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Characterisation of a red form of methanol dehydrogenase from the marine methylotroph *Methylophaga marina*

H.T.C. Chan and C. Anthony

SERC Centre for Molecular Recognition, Department of Biochemistry, University of Southampton, Southampton, UK

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1. SUMMARY

The quinoprotein methanol dehydrogenase (MDH) of the marine methylotroph *Methylophaga marina* is similar to that of other methylotrophs in being an $\alpha_2\beta_2$ tetramer containing two molecules of PQQ and a single atom of calcium. Its electron acceptor is cytochrome c_L and interaction of the two proteins is by way of carboxylates on the cytochrome and lysyl residues on the α subunit of MDH. The reaction was not, however, sensitive to high ionic strength as was the reaction in non-halophilic bacteria. A red form of the enzyme was sometimes produced which had a low specific activity and a low calcium content. Activity was restored by incubation with Ca^{2+} which also produced the typical (green) enzyme, with a typical absorption spectrum. This provides the first demonstration of reconstitution of active MDH from enzyme lacking calcium isolated from a wild-type methylotroph.

Correspondence to: C. Anthony, SERC Centre for Molecular Recognition, Department of Biochemistry, University of Southampton, Southampton SO9 3TU, UK.

2. INTRODUCTION

In Gram-negative methylotrophic bacteria, the first oxidation step during growth on methanol is catalysed by methanol dehydrogenase (MDH) which is an NAD^+-independent quinoprotein that is usually assayed in a dye-linked assay system [1]. The structure of MDH is an $\alpha_2\beta_2$ tetramer [2–5], and some MDHs have recently been shown to have a single Ca^{2+} per tetramer [5,6]. In the two cases studied there are two molecules of PQQ per tetramer [6,7]. The natural electron acceptor for MDH is a specific *c*-type cytochrome called cytochrome c_L [8]; both of these proteins are in the periplasm and so are exposed to the same conditions of pH and ionic strength as are present in the growth environment.

Methylophaga marina is a marine methylotroph which is similar in many ways to the obligate methylotroph *Methylophilus methylotrophus*; it differs in being moderately halophilic, thus requiring a growth medium containing NaCl (410 mM) [9]. A preliminary description of its MDH indicated that, although differing in its substrate specificity and isoelectric point, it is essentially similar to other MDHs; however, the reaction

with cytochrome c_L was not investigated, nor were the subunit structure, or the PQQ and calcium contents determined [10]. The system from *M. marina* is of particular interest because it has been shown recently that the interaction of MDH with cytochrome c_L is strongly inhibited by high ionic strength in *Methylophilus methylotrophus*, *Methylobacterium extorquens* and *Acetobacter methanolicus*; it was concluded from this that the interaction between MDH and cytochrome c_L is electrostatic in nature, and it was subsequently demonstrated that this interaction is by way of carboxylate groups on the cytochrome c_L and lysyl groups on the MDH α-subunit [3,4]. This raises a number of questions about the system for methanol oxidation in *M. marina*: does its MDH have the usual $\alpha_2\beta_2$ tetrameric structure; does it contain the usual complement of PQQ, does it contain Ca^{2+}; does it react with a *c*-type cytochrome corresponding to cytochrome c_L; if so, how do these proteins interact in a periplasm that contains about 400 mM NaCl? This paper reports our investigation of these questions together with some observations on the binding of Ca^{2+} and PQQ that are different in this organism in a way that probably relates to its marine environment.

3. MATERIALS AND METHODS

Two strains of *M. marina* were used. Most experiments described here used strain 42 which was a gift from Dr. F. Gasser (Pasteur Institute, Paris, France). The production of the red form of MDH was confirmed using strain 2244 obtained from NCIMB, Aberdeen, UK. Bacteria were grown aerobically on methanol in artificial seawater medium [9] in 15-l fermenters as described previously [10].

MDH was purified initially by the two-phase partition system using polyethyleneglycol, followed by ion-exchange chromatography, as described previously [10]. This sometimes produced enzyme containing impurities as judged by SDS-PAGE and further steps were included; these were cation-exchange chromatography on S-Sepharose (Pharmacia) and gel filtration on Sephacryl-200 (Pharmacia), as described previously for the enzyme from *Methylobacterium extorquens* [11]. MDH was assayed using the dye-linked assay [11] or the cytochrome-linked assay at pH 7.0; 2,6-dichlorophenolindophenol was used as the terminal electron acceptor for studies of the effect of salt on enzyme activity [4] and horse heart cytochrome *c* as the terminal electron acceptor for all other studies, as described previously [4].

The stoichiometry of the subunits of MDH and the PQQ content were exactly as shown for *Methylobacterium extorquens* previously [2]. Calcium was determined, and reconstitution of active MDH with Ca^{2+} was as described previously [6].

The effects of salts on MDH activities, the modification with 2,4,6-trinitrobenzenesulphonate, and the two-stage sulpho-*N*-hydroxysuccinimide-enhanced cross-linking with 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide was as described for *Methylobacterium extorquens* and *Acetobacter methanolicus* [3,4].

Cytochromes were purified and characterised, and SDS-PAGE and haem-staining done as described earlier [12]. Absorption spectra were recorded as previously described [6].

4. RESULTS AND DISCUSSION

4.1. Purification and reaction with cytochrome c_L

The MDH from methanol-grown *Methylophaga marina* was initially purified by a single-stage method based on that previously described [10], with additional chromatography steps as described in MATERIALS AND METHODS; these were necessary to remove some impurities present after the two-phase partition steps. Although the MDH produced was sometimes the typical green colour as previously described for this enzyme, occasionally a violet-red form was produced. We were unable to determine what led to the production or otherwise of this form.

Extracts of *Methylophaga marina* contained two major soluble *c*-type cytochromes as shown by haem-staining proteins after SDS-PAGE (M_r about 19 000 and 10 000). These were purified as described for cytochrome c_L and cytochrome c_H of *Methylobacterium extorquens*. Their behaviour on columns indicated that the smaller cytochrome

had a relatively high isoelectric point and the larger cytochrome had a relatively lower pI. These two cytochromes thus appear to be similar to the cytochrome c_L and cytochrome c_H from other methylotrophs [8].

The activity of the green active form of the enzyme in the dye-linked assay was as previously described (specific activity, 3.7 μmol min^{-1} mg^{-1}). Only the larger acidic cytochrome (cytochrome c_L) was active with MDH; its specific activity was slightly greater than that of other MDHs in the cytochrome-linked system and it showed a very high affinity for cytochrome c_L (V_{max}, 192 nmol min^{-1} mg^{-1}; K_m, 0.33 μM). When the protein was denatured in SDS and its components measured by gel filtration and SDS-PAGE as described for the MDH of *Methylobacterium extorquens* [2], it was shown to have two types of subunit (M_r, about 65 000 and 8500) arranged as an $\alpha_2\beta_2$ tetramer which contained two molecules of PQQ. When assayed by atomic absorption spectroscopy, the concentration of Ca^{2+} in the active enzyme was 0.85 Ca^{2+}/mol, suggesting that this MDH is similar to others that have been analysed in containing a single calcium atom per $\alpha_2\beta_2$ tetramer.

The only salt having any effect on MDH when it was measured in the dye-linked assay system was NH_4Cl, the usual activator of MDH [1]. In the cytochrome-linked assay system this MDH was remarkably resistant to inhibition by NaCl; no inhibition occurred up to 1.0 M NaCl whereas 10 mM NaCl gives about 50% inhibition with MDHs from other methylotrophs [3,4]. It may thus be concluded that interaction of these two proteins must involve a higher proportion of non-ionic bonds than are usually involved in this interaction. That similar groups on the surface of the two proteins are involved in the interaction is indicated, however, by the effect of chemical modification and cross-linking studies. When MDH was modified with TNBS (four lysine groups modified per mol of MDH) all activity was lost, thus indicating that the presence of highly reactive lysines is important in bonding to cytochrome c_L. That these lysines are on the α subunit was shown by cross-linking unmodified MDH with cytochrome c_L which had some carboxylates modified with sulpho-NHS/EDC. The interaction of MDH and cytochrome c_L was inhibited competitively by EGTA (I_{50}, 10 μM); this powerful inhibition, which was not observed with the dye-linked activity is similar to that shown for MDHs from other methylotrophs and has been interpreted as being due to binding of EGTA to lysine residues on MDH involved in reaction with cytochrome c_L [13].

4.2. The red form of MDH

Occasionally, after cell breakage the MDH was violet-red in colour. This red form of the enzyme of *M. marina* had a lower activity (42%) in the cytochrome-linked assay to the normal green form (V_{max}, 80 nmol cytochrome reduced min^{-1} mg protein^{-1}; K_m, 0.83 μM), but its activity in the dye-linked assay was very low (47 nmol min^{-1} mg^{-1}) (1% of green form). The red enzyme was usually stored in 50 mM-Tris buffer (pH 7.0) containing 100 mM NaCl. When the concentration of salt was diminished by dilution or dialysis, it became green in colour and started to precipitate; this was reversed by addition of 100 mM NaCl. The colour of the red enzyme was due to the relatively high absorbance by the prosthetic group at about 520 nm and the relatively low absorbance at 345 nm compared with the usual forms of MDH [1] (Fig. 1). The 'red' spectrum was seen at pH values of 7.0 or higher. When the pH was lowered to values below 6.0 the enzyme appeared relatively green, the shallow peak at about 520 nm was diminished and the absorbance between 390 and 420 nm increased. It can be seen from Fig. 1 that the pK value for this shift was about 6.5; this suggests that a histidine group on MDH or, more likely, a carboxyl group with a high pK on the MDH or the PQQ itself are involved in binding PQQ.

Although the spectrum was not identical, the absorbance at 520 nm was similar to that observed previously in the MDH of mutants of *Methylobacterium extorquens* which are altered in the biosynthesis of MDH. These MDHs were often reddish in colour, contained no calcium and could be reconstituted to active MDH having a typical absorption spectrum by incubation with Ca^{2+}. That the low activity and unusual spectrum

of the MDH was due to lack of calcium was confirmed by the demonstration that its calcium content was only 0.2 Ca^{2+} ions per $\alpha_2\beta_2$ tetramer of MDH. Incubation in 10 or 20 mM $CaCl_2$ for 2 h at pH 9 (but not pH 7) led to a small increase in activity in the cytochrome-linked assay (2.4-fold) but a very large increase (128-fold) in the dye-linked assay, giving a value similar to that previously published for the MDH from this organism. Concomitantly with this change in activity, the spectrum changed; it lacked the absorbance at 520 nm, and appeared similar to the spectrum of typical MDH (Fig. 2). This observation that the spectrum of the prosthetic group of MDH changes with addition of Ca^{2+} confirms the previous conclusion with MDH from mutants of *Methylobacterium extorquens* that a Ca^{2+} ion is involved in binding of PQQ at the active site. The fact that reconstitution of MDH was only observed at high pH suggests that a group on PQQ

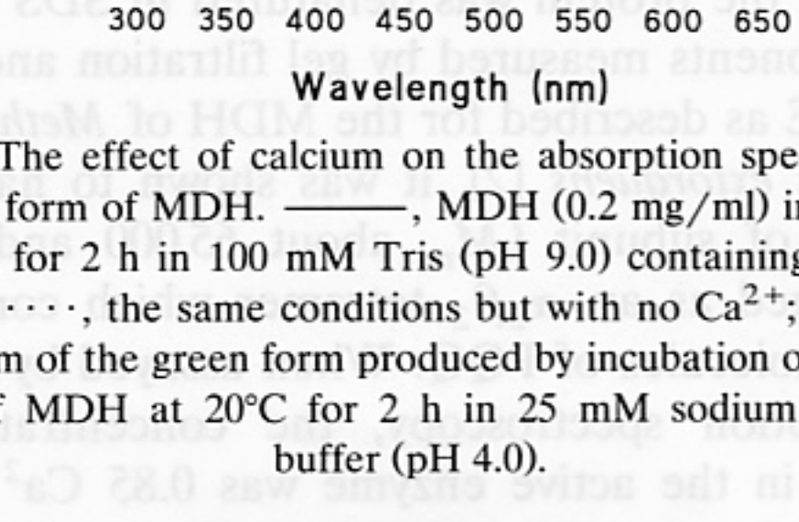


Fig. 2. The effect of calcium on the absorption spectrum of the red form of MDH. ———, MDH (0.2 mg/ml) incubated at 20°C for 2 h in 100 mM Tris (pH 9.0) containing 20 mM Ca^{2+}; · · · · ·, the same conditions but with no Ca^{2+}; - - - - - -, spectrum of the green form produced by incubation of the red form of MDH at 20°C for 2 h in 25 mM sodium formate buffer (pH 4.0).

or on the protein having a pK less than 8 must be in the Ca^{2+}-binding region and must be charged in the dissociated form. We cannot be certain whether or not this is the same group shown to have a pK of about 6.5 in the enzyme lacking calcium.

5. CONCLUSIONS

The experiments described in this paper demonstrate that the MDH from *Methylophaga marina* is similar to that in other methylotrophs in its subunit structure, PQQ content, reactivity with cytochrome c_L and presence of a single Ca^{2+} at the active site. It appears, however, that the MDH of *Methylophaga marina* may have two conformations one of which has a relatively low affinity for Ca^{2+}, and this may be correlated with its normal growth environment which is seawater containing about 10 mM Ca^{2+}. A result of this characteristic is that the red enzyme from *Methylophaga marina* is the only MDH so far described in which a calcium requirement can be

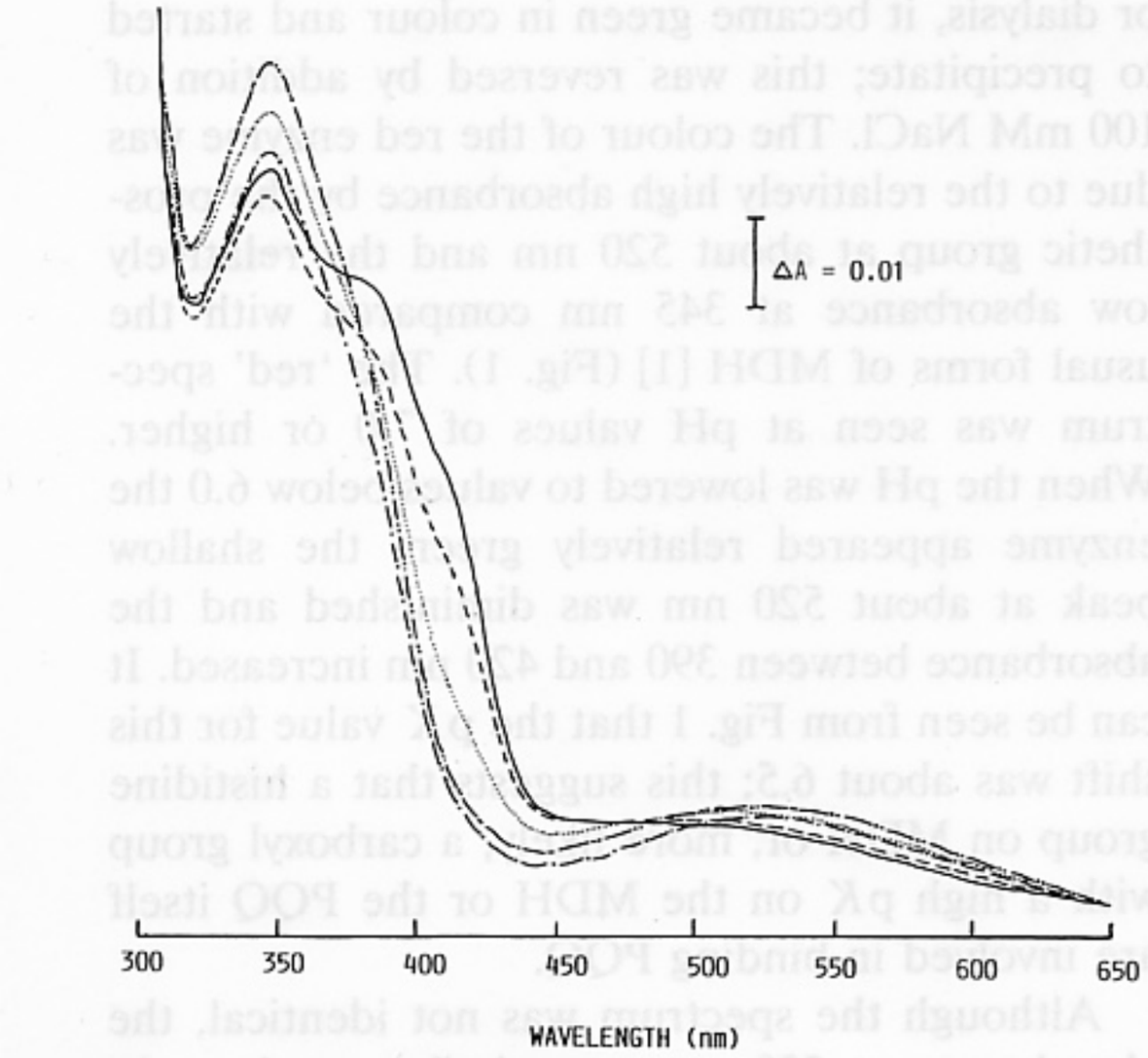


Fig. 1. The effect of pH on the absorption spectra of the red form of MDH from *Methylophaga marina*. The spectrum of MDH (0.2 mg/ml) was recorded in various buffers (25 mM): ———, formate (pH 4.0); - - - - - -, Mes (pH 6.0); · · · · · ·, Mops (pH 7.0); ·–·–·, Tris (pH 8.0); ··–··–··, Caps (pH 10.0). The spectrum at pH 5 (acetate buffer) was the same as that recorded at pH 4.0; the spectrum at pH 9.0 (Tris buffer) was the same as that recorded at pH 8.0. Spectra were recorded at 20°C as described in MATERIALS AND METHODS.

demonstrated by reconstitution of active enzyme from inactive MDH isolated from a wild-type organism. Such reconstitution is a common feature of the quinoprotein dehydrogenases for glucose and ethanol (see ref. 6) but it has not previously been demonstrated with MDH. It is worth noting that the reconstitution of MDH differs from that of the other quinoproteins in that it does not require added PQQ; this appears to be tightly bound to MDH even in the absence of the Ca^{2+}.

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