Formaldehyde dehydrogenase preparations from *Methylococcus capsulatus* (Bath) comprise methanol dehydrogenase and methylene tetrahydromethanopterin dehydrogenase

Ekundayo K. Adeosun,1,† Thomas J. Smith,1,‡ Anne-Mette Hoberg,1,§ Giles Velarde,2 Robert Ford2 and Howard Dalton1

1Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK
2Department of Biomolecular Sciences, PO Box 88, UMIST, Manchester M60 1QD, UK

In methylotrophic bacteria, formaldehyde is an important but potentially toxic metabolic intermediate that can be assimilated into biomass or oxidized to yield energy. Previously reported was the purification of an NAD(P)⁺-dependent formaldehyde dehydrogenase (FDH) from the obligate methane-oxidizing methylotroph *Methylococcus capsulatus* (Bath), presumably important in formaldehyde oxidation, which required a heat-stable factor (known as the modifin) for FDH activity. Here, the major protein component of this FDH preparation was shown by biophysical techniques to comprise subunits of 64 and 8 kDa in an $\alpha_2\beta_2$ arrangement. N-terminal sequencing of the subunits of FDH, together with enzymological characterization, showed that the $\alpha_2\beta_2$ tetramer was a quinoprotein methanol dehydrogenase of the type found in other methylotrophs. The FDH preparations were shown to contain a highly active NAD(P)⁺-dependent methylene tetrahydromethanopterin dehydrogenase that was the probable source of the NAD(P)⁺-dependent formaldehyde oxidation activity. These results support previous findings that methylotrophs possess multiple pathways for formaldehyde dissimilation.

INTRODUCTION

Methanotrophic bacteria such as the $\gamma$-proteobacterium *Methylococcus capsulatus* (Bath) grow using methane as their sole source of carbon and energy (Hanson & Hanson, 1996). Methane is first oxidized to methanol by action of methane monooxygenase and then to formaldehyde by methanol dehydrogenase (MDH) (Hanson & Hanson, 1996; Anthony, 1982). Formaldehyde lies at a metabolic branch point, where metabolic carbon can either be assimilated into biomass or oxidized to carbon dioxide to produce energy (Hanson & Hanson, 1996; Murrell et al., 2000).

Previous studies in our laboratory suggested that *Mc. capsulatus* (Bath) could oxidize formaldehyde to formate using a soluble formaldehyde dehydrogenase (FDH) that existed as a dimer of 57 kDa subunits, possessed NAD(P)⁺-dependent FDH activity and required a factor (apparently a low-molecular-mass protein) from heat-treated soluble extract (HTSE) (Stirling & Dalton, 1978). We conducted a subsequent investigation into the FDH of *Mc. capsulatus* (Bath) which suggested that the protein responsible for FDH activity was actually a homotetramer of 63 kDa subunits and that the factor in the HTSE required for activity with formaldehyde was a heat-stable 8-6 kDa protein, termed the modifin. The modifin preparation converted FDH from a general aldehyde dehydrogenase to a specific FDH and was thus a potential regulator of formaldehyde metabolism (Tate & Dalton, 1999). Other reports have also indicated the presence of a membrane-associated FDH (Zahn et al., 2001) and at least some of the enzyme activities that are necessary for oxidation of formaldehyde as a conjugate to tetrahydromethanopterin (H₄MPT) (Vorholt et al., 1999).

Here we report analysis of modifin-dependent FDH preparations similar to those which we prepared previously that show that the principal enzyme activities within these preparations are a quinoprotein MDH and a methylene tetrahydromethanopterin dehydrogenase (methylene H₄MPT-DH), which is one of the enzymes of the
H₄MPT-dependent formaldehyde oxidation pathway (Vorholt et al., 1998).

METHODS

**Coenzymes.** NAD⁺ was purchased from Sigma and H₄MPT was provided by Rudolf K. Thauer. Anoxic solutions of H₄MPT were prepared in 50 mM Tris/HCl (pH 7.0) containing 2 mM DTT. Methylene H₄MPT was generated by spontaneous reaction of H₂MPT with formaldehyde (Vorholt et al., 1998).

**Growth of bacteria.** *M. capsulatus* (Bath) was cultivated at 45 °C using methanol (15%, v/v, in air) as the growth substrate, in nitrate minimal salts medium (Dalton & Whittenbury, 1976) containing CuSO₄·5H₂O (0.5 or 1.5 mg L⁻¹, as stated for each experiment) as the sole source of added copper. Cultures were grown in a 100 litre batch fermentation vessel or continuously, as described previously (Tate & Dalton, 1999) except that the dilution rate was 0.05 h⁻¹. Cells were harvested according to the published method (Tate & Dalton, 1999) and stored at −80 °C.

**Enzyme assays.** All enzyme assays were performed at 45 °C. Modifin/HTSE-dependent FDH assays were performed by spectrophotometric quantification at 340 nm of the production of NADH, as described by Tate & Dalton (1999). NAD⁺-dependent methylene H₂MPT-DH activity was also monitored spectrophotometrically (Vorholt et al., 1998). Dye-linked oxidation of methanol and formaldehyde was similarly measured at 600 nm by a modification of the method of Anthony & Zatman (1967), as follows. The enzyme sample was incubated in 50 mM Tris/HCl (pH 9.0) containing substrate (10 mM), NH₄Cl (15 mM), KCN (5 mM) and dichloroindophenol (DCIP; 0.1 mM); the reaction was initiated by adding the mediator phenazine ethosulfate to 1 mM); the reaction was initiated by adding the mediator phenazine ethosulfate to 1 mM). The concentration of ammonium sulfate in the supernatant was adjusted to 60% saturation and, after a further 30 min incubation on ice, the precipitate, containing the FDH activity, was collected by centrifugation as above and resuspended in a minimal volume of buffer A (20 mM potassium phosphate buffer, pH 7.0). The active fractions, containing the partially purified FDH, were pooled and concentrated as above. This gave preparation FDH-1, which was drop-frozen in liquid nitrogen and stored at −80 °C.

**General protein characterization.** Protein concentration was determined by using the Bradford assay reagent (Bio-Rad) according to the manufacturer’s instructions. Bovine serum albumin was used as the standard. SDS- and native-PAGE were performed using the discontinuous buffer system of Laemmli (1970). Molecular masses of polypeptides analysed by SDS-PAGE were estimated by comparison with Dalton Mk VIII standards (Sigma). Electrolysis of protein samples from bands excised from polyacrylamide gels was performed in the gel-running buffer using a model 422 Electroeluter (Bio-Rad). Samples for N-terminal sequencing were subjected to SDS-PAGE and then electroblotted onto a PVDF membrane (Pharmacia-Amersham) and stained with Coomassie blue. Protein bands of interest were excised and the peptide sequence was determined using an Applied Biosystems 476A protein sequencer. Electrospray-mass spectrometry (ES-MS) was performed using a Quattro II QQQ tandem mass spectrometer (Micromass, Altrincham, UK) as detailed by Millar et al. (1998).

**Equilibrium sedimentation.** Sedimentation equilibrium analysis of preparation FDH-1 was performed at 4 °C in a Beckman Optima XL-A analytical ultracentrifuge. Samples were diluted to 0.34, 0.17 and 0.04 mg ml⁻¹ using 20 mM potassium phosphate buffer (pH 7.2). Each dilution (70 μl) was injected into a separate cell in the centrifuge rotor and the samples were centrifuged to equilibrium at 6000 and 9000 r.p.m. Scanning absorbance optics were used at wavelengths of 280 nm (for samples at 0.34 and 0.17 mg ml⁻¹) and 223 nm (for samples at 0.04 mg ml⁻¹). Sedimentation equilibrium was considered to have been achieved when a negligible difference between solute distributions was observed after a time interval of 3 h. After equilibrium at both rotor speeds, the rotor was accelerated to 45 000 r.p.m. to obtain a reading of non-redistributing absorbance, which was used as baseline reading in the analysis. The partial specific volume of the proteins and the density of the solvent were estimated from composition information using the program SEDINTERP (Laue et al., 1992). Solute distributions were analysed using non-linear least-squares fitting of exponential equations using the ORIGIN software package (MicroCal Software, Northampton, MA, USA). The data obtained were fitted to models that included association terms, in order to determine the stoichiometry of any intermolecular interactions.

**Electron microscopy and single-particle analysis.** Samples were added to freshly glow-discharged (rendered hydrophilic) copper/carbon mesh grids for 30 s, the sides of which were then blotted with Whatman grade 50 filter paper, before negative staining in 4% uranyl acetate (Rosenberg et al., 1997). Micrographs of the grids were taken with a Philips CM10 transmission electron microscope operating at 100 kV and scanned using a leaf microdensitometer (University of Leeds). Particles from electron micrographs were selected using the SPIDER software package on an Indigo workstation (Silicon Graphics) and the densities normalized. Reference-free alignment was carried out and the results were statistically analysed by sorting particles into groups for averaging using hierarchical clustering (Holzenburg et al., 1994).
Fourier ring correlation between two subaverages was used to assess the resolution. The point at which the Fourier ring correlation values dropped to 0.5, indicating higher resolution shells did not correlate significantly, was determined by fitting the data with a four-parameter Boltzman function (using ORIGIN). A threshold for each class that gave the best averages in terms of resolution was thus determined.

RESULTS

Initial preparation of the components of FDH: preparation FDH-1 and the modifin

In order to obtain sufficient of the modifier- and NAD$^+$-dependent FDH from *M. capsulatus* (Bath) for detailed characterization, we repeated the cell growth and purification procedures described by Tate & Dalton (1999). Despite repeated attempts, these yielded material with a mean specific activity of only 55 nmol min$^{-1}$ (mg protein)$^{-1}$, which was less than a quarter of the 234 nmol min$^{-1}$ (mg protein)$^{-1}$ obtained previously (Tate & Dalton, 1999) [NB: the FDH and modifin activities of Tate & Dalton (1999) that are presented here have been reduced by 1000-fold to compensate for a typographical error on the part of the authors]. However, by modifying the purification protocol to that described in Methods and by increasing the concentration of copper in the growth medium (from 0.1 to 1.5 mg l$^{-1}$ of CuSO$_4$5H$_2$O), higher NAD$^+$-dependent FDH activities were obtained (Table 1). The resulting preparations, which will be referred to as preparation FDH-1, consistently contained the 64 kDa polypeptide at a purity of about 80%. The major contaminant was an 8 kDa protein (Fig. 1) that was previously attributed to modifin co-purifying with the 64 kDa moiety. The specific activity of preparation FDH-1 [167 nmol min$^{-1}$ (mg protein)$^{-1}$] was 71% of that observed for the FDH of Tate & Dalton (1999) and considerably lower than the 4278 nmol min$^{-1}$ (mg protein)$^{-1}$ obtained in an earlier study (Stirling & Dalton, 1978). In a separate experiment the modifin was prepared from HTSE using the procedure described by Tate & Dalton (1999) (data not shown). The apparent specific activity of the purified modifin [26 nmol min$^{-1}$ (mg protein)$^{-1}$] was considerably less than that found previously (Tate & Dalton, 1999).

The low specific activity of preparation FDH-1 and the modifin, compared to the previous studies, suggested that inactivation or inhibition of the enzyme was occurring during the purification procedures. Alternatively, the preparations may have lacked a component, other than the 64 and 8 kDa polypeptides, that was necessary for NAD$^+$-linked FDH activity.

The major protein components of preparation FDH-1 are identical to the components of FDH isolated previously

The major polypeptides of preparation FDH-1 were subjected to N-terminal sequencing and accurate mass analysis by means of ES-MS in order to investigate whether degradation of the proteins has occurred that might account for the low FDH activity. The N-terminal sequences of the 64 and 8 kDa species from preparation FDH-1 (Fig. 2a, b) were very similar to those found previously (Tate & Dalton, 1999). The positions of the N termini in the two proteins were unchanged, which suggested that N-terminal degradation was not the cause of inactivation, although in the case of the 8 kDa moiety there was a discrepancy in the identification of the N-terminal residue. The molecular masses of the two major polypeptides in preparation FDH-1 were 63615 and 82125 Da, which were identical to the masses during the purification procedures. Alternatively, the preparations may have lacked a component, other than the 64 and 8 kDa polypeptides, that was necessary for NAD$^+$-linked FDH activity.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (nmol min$^{-1}$)</th>
<th>Specific activity [nmol min$^{-1}$ (mg protein)$^{-1}$]</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>3450</td>
<td>17300</td>
<td>5</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>554</td>
<td>1110</td>
<td>20</td>
<td>64</td>
<td>4.0</td>
</tr>
<tr>
<td>Ion exchange (Mono Q)</td>
<td>174</td>
<td>9740</td>
<td>56</td>
<td>56</td>
<td>11.2</td>
</tr>
<tr>
<td>Affinity chromatography (Hi-Trap Blue)</td>
<td>86</td>
<td>8940</td>
<td>104</td>
<td>52</td>
<td>20.8</td>
</tr>
<tr>
<td>(Gel filtration) Superdex 200; preparation FDH-1</td>
<td>42</td>
<td>7010</td>
<td>167</td>
<td>41</td>
<td>33.4</td>
</tr>
</tbody>
</table>

Table 1. Fractionation of cell-free extract from *Mc. capsulatus* (Bath) resulting in preparation FDH-1

Fig. 1. SDS-PAGE of preparation FDH-1 (lane 2) showing the 64 and 8 kDa polypeptides and minor contaminants. Molecular masses of standards (lane 1) are indicated in kDa.
The 64 and 8 kDa polypeptides form a complex with the same $\alpha_2\beta_2$ stoichiometry as known MDHs and similar molecular topology

Previously the native molecular mass of the major protein complex in FDH preparations was estimated at 250 kDa, based on gel filtration chromatography (Tate & Dalton, 1999). In order to obtain an accurate value for the native molecular mass that could be used to determine the stoichiometry of the complex, the molecular mass(es) of the protein complex(es) in preparation FDH-1 were determined in solution under native conditions by sedimentation equilibrium analysis. Analysis of residual plots after fitting of the experimental data from FDH-1 samples to specific models of association indicated that the data were best accounted for by a single species of molecular mass 140 600 ± 1200 Da. Models which included self-association terms and/or non-interacting species did not describe the data well (data not shown). Assuming that this species is composed only of the 8 and 64 kDa polypeptides, the complex must have an $\alpha_2\beta_2$ stoichiometry, with a molecular mass of 143 655 Da as calculated from the masses of the individual polypeptides determined by ES-MS. The slight discrepancy between sedimentation equilibrium results and the mass derived from the ES-MS data was attributed to inaccuracies in the estimation of solute partial specific volume and solvent density.

The topology of the major macromolecular constituents in the FDH preparations was investigated in electron micrographs of negatively stained particles of FDH by means of single-particle analysis. Two distinct species were identified by visual inspection of the micrographs. The first (species A) was small, thin and tubular; the second (species B) was broader and appeared to be a sandwich of the first. Particles of each species were separately selected by size into subpopulations before performing rotational and translational alignment. This process used an interactive procedure of cross-correlation and auto-correlation of images which were thereby (Frank, 1990) averaged to produce a refined image. Hierarchical cluster analysis of each aligned particle dataset (corresponding to species A and B, respectively) resulted in final averages from both datasets with two very different structures. The resolution of the species A image extends about 26 Å, and that of species B to 35 Å. Enlarged versions of the best averages of each species were compared with the 1-9 Å resolution crystal structure of the $\alpha_2\beta_2$ tetramer of MDH from Methylophilus W3A1 (Xia et al., 1999) (Fig. 3). Species A was similar in size and shape to the MDH crystal structure, consistent with the proposal that the 64 and 8 kDa FDH components, like the subunits of MDH from Methylophilus W3A1, existed in an $\alpha_2\beta_2$ configuration. Species B resembled a sandwich of two MDH heterotetramers and suggested that, under the conditions used for sample preparation, further aggregation of the $\alpha_2\beta_2$ complex in preparation FDH-1 was possible.

### The major components of preparation FDH-1 were similar to the subunits of known quinoprotein MDHs

The N-terminal sequences of the 64 and 8 kDa components of preparation FDH-1 (Fig. 2a, b) were longer than those obtained previously (Tate & Dalton, 1999). By performing sequence database searches using these extended sequences it was possible to identify significant similarities between the N termini of the 64 and 8 kDa polypeptides and equivalent regions of the $\alpha$ and $\beta$ subunits, respectively, of quinoprotein MDHs from methyloptrophic bacteria (Fig. 2c, d). The subunits of such MDHs have masses of 66 and 8-5 kDa (Anthony, 1992), further suggesting that preparation FDH-1 contained such an enzyme.

#### Fig. 2. N-terminal sequence comparisons. Comparisons of (a) the 64 kDa component of preparation FDH-1 with sequence of the similar polypeptide obtained by Tate & Dalton (1999); (b) the 8 kDa component of FDH-1 with the modifin of Tate & Dalton (1999); (c) the 64 kDa component of preparation FDH-1 with the large subunit of MDH from Methylobacter albus (Chistoserdova et al., 1994); (d) the 8 kDa component of preparation FDH-1 with the $\beta$-subunit of MDH from Hyphomicrobium methyllovorum (Tanaka et al., 1997). Numbers refer to the amino acids of the heterologous MDHs, which were the highest scoring matches obtained from BLAST searches (Altschul et al., 1997).

<table>
<thead>
<tr>
<th>Component</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>64 kDa FDH (Tate)</td>
<td>NSELDRLSKDDRNWMQTKD</td>
</tr>
<tr>
<td>8 kDa Modifin (Tate)</td>
<td>YDGTCHKAPGCNEPKEPG</td>
</tr>
<tr>
<td>64 kDa MDH (a)</td>
<td>NSELDRLSKDDRNWMQT</td>
</tr>
<tr>
<td>8 kDa MDH (b)</td>
<td>YDGTCHKAPGCNEPKEPG</td>
</tr>
</tbody>
</table>

The 64 and 8 kDa polypeptides form a complex with the same $\alpha_2\beta_2$ stoichiometry as known MDHs and similar molecular topology.
The $\alpha_2\beta_2$ heterotetramer has dye-linked dehydrogenase activity

Previously described pyrroloquinoline quinone (PQQ)-dependent dehydrogenases, including MDHs, do not use NAD(P)$^+$ as the electron acceptor (Anthony, 1992) and so it was a surprise to find an apparently PQQ-dependent enzyme complex as the major constituent of a preparation that exhibited NAD$^+$-linked dehydrogenase activity. The natural electron acceptor of quinoprotein MDHs is cytochrome $c$ (Anthony, 1992), but such enzymes can also utilize the artificial electron acceptor phenazine ethosulfate, which can be coupled to the reduction and concomitant decolourization of the dye 2,6-dichloroindophenol (DCIP) to produce a dye-linked spectrophotometric assay (Anthony & Zatman, 1967). The dehydrogenase activity of the $\alpha_2\beta_2$ heterotetramer was therefore assayed using the phenazine ethosulfate/DCIP electron acceptor system in order to determine whether it could be activated in the same manner as previously described quinoprotein MDHs. To exclude the possibility that any dye-linked dehydrogenase activity might be due to minor contaminating protein(s) and not to the 144 kDa $\alpha_2\beta_2$ heterotetramer, the tetramer was further purified by preparative native-PAGE followed by electroelution under non-denaturing conditions. The electroeluted $\alpha_2\beta_2$ heterotetramer showed a high level of dye-linked dehydrogenase activity with methanol ($k_{cat} = 17.8\ s^{-1}; K_m = 0.48\ mM$) and formaldehyde ($k_{cat} = 16.2\ s^{-1}; K_m = 0.77\ mM$). The $k_{cat}$ values were similar to those obtained with the quinoprotein MDH from *Methylophilus methylotrophus*. In comparison to the MDH from *M. methylotrophus* (Ghosh & Quayle, 1981), the $K_m$ values exhibited by the FDH-associated $\alpha_2\beta_2$ heterotetramer were elevated (by 24-fold in the case of methanol), probably because of the presence of KCN in the assays described here. KCN, which competitively inhibits MDHs with respect to the alcohol substrate, was included because it inhibits the endogenous reduction of phenazine ethosulfate that otherwise occurs even in the absence of substrate (Anthony & Zatman, 1964; Duine & Frank, 1980; Harris & Davidson, 1993).

Fig. 3. Single-particle analysis of the $\alpha_2\beta_2$ complex from preparation FDH-1, showing average images of the tubular species A and the broader species B. A space-filling molecular model derived from the X-ray crystal structure MDH from *Methylophilus W3A1* (Xia et al., 1999) is shown on the same scale (top left) for reference.
The FDH-1 preparations contain a methylene H₄MPT-DH that may be involved in NAD⁺-dependent FDH activity

The observation that the αβ₂ heterotetramer alone showed no NAD⁺-dependent FDH activity suggested that the enzyme(s) responsible for the NAD⁺-linked FDH activity must be among the minor contaminants in preparation FDH-1. The previous observation of enzyme activities associated with the H₄MPT-dependent formaldehyde dissimilation pathway in cell-free extracts of *M. capsulatus* (Bath) (Vorholt *et al.*, 1999) suggested one possible explanation. In *M. extorquens*, in which this pathway is well characterized, the formation of methylene H₂MPT is catalysed by a specific formaldehyde-activating enzyme (Vorholt *et al.*, 2000); however, formaldehyde and H₂MPT also react spontaneously to form methylene H₂MPT. Methylene H₂MPT is the substrate for the NAD(P)⁺-dependent enzyme methylene H₂MPT-DH (Chistoserdova *et al.*, 1998) and so, in the presence of H₂MPT, methylene H₂MPT would oxidize formaldehyde in an NAD⁺-dependent manner. Moreover, since H₂MPT is heat-stable (Romesser & Wolfe, 1982) it, rather than the 8 kDa polypeptide, could be the active component of HTSE and the modifin that stimulates oxidation of formaldehyde. Consistent with this hypothesis, it was found that the NAD⁺-linked FDH activity of preparation FDH-1 increased from 167 nmol min⁻¹ (mg protein)⁻¹ to 23 000 nmol min⁻¹ (mg protein)⁻¹ when the HTSE in the assay was replaced by H₂MPT (to 35 μM). Thus the FDH preparations contained an NAD⁺-dependent methylene H₂MPT-DH and it was likely that H₂MPT was at least one of the components of the modifin preparation that stimulated the NAD⁺-linked FDH activity.

DISCUSSION

The data presented here show that the major component of the FDH preparation of Tate & Dalton (1999), which was previously described as a homotetramer of 64 kDa subunits, is in fact a quinoprotein MDH with an αβ₂₂ arrangement of 64 and 8 kDa subunits, which has similar properties to quinoprotein MDHs from other methanotrophic bacteria (Anthony, 1992; Chistoserdova *et al.*, 1994; Tanaka *et al.*, 1997). The MDH from *M. capsulatus* (Bath) has similar catalytic properties to the dye-linked methanol (or primary alcohol) dehydrogenase of the closely related methanotroph *M. capsulatus* (Texas), which is a quinoprotein of comparable native molecular mass (Patel *et al.*, 1972; Wadzinski & Ribbons, 1975). The conclusion that the NAD⁺-linked FDH activity must be due to a minor protein in preparation FDH-1 also explained our difficulty in purifying active ‘FDH’, because the NAD⁺-dependent dehydrogenase activity that was being assayed did not reside in the polypeptide that was previously believed to contain it.

Our observation that the FDH preparation FDH-1, described here, contained a methylene H₄MPT-DH showed that such an activity might be responsible for the NAD⁺-dependent FDH activity of preparation FDH-1 and offered a possible explanation for the FDH-stimulating activity of the HTSE and modifin preparations, by providing the heat-stable coenzyme H₂MPT. These results confirm the previous observation of the activities of enzymes of the H₂MPT pathway in *M. capsulatus* (Bath) (Vorholt *et al.*, 1999) and demonstrate the importance of H₂MPT as a C₁-carrier during oxidation of formaldehyde by this methanotrophic bacterium.

The NAD⁺-dependent FDH activity observed by Tate & Dalton (1999), within a preparation that also contained principally the quinoprotein MDH, must likewise have been due to a minor enzyme in the preparation. It is possible that this was the same methylene H₂MPT-DH observed during the present study; however, the complex kinetics, the effect of the modifin of inhibiting oxidation of aldehydes larger than formaldehyde and the effect of NAD⁺ in changing the spectral properties of the preparation cannot be explained by the model presented here. If H₂MPT is indeed the active component of HTSE and the modifin preparations, the FDH activities in the presence of modifin and HTSE reported here and by Tate & Dalton (1999) are overestimated by about 4-4-fold, because of the increase in Δ₄₄₀ due to oxidation of methylene H₂MPT to methenyl H₂MPT (Vorholt *et al.*, 1998) that was not taken into account during calculation of these activities.

The available evidence suggests at least two routes via which formaldehyde can be oxidized in *M. capsulatus* (Bath): (1) via the H₂MPT-dependent pathway; (2) by oxidation to formate by the membrane-associated FDH described by Zahn *et al.* (2001). It is also intriguing that the soluble FDH purified from *M. capsulatus* (Bath) by Stirling & Dalton (1978) was clearly distinct from the enzymes identified in preparation FDH-1 because it used NAD⁺ as its electron acceptor but was dependent on a factor from HTSE which, since it was sensitive to trypsin, is unlikely to have been H₂MPT. Thus, although the preparation that Tate & Dalton (1999) classified as a soluble FDH is clearly principally composed of an MDH, the existence of a soluble NAD(P)⁺-dependent FDH in this organism cannot currently be excluded.

ACKNOWLEDGEMENTS

We thank Arthur Moir (University of Sheffield) for performing N-terminal sequencing, Neil Errington (National Centre for Macromolecular Hydrodynamics, University of Nottingham) for performing equilibrium sedimentation analysis, Rudolf K. Thauer (Max-Planck-Institute for Terrestrial Microbiology, Marburg, Germany) for the gift of a sample of tetrahydromethanopterin, Susan Slade for technical assistance during protein purification and Keith Jennings for advice about mass spectrometry. This work was supported by the BBSRC through a studentship to E.K.A.
REFERENCES


