEAE-cellulose treatment (step 4) (50 μ g protein). All samples were applied in 100 μ l and contained 6% (w/v) sucrose. Tracking dye is at the base (anodic end) of the gels.

Table 2. *Purification of heat-sensitive component of the formaldehyde dehydrogenase*

Formaldehyde dehydrogenase activity was measured spectrophotometrically as described in Methods.

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity [m-units (mg protein) ⁻¹]	Yield	Purification factor
Soluble extract	15.0	720	60.8	84.4	100	1.0
(NH ₄) ₂ SO ₄ (30 to 55% saturation precipitate)	2.8	466	45.3	97.2	74.5	1.2
Sephadex G-75	6.0	370	48.6	131.5	79.9	1.6
Sephadex G-200	44.0	88	89.1	1012.5	146.5	12.0
DEAE-cellulose	39.0	17.6	75.3	4278.4	123.8	50.7

Electron acceptors. Various artificial and physiological electron acceptors were tested with the purified formaldehyde dehydrogenase. Phenazine methosulphate (PMS) (0.7 mM), 2,6-dichlorophenolindophenol (DCPIP) (0.2 mM), PMS (0.7 mM) plus DCPIP (0.2 mM), K₃Fe(CN)₆ (2.0 mM), K₃Fe(CN)₆ (2.0 mM) plus PMS (1.0 mM), K₃Fe(CN)₆ (2.0 mM) plus NAD⁺ (0.67 mM), and cytochrome *c* (0.1 mM) did not act as electron acceptors. NAD⁺

and NADP⁺ both acted as electron acceptors and, with formaldehyde at 2.5 mM, gave apparent K_m values of 0.063 and 0.155 mM, respectively, as determined by the method of Eisenthal & Cornish-Bowden (1974).

Restoration of activity by thiol and other compounds. A number of thiol compounds were tested at 0.1, 2.0 and 20 mM to see if they could restore formaldehyde dehydrogenase activity to the purified HSC, i.e. replace the requirement for HTSE. The assays were performed as mentioned in the Methods, with the thiol compounds preincubated for 5 min with the enzyme protein (11 μ g). The specific activity of the HSC supplemented with HTSE (0.4 ml) was 4.28 units (mg protein)⁻¹. Reduced glutathione, sodium thioglycollate, dithiothreitol, 2,3-dimercaptopropanol, DL-cysteine and 2-mercaptoethanol all failed to restore any activity to the HSC.

Various other compounds were tested to see if they could restore formaldehyde dehydrogenase activity to crude soluble extract which had been dialysed at 4 °C for 24 h against 20 mM-potassium phosphate buffer. The compounds and final assay concentrations were: AMP, sodium salt (1.0 mM); ADP, sodium salt (1.0 mM); ATP, disodium salt (1.0 mM); cyclic AMP, sodium salt (1.0 mM); *N*⁶,*O*^{2'}-dibutyl cyclic AMP, sodium salt (1.0 mM); pyridoxal 5-phosphate (0.1 mM); *dl*-L-tetrahydrofolic acid (0.1 mM); coenzyme B₁₂ (0.05 mM). The specific activity of the dialysed extract supplemented with HTSE (0.4 ml) was 84.4 m-units (mg protein)⁻¹. None of the above compounds restored activity to the dialysed extract.

Effect of trypsin on heat-treated soluble extract. The HTSE which was kept on ice for 30 min (tube 1, see Methods), when supplemented with 11 μ g purified formaldehyde dehydrogenase in 0.4 ml, gave a specific activity of 4.28 units (mg protein)⁻¹. Incubation of the HTSE fraction at 37 °C for 30 min (tube 2) had no effect on its ability to complement with purified formaldehyde dehydrogenase to give the same specific activity. Incubation of the HTSE fraction at 37 °C for 30 min in the presence of trypsin (tube 3) completely abolished its ability to complement with formaldehyde dehydrogenase. Furthermore, the HTSE fraction that had been incubated at 37 °C for 30 min in the presence of trypsin plus an excess of trypsin inhibitor (tube 4) restored complete activity to the formaldehyde dehydrogenase enzyme. These results suggest that the cofactor in the HTSE fraction was trypsin-sensitive.

Activity stain. After polyacrylamide gel electrophoresis of the purified HSC of the formaldehyde dehydrogenase, the unstained gels were immersed in an activity stain or in Coomassie blue stain as described in the Methods. The gel incubated at 45 °C for 30 min in the complete active stain and the gel incubated at 30 °C for 90 min both showed a single active stained band. The active stained bands had identical electrophoretic mobility to the single band found on similar gels stained with Coomassie blue. The gel incubated with the activity stain at 30 °C required three times as long as a similar gel incubated at 45 °C to develop a band of equal intensity. The gel incubated at 45 °C with the activity stain minus formaldehyde gave no equivalent band.

Absorption spectrum. The visible and ultraviolet absorption spectrum of the purified HSC revealed no characteristic absorption peaks, other than the absorption at 280 nm.

Inhibition studies. Various metal-chelating agents were tested as potential inhibitors of enzyme activity at 2.5 mM: thiourea, thioacetamide, diethyldithiocarbamate, 2,2'-bipyridine, neocuproine, imidazole, 1,10-phenanthroline, acriflavin, 8-hydroxyquinoline and KCN. Only 2,2'-bipyridine (10% inhibition) and thiourea (10% inhibition) caused any inhibition.

A number of substrate analogues (methanol, ethanol, 1-propanol, acetaldehyde and propionaldehyde) were also tested as potential inhibitors at 2.5 mM, but only methanol (5% inhibition) had any inhibitory effect.

Of various thiol reagents tested (Table 3), iodoacetic acid and iodoacetamide caused little inhibition of activity, whereas *N*-ethylmaleimide caused around 60% inhibition at 2.0 mM. *p*-Hydroxymercuribenzoate completely inhibited activity at 1.0 mM but was totally ineffec-

Table 3. Inhibition of formaldehyde dehydrogenase activity by thiol reagents

Activity was measured spectrophotometrically as described in Methods; each potential inhibitor was preincubated with the enzyme for 5 min. The uninhibited specific activity of the enzyme was $4.28 \text{ units (mg protein)}^{-1}$.

Reagent	Final concn (mM)	Inhibition (%)
Iodoacetic acid	2.0	14
	1.0	8
	0.1	0
Iodoacetamide	2.0	14
	1.0	28
	0.1	0
<i>N</i> -Ethylmaleimide	2.0	59
	1.0	50
	0.1	0
<i>p</i> -Hydroxymercuribenzoate	1.0	100
	0.1	0
5,5'-Dithiobis(2-nitrobenzoic acid)	0.1	9

Fig. 2

Fig. 3

Fig. 2. Effect of pH on formaldehyde dehydrogenase activity. Activity was measured spectrophotometrically as described in Methods; the maximum specific activity was $4.28 \text{ units (mg protein)}^{-1}$. All buffers were 20 mM: ●, potassium phosphate; ○, Tris/HCl; □, tetrasodium pyrophosphate/HCl.

Fig. 3. Effect of temperature on formaldehyde dehydrogenase activity. Activity was measured spectrophotometrically as described in Methods; the maximum specific activity was $4.28 \text{ units (mg protein)}^{-1}$.

tive at 0.1 mM. 5,5'-Dithiobis(2-nitrobenzoic acid) caused little inhibition at 0.1 mM and could not be tested at a higher concentration due to absorption by the compound at 340 nm.

Effect of pH and temperature. The optimum pH for formaldehyde dehydrogenase activity was pH 7.2 (Fig. 2). The enzyme was equally active in phosphate buffer or Tris/HCl-buffer at pH 7.2. Maximum enzyme activity occurred at 45 °C (Fig. 3), with the reaction rate deteriorating rapidly at higher temperatures. After the usual preincubation of an assay mixture at 45 °C, no enzyme activity was lost on further incubation at 45 °C for at least 10 min before formaldehyde addition.

Molecular weight and subunit size. The molecular weight of the HSC of the formaldehyde dehydrogenase from *M. capsulatus* was estimated by gel filtration to be 115000 (Fig. 4). SDS-polyacrylamide gel electrophoresis (Fig. 5) gave a value of 57000 for the subunit molecular weight of the HSC, suggesting that the enzyme is a dimer.

Fig. 4

Fig. 4. Molecular weight estimation of the heat-sensitive component (HSC) of formaldehyde dehydrogenase on a Sephadex G-200 column (see Methods for details). Molecular weight standards were: (1) malate dehydrogenase (67000); (2) alkaline phosphatase (80000); (3) alcohol dehydrogenase (150000); (4) catalase (232000); (5) ferritin (443000).

Fig. 5

Fig. 5. Sodium dodecyl sulphate gel electrophoresis of the heat-sensitive component (HSC) of formaldehyde dehydrogenase (see Methods for details). Molecular weight standards were: (1) α -chymotrypsinogen A (23700); (2) alcohol dehydrogenase (37500); (3) ovalbumin (43000); (4) catalase (60000); (5) phosphorylase a (92500).

DISCUSSION

The formaldehyde dehydrogenase found in *M. capsulatus* is similar to the enzyme found in Bacterium 4B6 (Colby & Zatman, 1973), i.e. type 3 (Table 1), but *M. capsulatus* is the first methane-oxidizing bacterium found to possess a glutathione-independent, NAD(P)⁺-linked formaldehyde dehydrogenase. Possessing such an enzyme is of great advantage to the organism as its growth yield, when growing on methane as the sole source of carbon and energy, could be exclusively NAD(P)H-dependent (Anthony, 1978). This apparent deficiency of reducing power arises because NAD(P)H is required for methane mono-oxygenase activity (Colby & Dalton, 1976) and, theoretically, for the assimilation of formaldehyde into cell material (Van Dijken & Harder, 1975), but no NAD(P)H is generated from the oxidation of methane to formaldehyde. Although NAD(P)H cannot be generated from methanol dehydrogenase activity, reducing equivalents [other than NAD(P)H] are produced and could theoretically give a P/O ratio of 2. In some methane oxidizers, e.g. *Methylosinus trichosporium* OB3b, the methanol dehydrogenase coupled with cytochrome *c* acts directly as the reducing agent for methane mono-oxygenase activity negating the need for NAD(P)H (Tonge, Harrison & Higgins, 1977). This does not occur in *M. capsulatus* (Colby & Dalton, 1976). Therefore, in *M. capsulatus* the amount of reducing power generated by the complete oxidation of methane to carbon dioxide is critical, as this has to satisfy the deficiency of the cell's reducing power. In most methylotrophs, the only source of NAD(P)H from the complete oxidation of methane is the final oxidation step, formate to carbon dioxide and water, catalysed by formate dehydrogenase. Hence, an NAD(P)⁺-linked formaldehyde dehydrogenase would greatly reduce the percentage of the carbon substrate needing to be completely oxidized to carbon dioxide in order to generate the reducing power required for cellular biosynthesis. If a reverse electron transport system was operative in this organism, the problem of an NAD(P)H deficiency could be somewhat alleviated, due to the conversion of 'excess' ATP to NAD(P)H. However, reverse electron flow has not been unequivocally demonstrated in methylotrophs (see Dalton, 1977).

Patel & Hoare (1971) were unable to show the presence of either a DCPIP-linked or

NAD(P)⁺-linked formaldehyde dehydrogenase in extracts of *M. capsulatus* strain Texas, which supports the idea, previously suggested (Stirling & Dalton, 1977), that there is a more radical difference between the Bath and Texas strains than their taxonomic relationship implies.

The presence of potassium cyanide in the formaldehyde dehydrogenase assays totally inhibited formate dehydrogenase activity and partially inhibited the NAD(P)H oxidase activity present. It is thought that the apparent increase in the yield during purification of the HSC was due to the removal of the cyanide-resistant NAD(P)H oxidase activity originally present in the crude soluble extracts.

The substrate specificity results show that the NAD(P)⁺-linked formaldehyde dehydrogenase from *M. capsulatus* is not absolutely specific and can oxidize other aldehydes. However, as only formaldehyde, glyoxal, glycolaldehyde and glyceraldehyde are oxidized, the range of substrate aldehydes is limited compared with the variety of aldehydes oxidized by enzymes isolated from other methylotrophic bacteria. Generally, non-specific aldehyde dehydrogenases oxidize a much wider range of aldehydes in addition to those listed above, including acetaldehyde, propionaldehyde, butyraldehyde, glyoxylate and glyceraldehyde 3-phosphate.

The apparent substrate specificity of the formaldehyde dehydrogenase of *M. capsulatus* could be further restricted if the activity obtained with glyceraldehyde is a result of contamination by formaldehyde, since a 25 mM solution of DL-glyceraldehyde was found to contain approximately 60 μ M-formaldehyde, as determined by the method of Chrastil & Wilson (1975). The authors claim that no reactivity with DL-glyceraldehyde is obtained with their colorimetric assay.

The K_m and V_{max} values obtained with glyoxal could be greater than quoted because of the very narrow range of substrate concentrations which gave activity, apparently due to potent substrate or product inhibition. Hence, the apparently greater affinity for the enzyme shown by glyoxal compared with formaldehyde could be reduced or even reversed. On the evidence of the K_m values, glycolaldehyde and glyceraldehyde appear to be much poorer substrates than formaldehyde for the enzyme.

The lack of activity obtained when the purified HSC was supplemented with various thiol compounds suggests that there is no requirement for a thiol compound for enzyme activity and no formyl/thiol compound is produced during formaldehyde oxidation in *M. capsulatus*. Hence, *M. capsulatus* is unlike the yeast *Hansenula polymorpha* whose formaldehyde dehydrogenase requires glutathione for activity and produces 5-formylglutathione from the oxidation of formaldehyde (Van Dijken *et al.*, 1976).

The lack of formaldehyde dehydrogenase activity in dialysed, crude soluble extract assayed in the presence of various potential cofactors suggests that none of the common, low molecular weight physiological cofactors, such as AMP, ATP, cyclic AMP etc., are required for activity in *M. capsulatus*.

The sensitivity of the HTSE to the endopeptidase trypsin suggests that the enzyme component/cofactor present in HTSE and required to restore activity to the purified HSC is either a protein or a peptide. Since the component present in HTSE will restore activity to dialysed crude soluble extract, it is likely that the HTSE compound is itself dialysable and is presumably a polypeptide, although attempts to purify this component have so far met with little success. The enzyme showed almost uniform resistance to metal-binding/chelating agents, implying either the non-involvement of a metal(s) or that it contains a metal-ion complex well shielded from attack by such agents.

The effect of thiol reagents on formaldehyde dehydrogenase activity was variable. The total inhibition obtained with *p*-hydroxymercuribenzoate at 1 mM was not surprising, for this effective thiol reagent is known to inhibit enzymes at micromolar concentrations (Trudinger, 1969). The weak inhibitions observed in the presence of the less potent thiol reagents, iodoacetic acid and iodoacetamide, support the view that no formyl/thiol compound is

formed as an intermediate during the oxidation of formaldehyde, since one would expect potent inhibition of such a reaction to occur with the concentrations of reagents tested. However, there are obviously thiol groups present in the formaldehyde dehydrogenase enzyme which are vulnerable to the action of thiol reagents and this would not be unexpected with a major component of the enzyme having a molecular weight of over 10^5 .

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