A low-molecular-mass protein from *Methylococcus capsulatus* (Bath) is responsible for the regulation of formaldehyde dehydrogenase activity in vitro

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An 86 kDa protein, which the authors call a modifin, has been purified from *Methylococcus capsulatus* (Bath) and has been shown to alter the substrate specificity and kinetics of NAD⁺-linked formaldehyde dehydrogenase (FDH) isolated from the same organism. Purification methods for both the modifin and FDH are presented which reliably produced pure protein for further analysis. Analysis of the molecular mass and N-terminal sequence of both FDH and the modifin indicate that they are unique proteins and show no similarity to alcohol or aldehyde dehydrogenase enzymes isolated from methyloptrophic bacteria. Substrate specificity studies demonstrated that FDH oxidized formaldehyde exclusively in the presence of the modifin; a diverse range of aldehydes and alcohols were oxidized by FDH in the absence of the modifin. No formaldehyde oxidation was detected in the absence of the modifin. Attempts to replace the modifin with glutathione or high concentrations of methanol to stimulate formaldehyde oxidation failed. With acetaldehyde as substrate, FDH showed standard Michaelis-Menten kinetics; interaction of FDH with the modifin using formaldehyde as substrate altered the kinetics of the reaction to sigmoidal. Kinetic analysis during turnover experiments indicated that the FDH may be associated with bound formaldehyde following enzyme isolation and that NAD may also be associated with the enzyme but in a form that is less tightly bound than found with the methanol dehydrogenase from *Bacillus methanolicus*. Data are presented which indicate that the modifin may play an important role in regulating formaldehyde concentration in vivo.

Keywords: *Methylococcus capsulatus* (Bath), modifier protein, protein regulation, formaldehyde dehydrogenase

INTRODUCTION

Methylotrophic bacteria that grow on single-carbon compounds as their sole carbon and energy source produce formaldehyde as an intermediate from the oxidation of the primary carbon substrate (Anthony, 1982). Formaldehyde can then either be assimilated via one or more pathways into central metabolites or oxidized further to carbon dioxide, generating energy in the form of NADH₂. The oxidation of formaldehyde to formate is catalysed by formaldehyde dehydrogenase (FDH), of which there are two principal types depending on whether the electron acceptor is NAD⁺ or an artificial dye. Within these two groups eight different forms have been identified (Stirling & Dalton, 1978). These have been classified according to their requirements for secondary cofactors such as glutathione (Harrington & Kallio, 1960; Hampton & Zatman, 1973; Johnson & Quayle, 1964; Eggeling & Sahm, 1984; van Ophem et al., 1992), other thiol compounds (Poels & Duine, 1989), other proteins (Stirling & Dalton, 1978), or their need for an activator, such as tetrahydrofolate (Large & Quayle, 1963). They have also been classified according

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Abbreviations: CV, column volume; ESI-MS, electrospray ionization mass spectrometry; FDH, formaldehyde dehydrogenase; gFDH, GSH-dependent formaldehyde dehydrogenase; HTSE, heat-treated soluble extract; MDH, methanol dehydrogenase; sMMO, soluble methane monoxygenase.
to their substrate specificity (Johnson & Quayle, 1964; Patel & Hoare, 1971; Boulton & Large, 1977).

The FDH enzyme isolated from *Methylcoccus capsulatus* (Bath) is particularly unusual in that it required a heat-stable (70 °C/12 min) dialysable protein for activity. No formaldehyde oxidation activity was detected if this heat-stable factor was replaced by thiol compounds (Stirling & Dalton, 1978). Attempts to purify the heat-stable protein met with little success. Subsequent attempts to purify this component by J. Green in our laboratory (unpublished observations) led to the observation that the FDH component of the complex would only oxidize formaldehyde in the presence of the heat-stable protein. In the absence of the heat-stable protein FDH was able to oxidize other aldehydes and some alcohols.

This communication reports our findings concerning the interaction of the heat-stable modifier protein (which we prefer to call a modifin since it is able to modify the substrate specificity of the dehydrogenase) with FDH. In particular we describe reliable purification methodologies for both the modifin and FDH. Kinetic data are also presented on the interaction between the modifin and FDH which suggest how the alteration in the substrate specificity may be effected.

**METHODS**

All reagents, unless otherwise stated, were purchased from Sigma and were of the highest grade available. All spectrophotometric measurements were made using a Hewlett Packard HP8452A diode array spectrophotometer (Hewlett Packard).

**Formaldehyde determination.** The acetylacetone method for formaldehyde concentration determination (Nash, 1953) was used. In summary, the Nash reagent (2 ml) and the analyte (0-1 ml) were incubated at 60 °C for 5 min and then diluted to a total volume of 10 ml with water. The absorbance of the diluted reaction mixture was read at 412 nm. A calibration curve was prepared using a standard solution of 1 M formaldehyde (produced by the hydrolysis of the more stable polymer paraformaldehyde in a sealed bottle for 12 h at 100 °C).

**Enzyme assays.** The amounts of formate and acetate produced in enzyme assays were determined by HPLC analysis. A 50 µl sample was taken from an enzyme reaction mixture and diluted with 50 µl concentrated phosphoric acid. This sample was centrifuged at 16,500 g for 5 min prior to a 25 µl aliquot being loaded onto an Hichrom Licrosphere HPLC column equilibrated with an aqueous solution of 0.01 M octylamine adjusted to pH 6.6 with phosphoric acid. The sample was then desalted on a Pharmacia Fast Desalt column at a flow rate of 1 ml min⁻¹ in 15 column volumes (CV) at pH 8.0, containing 6 M urea. The salt fraction (2 ml) was loaded onto a Pharmacia Mono Q (HR5/5) column equilibrated with the desalting buffer. The sample was eluted in a linear gradient of 0–1 M KCl in 15 column volumes (CV) at a flow rate of 0.5 ml min⁻¹. Standard NAD⁺ and NADH were also urea-treated and served as standards for the identification of NAD⁺ in the test sample.

**Growth of organism.** *M. capsulatus* (Bath) was grown in a continuous culture vessel (2 l) under oxygen-limiting conditions on nitrate mineral salt medium buffered with HCl at pH 7.2 (Dalton & Whittenbury, 1976). Cells were grown at 45 °C at a dilution rate of 0.024 h⁻¹ with methane (25% v/v in air) as the sole carbon and energy source. Bacteria were harvested from a 201 overflow vessel by centrifugation at 10,000 g for 10 min. The resulting cell pellet was washed by resuspending in 100 ml 20 mM phosphate buffer, pH 7.2. The resuspended cells were centrifuged at 10,000 g for 10 min, the supernatant removed and the cell paste resuspended in 20 ml of the same buffer. The cells were stored drop-frozen in liquid nitrogen at −80 °C.

**Protein purification.** Cell extract containing both FDH and the modifin was prepared by passing defrosted cell paste through a Constant Systems (Holly Farm Business Park, Honley, UK) cell disrupter at 146 MPa and centrifugation at 68,500 g for 1.5 h. The supernatant was retained as the crude extract. Protein purification was performed using either a Pharmacia FPLC system or a Pharmacia BioPilot system.

**FDH purification procedure**

Step 1. 30% (w/v) ammonium sulphate was added to crude extract and left to stand on ice for 30 min. Precipitated material was removed by centrifugation at 48,500 g. Ammonium sulphate was increased in the supernatant to 55% (w/v) and the precipitate again removed by centrifugation. The pellet was redissolved in a minimum volume of 25 mM Tris/HCl, pH 7.2, containing 50 mM KCl.

Step 2. The FDH active solution from Step 1 was loaded onto a custom-packed Pharmacia Superdex 200 pg (XK 50/1000) gel filtration column, previously equilibrated with 2 CV
precipitated material was removed by centrifugation at 30,000 g for 10 min. The supernatant was used as a heat-treated soluble extract (HTSE).

Step 2. The HTSE solution was loaded onto a Pharmacia Q-Sepharose (HR 26/10) anion-exchange column equilibrated with 25 mM Tris/HCl buffer, pH 7.2, containing 100 mM KCl. Protein was eluted at a flow rate of 0.75 CV h⁻¹. Fractions were collected and those showing modifin activity were pooled, drop-frozen in liquid nitrogen and stored at −80°C.

Preparation of heat-treated soluble extract and purification of the modifin

Step 1. Crude extract was heated to 70°C for 12 min. The precipitated material was removed by centrifugation at 10,000 g for 20 min. The supernatant was used as heat-treated soluble extract (HTSE).

Step 2. The HTSE solution was loaded onto a Pharmacia Q-Sepharose (HR 26/10) anion-exchange column equilibrated with 25 mM HEPES buffer, pH 7.2, containing 1 mM benzamidine. After sample loading, the column was washed with 1 vol. equilibration buffer and then the concentration of KCl was increased to 0.3 M in the same buffer, over 0.25 CV. The FDH protein was eluted in a linear gradient of 0.3–0.5 mM KCl in the same buffer, over 3 CV. The column was regenerated by increasing the KCl concentration to 1 M for 0.75 CV. Collected fractions were tested for formaldehyde oxidation activity. Active fractions were pooled and concentrated over a 30 kDa exclusion limit at 4°C.

Step 3. The concentrated active protein from the gel filtration column was loaded onto a Pharmacia Superdex 75 (HR 10/30) column equilibrated with 25 mM Tris/HCl buffer, pH 7.2, containing 100 mM KCl. Protein was eluted at a flow rate of 0.75 CV h⁻¹. Fractions were collected and those which demonstrated FDH activity were pooled, drop-frozen in liquid nitrogen and stored at −80°C.

Step 4. The active FDH material from Step 3 was loaded onto a Pharmacia Superdex 75 (HR 26/60) column equilibrated with 20 mM HEPES buffer, pH 7.2, containing 1 mM benzamidine. After sample loading, the column was washed for 2 CV with equilibration buffer at a flow rate of 6 CV h⁻¹. The sample was eluted from the column using a linear gradient of 0–1 M KCl in 5 CV equilibration buffer. Fractions were collected and those showing modifin activity were pooled and concentrated in an Amicon ultrafiltration unit over a membrane with an exclusion limit of 3 kDa.

Step 3. The concentrated protein from step 2 was loaded onto a Pharmacia Superdex 75 pg (HR 26/60) column. The fractions were eluted with 20 mM HEPES buffer, pH 7.2, containing 1 mM benzamidine at a flow rate of 0.75 CV h⁻¹. Collected fractions were assayed for modifin activity. Those exhibiting activity were pooled and concentrated over a membrane with an exclusion limit of 3 kDa ultrafiltration membrane. The concentrated material was sterile filtered through a 0.2-μm Whatman PVDF filter and stored at room temperature.

UV–visible spectra of proteins. All proteins were diluted in 25 mM phosphate buffer prior to measuring the absorbance over a wavelength range 220–800 nm.

Molecular mass determinations. The molecular mass of FDH was estimated by SDS-PAGE using a 15% (w/v) gel system with low-molecular-mass protein standard markers (Novex). Accurate molecular mass measurements of FDH and modifin were made using electrospray ionization mass spectrometry (ESI-MS) on a Quattro II tandem mass spectrometer (Micromass, Altrincham, UK). ESI-MS experiments were carried out in the nebulizer-assisted electrospray mode. The potential of the electrospray needle was set at 3.5 kV and the extraction cone voltage was set at 50 V. FDH was diluted to 40 pmol ml⁻¹ in Tris buffer, pH 7.2, containing 5% (v/v) acetic acid. Aliquots of 10 μl were introduced into the source at a flow rate of 5 μl min⁻¹. Mass spectra were acquired over the range m/z 600–1700. Calibration was carried out using a solution of horse heart myoglobin.

N-terminal sequence analysis. Protein samples for N-terminal sequence analysis were prepared as described by Packman (1993). Protein bands visualized on PVDF membranes were excised and sent for N-terminal sequence analysis at the University of Southampton Protein Sequencing Unit.

RESULTS

Purification of protein components

Attempts to purify FDH by the procedure previously reported by Stirling & Dalton (1978) resulted in heavy contamination by the hydroxylase component of the soluble methane monooxygenase (sMMO). Table 1 summarizes the procedure developed in this study which gave a reliable purification of FDH from M. capsulatus (Bath) soluble extract without sMMO contamination (which was removed by the Q-Sepharose chromatographic step). During purification of FDH, stabilization of formaldehyde oxidation activity proved difficult. Attempts to stabilize activity by inclusion of protease inhibitors such as benzamidine, or glycols such as mannitol or glycerol, had no effect on formaldehyde oxidation activity. Although a large amount of FDH activity was lost during the S200 gel filtration chromatography step, by using this step to remove the ammonium sulphate from the active fractions, purification time and the loss of enzyme activity were minimized.

A small (10 kDa) protein was identified by SDS-PAGE (Fig. 1, lanes D and E) in the last two stages of the FDH purification scheme. This contaminating molecule was removed by the final gel filtration step and corresponded to a loss in background formaldehyde oxidation activity. When this small protein was collected from the S75 gel filtration column and added back to FDH preparations, the background rate of formaldehyde oxidation activity was restored. It was concluded that the small protein identified was the modifin which copurified with the FDH.

Purification of the modifin has not been reported previously and Table 2 summarizes the procedure developed. Heat treatment of the soluble extract was required for the activity of the modifin to be discernible in crude soluble extract. Therefore no data are given for the activity of modifin in crude extract. This heat treatment was a useful initial step in the purification, removing approximately 75% of contaminating soluble protein, whilst attempts to purify the modifin without heat treatment were unsuccessful. It is possible that the modifin may be associated with another protein which is liberated upon heat treatment. An increase in the yield of modifin was also observed during the purification. It is possible that the modifin was removed from a component which interfered with the activity of the modifin or FDH. The purified material was active for...
S. TATE and H. DALTON

**Table 1. Purification of FDH**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (μmol min⁻¹ mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>190</td>
<td>9500</td>
<td>36100</td>
<td>4</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>60</td>
<td>3720</td>
<td>33500</td>
<td>9.3</td>
<td>92</td>
<td>2.3</td>
</tr>
<tr>
<td>Gel filtration chromatography</td>
<td>40</td>
<td>134</td>
<td>4600</td>
<td>34</td>
<td>12.7</td>
<td>8.5</td>
</tr>
<tr>
<td>Ion-exchange Q-Sepharose</td>
<td>24</td>
<td>16.8</td>
<td>3900</td>
<td>232</td>
<td>10.8</td>
<td>58</td>
</tr>
<tr>
<td>Superdex 75</td>
<td>10</td>
<td>16.4</td>
<td>3850</td>
<td>234</td>
<td>10.7</td>
<td>58.5</td>
</tr>
</tbody>
</table>

The subunit molecular mass of FDH was determined as 60 kDa by SDS-PAGE (Fig. 1) and as 63615 Da by ESI-MS. The molecular mass of the native FDH complex was determined as 250 kDa by gel filtration (data not shown) using a Pharmacia Superdex 200 gel filtration column. We assume therefore that FDH exists as a tetramer of 63 kDa subunits; this contrasts with the data published by Stirling & Dalton (1978), who reported that FDH was a dimer of 57 kDa subunits.

**Modifin.** The molecular mass of the modifin, as determined by ESI-MS, gave two species of molecular mass 8211 and 16421 Da, indicating either that the protein sample was heterogeneous or that the modifin existed as a dimer. The native molecular mass of the modifin was determined by gel filtration chromatography using a Pharmacia (HR10/30) S12 column. Pure modifin eluted as two species corresponding to approximately 10 and 20 kDa molecular mass (data not shown). This confirms that the two species identified by ESI-MS are both modifin and it is concluded from these data that the modifin exists as both a monomer and a dimer, of molecular mass 8211 Da and 16421 Da, respectively.

Purified samples of FDH and modifin were blotted onto PVDF membranes and their N-terminal sequences determined. Fig. 2 shows the sequence data generated from the two samples. These data were used to search the EMBL and SWISS-PROT databases for similar sequences using the EBI BLAST alignment program. No matching sequences were identified for either sequence. Comparison of the N-terminal sequence for FDH with published sequences of other FDH enzymes also showed no similarity with FDH enzymes which have been previously isolated from methylotrophic organisms. Searching the databases for proteins with similar amino

**Table 2. Purification of the modifin**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (μmol min⁻¹ mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat treatment</td>
<td>75</td>
<td>2250</td>
<td>49.5</td>
<td>22</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ion-exchange Q-Sepharose</td>
<td>220</td>
<td>2046</td>
<td>94.6</td>
<td>46</td>
<td>190</td>
<td>2.0</td>
</tr>
<tr>
<td>Gel filtration S75</td>
<td>60</td>
<td>24</td>
<td>72.6</td>
<td>3025</td>
<td>145</td>
<td>138</td>
</tr>
</tbody>
</table>
Regulation of formaldehyde oxidation

Modifin: S D G H X V A P X N

N-FDH: N S E L D R L X K P X R N V V
NMFD-FDH: K T K A A V L H S A G K P F E 1 2 E E L D G P R E H V
PD-FALDH: M R T R A A V A E A G K P L E V M E V N
HORSE FDH: S T A G V K I K C K A A V L W E
S.CER-FDH: S T A G V I K C R K A V T W E
E.COLI-FDH: N K S R A A V A F A P G R F L

Table 3. Substrate specificity of FDH in the presence and absence of the modifin

100% activity is equivalent to 240 nmol NADH formed min⁻¹ from 0.6 μM FDH in the presence of 20 μM modifin. Each substrate tested was added to the reaction mixture to a final concentration of 2 mM.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (%)</th>
<th>With modifin</th>
<th>Without modifin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>100</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td></td>
<td>0</td>
<td>57</td>
</tr>
<tr>
<td>Propanal</td>
<td></td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>Butanal</td>
<td></td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Pentanal</td>
<td></td>
<td>0</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Methanol</td>
<td>0</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>0</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Propanol</td>
<td>0</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Butanol</td>
<td>0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

(continued)

acid sequences and molecular masses also generated no matching proteins.

Substrate specificity of FDH and kinetics of formaldehyde/acetaldehyde oxidation

It was reported previously that FDH, in the presence of a heat-stable cofactor, was able to catalyse the oxidation of formaldehyde and a range of complex aldehydes (Stirling & Dalton, 1978). The activity of FDH in the absence of the heat-stable cofactor was not determined at that time. In this study the active component of the heat-stable extract (the modifin) was used to further characterize the substrate specificity of FDH. Table 3 shows that FDH is able to oxidize a range of aldehydes and alcohols in the absence of the modifin. Furthermore, formaldehyde was only oxidized by FDH when the modifin was present. This demonstrates that the modifin is acting as a powerful control protein to regulate formaldehyde oxidation. This is graphically demonstrated in Fig. 3, where the rate of formaldehyde and acetaldehyde oxidation are displayed as a function of the modifin concentration. Increasing the concentration of the modifin inhibits acetaldehyde oxidation with a concomitant increase in the rate of formaldehyde oxidation. This figure also shows that for maximum formaldehyde oxidation activity the FDH:modifin ratio should be 1:4 or greater.

The ability of FDH to oxidize alcohols in the absence of modifin (Table 3) has also been observed for FDH enzymes purified from Amycolatopsis methanolica and Hyphomicrobi um X (van Ophem et al., 1992; Poels & Duine, 1989). These enzymes were able to catalyse the oxidation of alcohols and aldehydes, but required the presence of reduced glutathione for the catalysis of
formaldehyde oxidation. The effect of reduced glutathione on the rate of formaldehyde oxidation by FDH isolated from *M. capsulatus* (Bath) was therefore investigated. Glutathione concentrations up to 2 M were added to the FDH reaction mixture in the absence of modifin but in no instance was formaldehyde oxidation detected.

van Ophem *et al.* (1992) demonstrated that high concentrations of methanol had a similar effect as glutathione in FDH enzymes isolated from *A. methanolicum*. Methanol was therefore added to the *M. capsulatus* FDH reaction mixture (up to a final concentration of 2 M) in the absence of modifin and formate production determined. No restoration of formaldehyde oxidation activity to FDH was observed.

The stoichiometry for the catalysis of formaldehyde and acetaldehyde oxidation in the presence and absence of modifin was investigated. A known amount of formaldehyde (100 nmol) and NAD$^+$ (2 mmol) was added to the FDH/modifin reaction mixture and the concentrations of formate and NADH formed were determined after 30 min. This experiment was repeated using FDH in the absence of the modifin with 100 nmol acetaldehyde as substrate and determining the amounts of acetate and NADH formed after 30 min. The oxidation of 100 nmol formaldehyde produced 94 nmol formate and 116 nmol NADH whilst the oxidation of 100 nmol acetaldehyde, in the absence of modifin, produced 107 nmol acetate and 119 nmol NADH. In both cases there is a stoichiometric relationship between substrate consumption and product formation, indicating a dehydrogenase activity. If FDH could perform a dismutase reaction, then the ratio of substrate to product would not be that observed, as the products of the dismutase reaction would be formate and methanol; no methanol was detected in this reaction.

Investigation of the kinetic constants for formaldehyde and acetaldehyde oxidation showed another important property of the modifin. Formaldehyde oxidation, in the presence of the modifin, showed a sigmoidal increase in the rate of NADH formation with increasing formaldehyde concentrations (Fig. 4a). However, a minimum concentration of acetaldehyde was required before substrate oxidation would occur. Acetaldehyde oxidation followed standard Michaelis-Menten kinetics thereafter (Fig. 4b). No oxidation of acetaldehyde was observed at substrate concentrations less than 1.5 mM, suggesting that there may be an inhibitor of acetaldehyde oxidation bound to the purified FDH.

To determine the nature of the molecule bound to FDH the enzyme was allowed to turn over in the presence of...
NAD⁺ molecule bound to each subunit of FDH.

During purification of the modifin, its yield, after the anion-exchange step, increased to 190 %, suggesting that an inhibitor of formaldehyde oxidation was being removed at this stage. Stirling & Dalton (1978) reported an increase in the yield of the FDH component (they did not purify the modifin) which they attributed to the substrate specificity of \( \gamma \)-galactosyltransferase, causing it to use glucose, rather than \( N \)-acyethylglucosamine, as a substrate in lactating mammary glands (Hill & Brew, 1975).
the inhibitory component in the present investigation, it is tempting to speculate that this factor may play a role in regulating the activity of the modifin itself (an anti-modifin).

Purified FDH was shown to have one formaldehyde molecule tightly bound to the enzyme; this may be important for stabilization of FDH in vivo or it could act as a regulator of activity by preventing the enzyme from oxidizing other substrates such as alcohols or higher aldehydes. Although we have not identified this molecule unequivocally, we believe it is likely to be formaldehyde, based on the observation that it can only be removed following turnover of the enzyme by addition of the modifin and NAD+. Once this substrate is removed, bound NAD+ can be detected on FDH. The association of NAD+ with FDH does not appear to be as strong as that of NAD+ in methanol dehydrogenase isolated from *Bacillus methanolicus* (Arfman et al., 1997). In the case of methanol dehydrogenase, an activator protein is believed to facilitate the cycling of the permanently bound NAD+ between oxidized and reduced states (Arfman et al., 1997). If NAD+ was bound to FDH in this manner, an absorbance maximum of the purified enzyme at 265 nm should be visible, but no such absorbance was detected (Fig. 6). It is assumed that formaldehyde binds preferentially before NAD+, or that the modifin is required for NAD+ binding.

The observed co-operativity and high affinity of formaldehyde binding in vitro suggests a detoxification role for FDH in preventing the accumulation of formaldehyde within the cell, in a manner similar to that proposed for other FDH enzymes (Fernandez et al., 1995; Harms et al., 1996). Such a ‘safety valve’ system is extremely important in methylotrophic bacteria since the formation of formaldehyde is an integral step in the growth of these organisms (Anthony, 1982). Without a method for the removal of any freely available formaldehyde, cell death would rapidly ensue.

There are three potential enzymes responsible for formaldehyde oxidation in *M. capsulatus* (Bath): methanol dehydrogenase (MDH) (Long & Anthony, 1991), FDH (Stirling & Dalton, 1978) and a cytochrome-dependent FDH (Hay, 1990). Both the cytochrome-dependent FDH and the MDH are not reactive with NAD/NADH. Thus the function of the FDH and modifin characterized here may be to serve not only as a ‘safety valve’ but also to supply NADH. As NADH is required for the hydroxylation of methane by sMMO (Colby & Dalton, 1978) without an NAD+-linked FDH the cells would almost certainly be NADH-limited during growth (Anthony, 1982). An earlier proposition that tetrahydrofolate-linked enzymes may be involved in formaldehyde oxidation by methylotrophs (Attwood & Quayle, 1984) has recently been verified by Chistoserdova et al. (1998) for *Methylbacterium extorquens* AM1, but these enzymes have not been identified in *M. capsulatus* (Bath) as yet.

In conclusion, it is proposed that the modifin is a novel control protein which regulates formaldehyde oxidation in *M. capsulatus* (Bath) in vitro. Data presented in this paper demonstrate the ability of the modifin to alter both the kinetics and the substrate specificity of alcohol/ aldehyde dehydrogenase. It is possible that the physiological function of the modifin is both to reduce formaldehyde concentrations and to generate NADH for methane hydroxylation by sMMO. The precise signals which stimulate expression and activity of the modifin are unknown but the data presented indicate that both high formaldehyde and NAD+ concentrations are probable signals. The identification of mutants or deletion of the modifin gene will allow its impact on the physiology of *M. capsulatus* (Bath) to be dissected and verify the intracellular signals required to stimulate activity.

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REFERENCES


