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Characterization of mutant forms of the quinoprotein methanol dehydrogenase lacking an essential calcium ion

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Methanol dehydrogenase (MDH) from *Methylobacterium extorquens*, *Methylophilus methylotrophus*, *Paracoccus denitrificans* and *Hyphomicrobium* X all contained a single atom of Ca^{2+} per $\alpha_2\beta_2$ tetramer. The role of Ca^{2+} was investigated using the MDH from *Methylobacterium extorquens*. This was shown to be similar to the MDH from *Hyphomicrobium* X in having 2 mol of prosthetic group (pyrroloquinoline quinine; PQQ) per mol of tetramer, the PQQ being predominantly in the semiquinone form. MDH isolated from the methanol oxidation mutants MoxA$^-$, K$^-$ and L$^-$ contained no Ca^{2+}. They were identical with the enzyme isolated from wild-type bacteria with respect to molecular size, subunit configuration, pI, *N*-terminal amino acid sequence and stability under denaturing conditions (low pH, high urea and high guanidinium chloride) and in the nature and content of the prosthetic group (2 mol of PQQ per mol of MDH). They differed in their lack of Ca^{2+}, the oxidation state of the extracted PQQ (fully oxidized), absence of the semiquinone form of PQQ in the enzyme, reactivity with the suicide inhibitor cyclopropanol and absorption spectrum, which indicated that PQQ is bound differently from that in normal MDH. Incubation of MDH from the mutants in calcium salts led to irreversible time-dependent reconstitution of full activity concomitant with restoration of a spectrum corresponding to that of fully reduced normal MDH. It is concluded that Ca^{2+} in MDH is directly or indirectly involved in binding PQQ in the active site. The MoxA, K and L proteins may be involved in maintaining a high Ca^{2+} concentration in the periplasm. It is more likely, however, that they fill a 'chaperone' function, stabilizing a configuration of MDH which permits incorporation of low concentrations of Ca^{2+} into the protein.

INTRODUCTION

In methylotrophic bacteria, methanol is oxidized to formaldehyde by a periplasmic quinoprotein methanol dehydrogenase (MDH), the primary electron acceptor being the specific acidic cytochrome c_L (Anthony, 1986, 1988, 1992*a*,*b*; Nunn & Anthony, 1988). MDH has an $\alpha_2\beta_2$ structure (Nunn *et al.*, 1989; Chan & Anthony, 1991; Cox *et al.*, 1992), each $\alpha_2\beta_2$ tetramer having two molecules of tightly bound prosthetic group, pyrroloquinoline quinone (PQQ), which is usually present in the semiquinone free-radical form (PQQ·H) (Salisbury *et al.*, 1979; Duine *et al.*, 1981).

Studies of the genetics of methanol oxidation in *Methylobacterium* (*Mb.*) *extorquens* AM1 have revealed that at least 22 genes play a role in methanol oxidation (Nunn & Lidstrom, 1986*a*,*b*; Nunn & Anthony, 1988; Nunn *et al.*, 1989; Anderson & Lidstrom, 1988; Anderson *et al.*, 1990; Lidstrom, 1990; Lee *et al.*, 1991). These include *moxF* and *moxI*, which code for the α- and β-subunits of MDH. In addition, three genes have been identified that map apart from the *moxF–moxI* operon, but which also give rise to inactive MDH (Nunn & Lidstrom, 1986*a*,*b*). These genes map closely together and were originally categorized as the *moxA* class; they have subsequently been renamed *moxA*, *moxK* and *moxL* (Lidstrom, 1990). MoxA$^-$, K$^-$ and L$^-$ mutants produced wild-type levels of MDH protein (only the α-subunit was then identified), but the enzymes were inactive and their spectra were markedly different from those of the wild-type bacteria (Nunn & Lidstrom, 1986*b*). These observations suggest that these mutants are unable to synthesize normal PQQ or that these genes code for proteins responsible for the proper association of the PQQ with the MDH apoprotein.

The present paper demonstrates that the prosthetic group in these mutants is normal PQQ, and that mutations in the *moxA*, *moxK* and *moxL* genes lead to synthesis of MDH lacking an essential Ca^{2+} which is involved, directly or indirectly, in binding PQQ to the enzyme.

METHODS

Chemicals

All chemicals were of analytical grade and were obtained from either BDH, Poole, Dorset, U.K., or from Sigma Chemical Co., Poole, Dorset, U.K., except for the following: cyclopropyl methyl ketone and 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide (EDC), both from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.; Econo-pac 10DG desalting column (P-6 column), Bio-Gel HT hydroxyapatite (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.; sulpho-*N*-hydroxysuccinimide (sulpho-NHS) (Pierce Europe, Oud-Beijerland, The Netherlands); PQQ from Fluka.

Growth, harvesting and breakage of bacteria

Mutant strains of *Mb. extorquens* AM1 (N.C.I.M.B. 9133) were provided by Dr. D. N. Nunn; MoxA was previously known as MoxA1, isolated (as mutant PG1) by Tatra & Goodwin (1983); MoxK was known as MoxA2 and was isolated (as mutant UV21) by Nunn & Lidstrom (1986*a*); MoxL was previously known as MoxA3 and was isolated (as mutant M15A) by Heptinstall & Quayle (1970). These strains were fully described by Nunn & Lidstrom (1986*a*,*b*) and renamed by Lidstrom (1990). All these *Mb. extorquens* strains were maintained on nutrient-agar plates kept at 4 °C or, for long-term storage, in nutrient broth with 50% (v/v) glycerol kept at −20 °C. The authenticity

Abbreviations used: MDH, methanol dehydrogenase; ADH, alcohol (ethanol) dehydrogenase; GDH, glucose dehydrogenase; PIP, 2,6-dichlorophenol-indophenol; PES, phenazine ethosulphate; PQQ, pyrroloquinoline quinone; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide; sulpho-NHS, sulpho-*N*-hydroxysuccinimide.

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of the mutant strains was checked by complementation using the tri-parental mating method (Nunn & Lidstrom, 1986*a*). *Mb. extorquens* was grown, harvested and disrupted as described by Day & Anthony (1990*a*). Wild-type bacteria were grown on 0.5% (v/v) methanol, and mutant strains were grown on 0.4% (w/v) methylamine hydrochloride with 0.5% (v/v) methanol present to act as an inducer of the methanol-oxidizing system.

Paracoccus (*Pa.*) *denitrificans* (N.C.I.M.B. 8944 Oxford strain) was maintained, grown, harvested and periplasmic fractions prepared as described previously (Long & Anthony, 1991).

Hyphomicrobium X was grown aerobically on methanol, harvested and disrupted as described by Frank & Duine (1990).

Methylophilus (*Mp.*) *methylotrophus* (N.C.I.M.B. 10515), grown on methanol, was obtained in the form of cell paste from ICI Biological Products Division, Billingham, U.K., and disrupted as described by Cross & Anthony (1980).

Escherichia coli (A.T.C.C. 10798) was grown on glucose, harvested, disrupted and membranes prepared as described by Geiger & Görisch (1987).

Pseudomonas (*Ps.*) *testosteroni* (A.T.C.C. 15667) was grown aerobically on ethanol, harvested and disrupted as described by Groen & Duine (1990).

Purification and assay of MDH and cytochrome c_L

The MDHs from *Mb. extorquens*, *Mp. methylotrophus* and *Hyphomicrobium* X were all purified by using the procedure described by Day & Anthony (1990*a*). MDH from *Pa. denitrificans* was purified from the periplasmic fraction as described by Long & Anthony (1991). MDH activity was assayed as described by Day & Anthony (1990*a*). Cytochrome c_L was purified as described by Day & Anthony (1990*b*). Protein was determined using the bicinchoninic acid method as described by Smith *et al.* (1985).

PAGE and Western blotting

SDS/PAGE and measurement of protein on gels was carried out as described by Day & Anthony (1990*b*). Non-denaturing PAGE was performed using the high-pH (pH 8.9) discontinuous system described by Goldenberg (1989). Antisera raised against holo-MDH from *Mb. extorquens* were prepared, and Western blotting performed, as described by Cox *et al.* (1992).

Spectrophotometric measurements

Absorption spectra were recorded at 20 °C on a SLM-AMINCO DW-2000 UV-VIS spectrophotometer using a scan speed of 100 nm/min with a 2 nm spectral band width and a 10 mm light path.

Fluorescence spectra were recorded at 20 °C on a Perkin–Elmer LS-3B fluorescence spectrophotometer at a scan speed of 120 nm/min with fixed excitation and emission slits of 10 nm nominal bandpass.

E.s.r. spectra of MDH (100 μM) in 10 mM-phosphate buffer, pH 7.0, were recorded on a Bruker ESP 300 *X*-band e.s.r. spectrometer with the help of Dr. David Lowe (Nitrogen Fixation Laboratory, University of Sussex, Falmer, Brighton, Sussex, U.K.). Spectra were recorded at 120 K using the following parameters: time constant, 81.92 ms; modulation amplitude, 0.4166 mT (4.166 G); receiver gain, 2×10^4 s; microwave power, 2×10^{-2} mW; centre field, 0.334 T (3340 G); sweep width, 5 mT (50 G); microwave frequency, 9.37 GHz. The instrument was calibrated using a diphenyl picryl hydrazyl standard (g value = 2.034).

Determination of PQQ

PQQ was determined by reconstitution of active quinoproteins from their apo forms. The first method used the membrane-bound apo-glucose dehydrogenase from *E. coli* as described by Geiger & Görisch (1987). The second method used the soluble apo-alcohol dehydrogenase quinohaemoprotein from *Ps. testosteroni* as described by van der Meer *et al.* (1990).

The prosthetic group was extracted from MDH (250 μg) in methanol at pH 1.0, and PQQ was determined using reverse-phase h.p.l.c. as described by van der Meer *et al.* (1990). Peaks were detected by absorption at 313 nm, and by fluorescence (excitation at 365 nm, total emission at over 418 nm). Identification of the peaks was achieved by performing identical runs with authentic PQQ and the quinol ($PQQH_2$). $PQQH_2$ was prepared by reduction of PQQ with phenylhydrazine, and oxidation of the extracted prosthetic group was achieved by addition of a slight excess of 2,6-dichlorophenol-indophenol (PIP) as described by Duine *et al.* (1981).

Dissociation of MDH

Dissociation of MDH into its component subunits and prosthetic group using gel filtration in the presence of SDS was carried out as described by Nunn *et al.* (1989). Dissociation of MDH at low pH was carried out as described by Anthony & Zatman (1967). The dissociation of MDH using the chaotropic agents urea and guanidinium chloride was based on a general protocol described by Jaenicke & Rudolph (1989). Dissociation in these methods was monitored by measuring the increase in fluorescence at two wavelength pairs: 282 nm excitation/340 nm emission (to detect fluorescence due to tryptophan; Schmid, 1989) and 365 nm excitation/470 nm emission (to detect fluorescence due to PQQ; Anthony & Zatman, 1967). In order to detect PQQ fluorescence effectively, the sensitivity of the fluorimeter was increased 100-fold for the second wavelength pair.

Oxidation and reduction of MDH

MDH is usually isolated with its prosthetic group in the semiquinone or reduced form. Oxidation of MDH was attempted using the method of Duine & Frank (1980). This method relies on the presence of KCN to protect the enzyme, whereas oxidation is achieved by the electron acceptor phenazine ethosulphate (PES)/PIP. Enzyme (10 nmol) in a total volume of 1 ml of 100 mM-tetrasodium pyrophosphate buffer, pH 9.0, or 100 mM-sodium borate buffer, pH 9.0, containing 50 mM-NH_4Cl, 10 mM-KCN and 100 μM-PIP, was mixed with PES at a final concentration of 1 mM. The mixture was passed down a P-6 desalting column (Bio-Rad) equilibrated in the pyrophosphate or borate buffer, to remove low-molecular-mass compounds. An alternative method, described by Dijkstra *et al.* (1984), was also used. Purified MDH (10 nmol) in 1 ml of 100 mM-tetrasodium pyrophosphate buffer, pH 9.0, or 100 mM-sodium borate buffer, pH 9.0, containing 20 mM-NH_4Cl (activator) was mixed with 5 μl of 200 mM-KCN, pH 9.0. After the addition of Wurster's Blue (equimolar with active-site concentration of MDH), endogenous substrate and substrate present in the buffer was oxidized by adding 0.1 μl aliquots of 100 mM-$K_3Fe(CN)_6$ until the blue colour persisted. Typically 0.5 μl of ferricyanide was sufficient to restore the blue colour. After oxidation, the solution was passed down a P-6 column equilibrated in the pyrophosphate or borate buffer.

By using both methods described here, it was found that endogenous substrate, whose nature is unknown but which is always present on MDH (Anthony, 1986), always rapidly reduced the oxidized MDH. When methanol was present throughout the oxidation procedure, the reduced form of enzyme was also produced.

Reaction of oxidized MDH with cyclopropanol

Cyclopropanol reacts with the oxidized form of MDH

(Dijkstra *et al.*, 1984), oxidation being achieved by reaction with Wurster's Blue. This was prepared from *NNN'N'*-tetramethyl-*p*-phenylenediamine hydrochloride as described by Michaelis & Granick (1944). Cyclopropanol was added to the MDH in the presence of Wurster's Blue, as described by Dijkstra *et al.* (1984), in order to facilitate reaction by cyclopropanol before reduction of the oxidized PQQ by endogenous substrate. Cyclopropanol was prepared by the enzymic hydrolysis of cyclopropyl acetate using porcine liver esterase as described by Jongejan & Duine (1987). Cyclopropyl acetate was prepared from cyclopropyl methyl ketone as described by Emmons & Lucas (1955).

Cross-linking of MDH and cytochrome c_L

The two-stage sulpho-NHS-enhanced cross-linking with EDC (Grabarek & Gergely, 1990) was as described previously (Chan & Anthony, 1991; Cox *et al.*, 1992).

Determination of free thiol groups and *N*-terminal sequences

MDH in 25 mM-Hepes buffer, pH 7.0, was assayed for free thiols by using the method described by Riddles *et al.* (1983). *N*-Terminal sequencing of MDH was carried out using an Applied Biosystems 407A 'gas phase' (pulsed liquid) protein sequencer coupled to a model 120 phenylthiohydantoin-derivative analyser as described by Cox *et al.* (1992).

Determination of metal ions in MDH

MDH (100 μM) from wild-type and mutant bacteria was passed through a P-6 column equilibrated with calcium-free 10 mM-Mops buffer, pH 7.0, and Ca^{2+} was determined by using a Perkin–Elmer 280 atomic-absorption spectrometer. Ca^{2+}-free buffers were prepared in water purified by passage through a Millipore Milli Q plus purification system, followed by passage through a column (16 mm × 150 mm) of Chelex-100 chelating resin (Bio-Rad). The concentration of Ca^{2+} in this buffer was less than 1 μM. MDH was assayed for the presence of other metal ions by plasma source spectrometry; the limit of detection for all metals by this method was 1 μg/l.

Reconstitution of active MDH by addition of Ca^{2+} to MDH from MoxA$^-$, K$^-$ and L$^-$ mutant strains

Purified MDH (70–700 pmol) was incubated at 20 °C for 60 min with various concentrations of $CaCl_2$ in 1 ml of 100 mM-Tris buffer, pH 9.0. After incubation the mixtures were used directly to measure activity after the addition of artificial electron acceptors (PES and PIP), substrate (methanol) and activator (NH_4Cl) in the standard dye-linked assay described above.

For the time-dependent reconstitution, MDH (3.7 nmol) was incubated in 500 μM-$CaCl_2$ in 2 ml of 100 mM-Tris buffer, pH 9.0. At regular intervals absorption spectra of the incubation mixture were recorded, and samples (50 μl) were removed and assayed in the standard dye-linked assay system. For measurement of activity in the cytochrome *c*-linked assay system, MDH (1.7 nmol in 1 ml) was incubated in 500 μM-$CaCl_2$ in 100 mM-Tris buffer, pH 9.0. After incubation, excess Ca^{2+} was removed by passage through a P-6 column equilibrated with Ca^{2+}-free 10 mM-Mops buffer, pH 7.0, and activity was measured in the cytochrome *c*-linked assay system. It was necessary to remove the excess Ca^{2+} by this method before assay because activity with cytochrome c_L is very sensitive to high ionic strength (Chan & Anthony, 1991; Cox *et al.*, 1992).

RESULTS

Absorption spectra of MDHs from wild-type *Mb. extorquens* and from MoxA$^-$, K$^-$ and L$^-$ mutants

The spectra shown in Fig. 1 are the typical absorption spectra due to the prosthetic group in MDH from wild-type bacteria; these include spectra of the reduced state and of the semiquinone form in which MDH is usually isolated. Also included is a spectrum of MDH containing oxidized PQQ from *Hyphomicrobium* X. We were unable to generate this form of enzyme from MDH of *Mb. extorquens*. The spectra in Fig. 2 demonstrate that the prosthetic groups in the MDHs from the mutants differ from each other and from the wild-type enzyme. Key features are the absence of an absorption maximum (due to PQQ) at 350 nm and the presence of an absorbing band at about 520 nm that is absent from the wild-type enzyme. This higher absorption band has been observed previously in some samples of MDH that had become inactive after prolonged storage at −20 °C. Comparison of the spectra in Fig. 1 and Fig. 2 shows that the spectrum of the prosthetic group in the altered MDHs does not correspond to that of the prosthetic group in any of its usual redox states. The altered spectra indicate either that the prosthetic group is not normal PQQ, or that it is PQQ, but found so differently in the enzyme that its spectrum is altered.

Prosthetic group of MDH from MoxA$^-$, K$^-$ and L$^-$ mutants

The prosthetic groups of the mutant forms of MDH were extracted with acid or separated by gel filtration in SDS as described in the Methods section. In all cases the absorbance and fluorescence characteristics were indistinguishable from those of authentic PQQ. The total content of PQQ (quinone plus quinol) was determined by h.p.l.c., analysis of spectra and by activity in reconstituting active glucose dehydrogenase (GDH) from apo-enzyme in membranes of *E. coli* or soluble alcohol dehydrogenase (ADH) from the apoprotein from *Ps. testosteroni*. H.p.l.c. analysis under acid conditions was used to determine the relative proportions of oxidized and reduced forms present in the MDH. As summarized in Table 1, the total PQQ content was the same as in wild-type MDH, but it was predominantly in the oxidized form instead of the usual mixture of oxidized and reduced forms which arises from the semiquinone form of the isolated enzyme.

E.s.r. spectroscopy confirmed the presence of PQQ in the semiquinone form on the normal MDH of *Mb. extorquens*, the *g* value was 2.0054, almost identical with the value first recorded for the enzyme from *Hyphomicrobium* (Duine *et al.*, 1981). The spectra for the mutant forms of MDH were identical with each other, but showed no signal whatsoever (after accumulation of 40 scans), indicating the complete absence of semiquinone form,

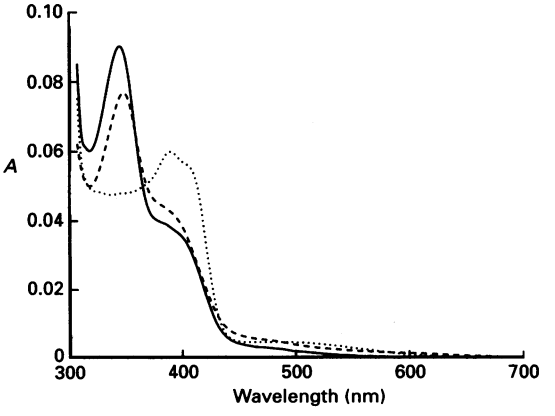


Fig. 1. Absorption spectra of MDH

Spectra of MDH (1 mg/ml) were recorded in 100 mM-potassium phosphate buffer, pH 7.0, at 20 °C. ——, Fully-reduced MDH of *Mb. extorquens*; – – –, semiquinone form of MDH of *Mb. extorquens*; · · · · · ·, fully-oxidized MDH of *Hyphomicrobium* X (taken from Frank *et al.*, 1989).

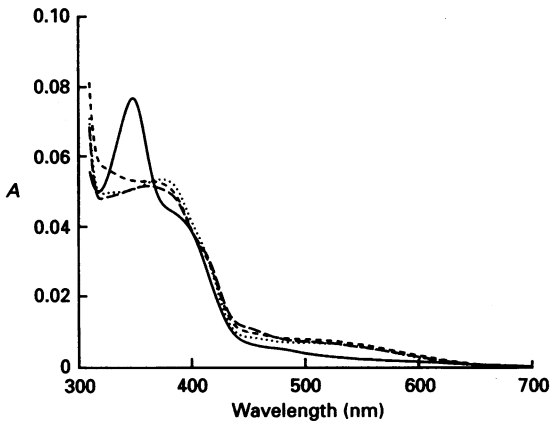


Fig. 2. Absorption spectra of MDH from *Mb. extorquens* and from MoxA$^-$, K$^-$ and L$^-$ mutant strains

Spectra of MDH (1 mg/ml) were recorded in 100 mM-potassium phosphate buffer, pH 7.0, at 20 °C. ——, wild-type; — — —, MoxA$^-$; - - - -, MoxK$^-$; · · · · · ·, MoxL$^-$.

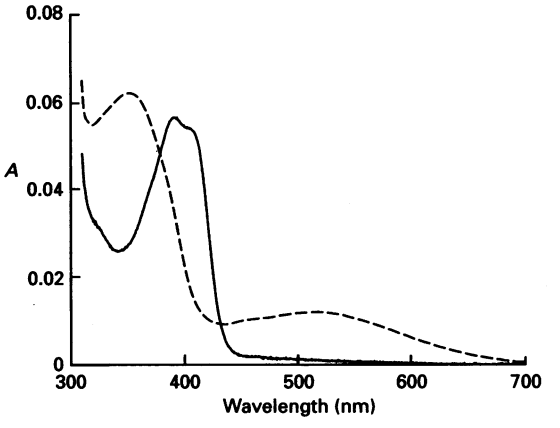


Fig. 3. Absorption spectra of the cyclopropanol-treated MDH from *Mb. extorquens* and MoxA$^-$ mutant strain

MDH (1 mg/ml) was oxidized with Wurster's Blue in 100 mM-tetrasodium pyrophosphate, pH 9.0, and treated with a 5-fold molar excess of cyclopropanol. After removal of excess reagents by gel filtration, the spectra were recorded in 100 mM-potassium phosphate buffer, pH 7.0, at 20 °C. ——, wild-type; ——, MoxA$^-$.

Table 1. Prosthetic group of MDH from wild-type *Mb. extorquens* and MoxA$^-$, K$^-$ and L$^-$ mutants

The PQQ content in MDH was determined by h.p.l.c. and by reconstitution of active enzyme from the apoquinoproteins of ADH and GDH as described in the Methods section. For the reconstitution of enzyme activity, the prosthetic group was released from MDH by boiling for 10 min. Denatured protein was removed by centrifugation, and samples of the supernatant were used directly in the reconstitution assays. The values shown here are averages of three determinations from the same batch of protein. For the h.p.l.c. analysis, the prosthetic group was released from the protein by acid treatment, extracted into methanol and loaded on to a Novapak C_{18} reverse-phase column. Peaks were detected by absorbance and fluorescence as described in the Methods section. After h.p.l.c. the proportions of quinone and quinol were estimated from the integral of the quinone peak in the fluorescence profile of unoxidized extracts; similar proportions were observed using the absorbance profile. The total amount of PQQ was estimated from the integral of the peak due to quinone in the fluorescence profile of oxidized extracts. The values shown here are averages of two determinations from the same batch of protein. Different batches of protein were used for the reconstitution assays and the h.p.l.c. analysis.

Source of MDH	PQQ (mol/mol of MDH) Apo-ADH	Apo-GDH	H.p.l.c.	Quinone (%)	Quinol (%)
Wild-type	2.01±0.18	2.01±0.30	1.88±0.20	40	60
MoxA$^-$	1.85±0.26	2.02±0.40	2.04±0.15	84	16
MoxK$^-$	1.97±0.20	2.14±0.45	2.13±0.10	96	4
MoxL$^-$	1.98±0.21	2.28±0.27	2.05±0.13	83	17

which is consistent with the demonstration that only the oxidized quinone form of PQQ could be extracted from the mutant enzymes.

The observation that the mutant forms of MDH have predominantly oxidized PQQ as prosthetic group, but have absorption spectra different from that of oxidized MDH, suggests that the PQQ is bound differently at the active site of the enzyme. This conclusion is supported by the observation that these MDHs did not react with cyclopropanol, a suicide inhibitor that reacts only with the quinone form of PQQ on MDH. The mechanism of reaction with cyclopropanol involves a basic residue at the active site and consists of a concerted proton abstraction followed by rearrangement of the cyclopropoxy anion to a ring-opened carbanion, and attack of this on the electrophilic C-5 of the oxidized (quinone) form of PQQ (Dijkstra *et al.*, 1984; Frank *et al.*, 1989). The spectra shown in Fig. 3 demonstrate that, although present in the oxidized form, the PQQ in the mutant enzyme was not able to react with cyclopropanol. Treatment with an oxidizing agent (Wurster's Blue), as is usually necessary before reaction with cyclopropanol, did not lead to enzyme able to react with this inhibitor. These results are consistent with the suggestion that the PQQ in the mutant MDH is bound differently, such that it is not amenable to attack by the activated cyclopropanol, or that the structure at the active site is sufficiently different from that in the wild-type enzyme that the initial ring-opening of the cyclopropanol is no longer catalysed.

Structure of MDH from MoxA$^-$, K$^-$ and L$^-$ mutants

The following experiments demonstrated that although these altered MDHs may be structurally different from the normal enzyme, any such differences must be relatively small. No free thiols were detected either in wild-type or mutant proteins. Gel filtration in SDS or guanidinium chloride showed that the proportion of α- and β-subunits was identical and the total relative molecular mass was unaltered; i.e. the mutant forms have retained the $\alpha_2\beta_2$ configuration. The *N*-terminal amino acid analysis (first five amino acids) was identical, as was the mobility determined during gel electrophoresis at pH 7.0 under non-denaturing conditions (related to pI and molecular size). Similarly, the rates of denaturation were identical in urea (5 or 7 M) or guanidinium chloride (2 or 3 M), or low pH (pH 2.6 or 3.1); this was determined by measuring the increase in fluorescence due to release of PQQ and due to tryptophan on denaturation. It has been shown previously, by cross-linking studies, that MDH 'docks' with the electron acceptor cytochrome c_L by way of the α-subunit of MDH (Chan & Anthony, 1991; Cox *et al.*, 1992). Similar cross-linking studies with the altered enzymes demonstrated that they were unaltered in this regard.

Presence of a Ca^{2+} ion in MDH and its absence from MDH from the MoxA$^-$, K$^-$ and L$^-$ mutants

Each $\alpha_2\beta_2$ tetramer of MDH from wild-type *Mb. extorquens* contains a single Ca^{2+} ion. This was also demonstrated in the MDH from the facultative methylotroph *Hyphomicrobium* X,

Table 2. Presence of Ca^{2+} in MDH from methylotrophic bacteria

Pure MDH was exchanged into Ca^{2+}-free 10 mM-Mops buffer, pH 7.0, by gel filtration. MDH at three dilutions (50, 100 and 150 μM) was then analysed for Ca^{2+} by using atomic-absorption spectrometry. The detection limit for Ca^{2+} was 1 μM. The values shown here for *Mb. extorquens* are averages for three different amounts of MDH, each from two different batches of protein. The values for *Hyphomicrobium* X, *Pa. denitrificans* and *Mp. methylotrophus* are averages for three different amounts of MDH from a single batch of protein from each organism.

Source of MDH	Ca^{2+} (mol/mol of MDH)
Mb. extorquens	
Wild-type	1.04±0.06
$MoxA^-$	0.03±0.04
$MoxK^-$	0.05±0.07
$MoxL^-$	0.01±0.03
Hyphomicrobium X	1.40±0.13
Pa. denitrificans	1.12±0.10
Mp. methylotrophus	1.05±0.08

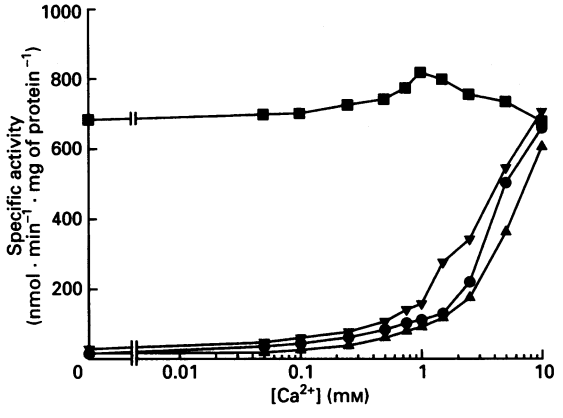


Fig. 4. Effect of Ca^{2+} concentration on reconstitution of active MDH from Ca^{2+}-free MDH purified from $MoxA^-$, K^- and L^- mutant strains

MDH was incubated at 20 °C for 60 min in various concentrations of $CaCl_2$ in 100 mM-Tris buffer, pH 9.0. After incubation, artificial electron acceptors were added together with substrate (methanol) and activator (NH_4Cl), for determination of enzyme activity as described in the Methods section. The concentration of MDH (wild-type bacteria) was 10 μg/ml. For the MDH from mutants, 100 μg/ml was used for lower concentrations of Ca^{2+} (0–500 μM) and 10 μg/ml for higher concentrations. ■, Wild-type; ▲, $MoxA^-$; ●, $MoxK^-$; ▼, $MoxL^-$.

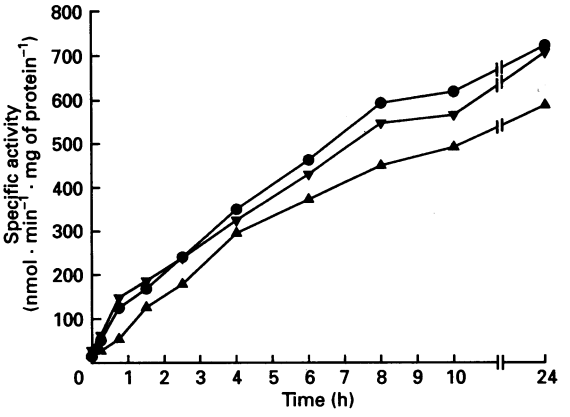


Fig. 5. Time-dependent reconstitution of MDH by Ca^{2+} addition

Addition (100 μg/ml) from the $MoxA^-$, K^- and L^- mutant strains was incubated with 500 μM-$CaCl_2$ in 100 mM-Tris buffer, pH 9.0, at 20 °C. Samples (50 μl) were removed from the incubation mixture and assayed in the standard dye-linked assay system. ▲, $MoxA^-$; ●, $MoxK^-$; ▼, $MoxL^-$.

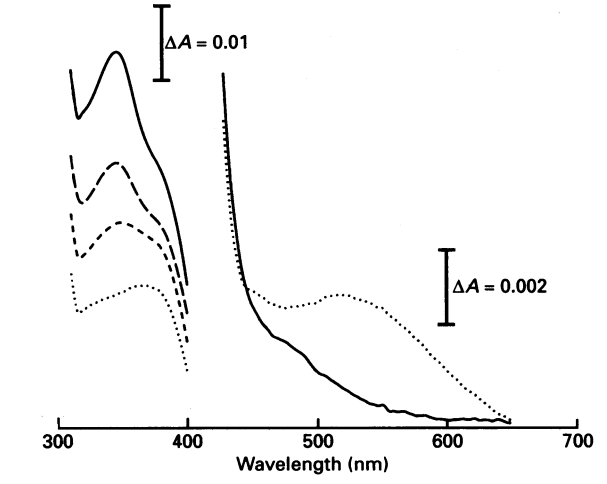


Fig. 6. Absorption spectra of MDH from the $MoxA^-$ mutant strain during reconstitution with Ca^{2+}

The absorption spectra of MDH in the incubation mixtures described in Fig. 5 were recorded at the following time intervals: ·····, 0 min; ----, 45 min; – –, 240 min; ——, 600 min.

which we confirmed has MDH with the usual $\alpha_2\beta_2$ tetrameric configuration. The same stoichiometry for Ca^{2+} was also demonstrated in the MDHs from the obligate methylotroph *Mp. methylotrophus* and the autotrophic methylotroph *Pa. denitrificans*. The following metal ions were not detectable in MDH: Mg^{2+}, Al^{3+}, Ni^{2+}, Zn^{2+}, Ba^{2+} and Sr^{2+}. This confirms the similar conclusion published in a recent paper on the MDH of the obligate methylotroph *Mb. glycogenes* (Adachi *et al.*, 1990). The results in Table 2 demonstrate that the MDH from the $MoxA^-$, K^- and L^- mutants contain virtually no detectable Ca^{2+}.

Reconstitution of active MDH by addition of Ca^{2+} ions to MDH from the mutants

Figs. 4, 5 and 6 and Table 3 demonstrate that incubation of mutant MDH in the presence of Ca^{2+} led to reconstitution of active enzyme; this was dependent on the Ca^{2+} concentration and on the time of incubation. Neither the substrate (methanol) nor the activator (ammonia) were necessary for reconstitution, although they were necessary for subsequent assay of the holoenzyme. During the reconstitution process the absorption spectrum returned to normal, the peak at 345 nm appearing, and the peak at 520 nm disappearing, from the spectrum of the mutant MDHs (Fig. 6). After reconstitution of active enzyme by incubation in Ca^{2+}, the absorption spectrum was characteristic of MDH in which the PQQ is present entirely in the reduced form; this was confirmed by h.p.l.c. analysis of the extracted prosthetic group. These results are consistent with the demonstration (above) that the prosthetic group on the MDH from the mutants was originally in the oxidized form. After reconstitution of active enzyme by incubation in Ca^{2+} the PQQ becomes available for reduction by the endogenous substrate on the enzyme, leading to the quinol prosthetic group. The quinol (reduced form) is not usually seen on the enzyme because the semiquinone form that is usually isolated is unable to react directly with substrate.

The reconstituted MDH was able to react with cyclopropanol under oxidizing conditions, giving a spectrum identical with that

Table 3. Reconstitution of active MDH by addition of Ca^{2+} ions to MDH from the MoxA$^-$, K$^-$ and L$^-$ mutants

MDH (1.5 μM) was exchanged by gel filtration into Ca^{2+}-free 100 mM-Tris buffer, pH 9.0, and then incubated in the presence or absence of Ca^{2+} (500 μM) at 20 °C for 24 h. After incubation, any excess Ca^{2+} was removed by gel filtration of MDH in Ca^{2+}-free 10 mM-Mops buffer, pH 7.0. Specific activities are in nmol/min per mg. Abbreviation: cyt, cytochrome.

	Before Ca^{2+} addition				After Ca^{2+} addition			
	Absorbance peaks (nm)		Specific activity		Absorbance peaks (nm)		Specific activity	
Source of MDH	345–350	520	Dye	Cyt. *c*	345–350	520	Dye	Cyt. *c*
Wild-type	+	−	686	90.0	+	−	808	102
MoxA$^-$	−	+	16	0.2	+	−	712	92
MoxK$^-$	−	+	15	0.1	+	−	840	89
MoxL$^-$	−	+	28	0.4	+	−	794	95

of cyclopropanol-inactivated MDH from wild-type bacteria shown in Fig. 3.

Ca^{2+} could not be removed from the reconstituted MDH, nor was the spectrum changed, nor the specific activity diminished by the following procedures: gel filtration, extensive dialysis against buffer containing Chelex-100 (a resin chelator of bivalent cations) (Bio-Rad) or treatment with 5 mM-EDTA at pH 7.0 (10 mM-Mops buffer), followed by gel filtration or dialysis against the same buffer (1000 vol.) containing Chelex-100 (Bio-Rad).

DISCUSSION

This paper records the first demonstration of the presence of Ca^{2+} in the MDHs from *Mb. extorquens*, *Mp. methylotrophus*, *Pa. denitrificans* and *Hyphomicrobium* X, adding further examples to the recent report of its presence in the MDH of a newly described methylotroph, *Mb. glycogenes*, by Adachi *et al.* (1990). In all cases there was a single atom of Ca per $\alpha_2\beta_2$ tetramer.

Other quinoproteins have been shown previously to contain Ca^{2+}; these are the soluble ADH from *Ps. aeruginosa* (Mutzel & Gorisch, 1991) and the soluble glucose dehydrogenase (GDH-B) from *Acinetobacter calcoaceticus* (Geiger & Gorisch, 1989). By contrast with the MDHs, these enzymes can be prepared in the apoenzyme form, and the active holoenzyme can be reconstituted with PQQ; Ca^{2+} or Sr^{2+} were essential for this reconstitution and could not be replaced with Mg^{2+}. Similar reconstitution experiments have implicated Ca^{2+} (not Mg^{2+}) in the structure of the quinoprotein ADH from membranes of *Gluconobacter suboxydans* (Shinagawa *et al.*, 1989) and in the soluble quinohaemoprotein ADH from *Ps. testosteroni* (Groen *et al.*, 1986). Bivalent cations have also been shown to be required for reconstitution of active holoenzyme from apoenzyme plus PQQ using the following quinoproteins: membrane glucose dehydrogenase from *E. coli* and *Ps. fluorescens* (Mg^{2+} more effective than Ca^{2+}) (Ameyama *et al.*, 1985), membrane glycerol dehydrogenase from *Gluconobacter industries* (Co^{2+} and Ni^{2+} were most effective) (Adachi *et al.*, 1988), glycerol dehydrogenase from membranes of *Gluconobacter suboxydans* (Ca^{2+} was most effective) (Adachi *et al.*, 1988) and the membrane-bound quinohaemoprotein aldehyde dehydrogenase from *Acetobacter rancens* (Mg^{2+} and Mn^{2+} were more effective than Ca^{2+}) (Hommel & Kleber, 1990). Insufficient information is available at present to determine the role of the metal ions in most of these quinoproteins because, although essential for reconstitution, neither the ion present in native holoenzyme, nor the number of ions per subunit, has been determined. In MDH from all the bacteria examined there was a single atom of Ca per $\alpha_2\beta_2$ tetramer. By contrast, the soluble ADH from *Ps. aeruginosa* contained two Ca atoms per α_2 dimer (Mutzel & Gorisch, 1991), and the soluble GDH-B from *Acinetobacter calcoaceticus* contained four Ca atoms per α_2 dimer (Geiger & Gorisch, 1989). This would suggest that the roles of Ca^{2+} in these three types of soluble quinoproteins might be different.

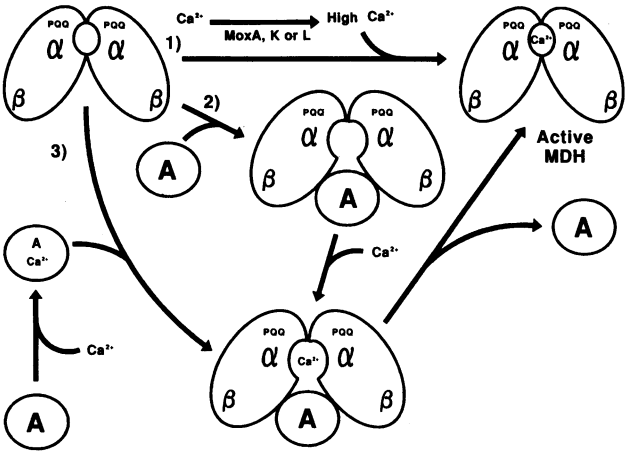
The demonstration that the quinoproteins discussed here contain Ca^{2+} is noteworthy, because involvement of Ca^{2+} as a structural element in enzymes is rather unusual, an important example being DNAase I, in which the Ca^{2+} is essential for stabilization of loop regions within the enzyme (Oefner & Suck, 1986). The presence of Ca^{2+} in these quinoproteins is likely to be relevant to the one feature that they have in common, namely their prosthetic group, PQQ. Analysis of the primary amino acid sequence of quinoproteins shows a conserved region that has been suggested to be the domain for binding the prosthetic group (Cleton-Jansen *et al.*, 1990; Inoue *et al.*, 1990; Anthony, 1992*a*). The Ca^{2+} might be involved in binding to acidic amino acids (or amides) in this region to stabilize a PQQ-binding conformation, or it may form a bridge between specific amino acid carboxylates and carboxylates of PQQ.

Although the putative PQQ-binding domain is seen in the four types of quinoprotein that have been analysed (MDH, ADH and two types of GDH), it was noted that there was sufficient difference between these regions for the PQQ binding to be different in the GDHs from that in the MDH or ADH. The possibility that bivalent cations other than Ca^{2+} might be involved in the membrane GDH supports this suggestion. Further, it can be seen that, although highly conserved in the MDH sequences, the carboxylates and amides likely to be involved in ligand formation with Ca^{2+} are not so well conserved between the four types of quinoprotein. This would be consistent with the demonstration that PQQ is readily released from GDHs and ADHs, but not from MDH. It should also be noted that metal ions can be removed from other quinoproteins by chelating agents, and this leads to loss of PQQ. By contrast, treatment of MDH with EDTA did not remove Ca^{2+} and did not lead to loss of PQQ or loss of activity.

In the case of MDH the presence of a single atom of Ca per $\alpha_2\beta_2$ tetramer suggests that the Ca^{2+} might be located at the interface between the two $\alpha\beta$ dimers. This does not necessarily eliminate the possibility that Ca^{2+} is also involved in the binding of PQQ to the enzyme.

The characterization of MDH isolated from MoxA$^-$, K$^-$ and L$^-$ mutants sheds some light in the role of Ca^{2+}. In these mutants the structural genes *moxF* and *moxI* code for the normal α- and β-subunits, which were present in the normal $\alpha_2\beta_2$ tetrameric configuration. Beside the absence of Ca^{2+}, the only observable difference in the MDH from the mutants was in their absorption and e.s.r. spectra and their activity with cyclopropanol. This is consistent with the proposal that the Ca^{2+} in normal MDH is responsible either for binding PQQ directly in the active site or for maintaining the correct conformation of MDH responsible for binding PQQ.

The *moxA*, *K* and *L* genes are clearly implicated in the insertion of Ca^{2+} into MDH. Scheme 1 summarizes possible roles for the proteins coded by these genes. A key feature of the three models included in Scheme 1 is that they are based on the observation that PQQ is tightly bound to the $\alpha_2\beta_2$ tetramer before insertion of Ca^{2+}, and that the conformation in the absence of Ca^{2+} is sufficiently similar to that of the holoenzyme for this to be formed when the concentration of Ca^{2+} in the medium is artificially high. The concentration of free Ca^{2+} in the



Scheme 1. Role of the *moxA*, *K* and *L* gene products in MDH assembly

The three possible roles summarized here are for the MoxA protein; an identical role could be described for MoxK and/or MoxL proteins. Nothing in the present work has distinguished the phenotype of the three mutants. (1) the three genes are involved merely in maintaining a sufficiently high local concentration of Ca^{2+} in the periplasm. (2) The MoxA protein stabilizes the $\alpha_2\beta_2$ tetramer together with the two molecules of PQQ in a conformation able to bind low concentrations of Ca^{2+}, after which the MoxA protein dissociates. (3) This proposal is the same as the second, except that the MoxA protein carries the Ca^{2+} to its location in the MDH.

periplasm, where the MDH is assembled, is presumably the same as that in the surrounding growth medium. The concentration in normal growth media is at least 20 μM. Incubation of pure MDH from the MoxA$^-$ mutant in this concentration of Ca^{2+} led to reconstitution of active MDH, but only 25% of maximum activity was achieved and the rate was extremely low (10% reconstitution after 2 days). The rate with higher concentrations was very much greater, and full activity could be achieved (100% activity in 1 h in 10 mM-Ca^{2+}). The simplest description of the role of the MoxA, K and L proteins might therefore be in providing a high local concentration of Ca^{2+} in the periplasm. However, as it is probable that Ca^{2+} flux in and out of the periplasm by way of porins in the outer membrane would be rapid, it is difficult to conceive of such a mechanism. Alternative functions of the proteins are suggested in Scheme 1. These include a Ca^{2+}-binding role in which the protein carries Ca^{2+} to the active site and is then released. Alternatively, the MoxA protein (for example) might be involved in stabilizing a conformation of MDH that is then able to bind Ca^{2+}, after which the MoxA protein is released and does not form part of the final structure. In this respect these proteins would be fulfilling a chaperone function (see Ellis, 1990).

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