The role of the novel disulphide ring in the active site of the quinoprotein methanol dehydrogenase from *Methylobacterium extorquens*

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All cysteines in methanol dehydrogenase (MDH) from *Methylobacterium extorquens* are involved in intra-subunit disulphide bridge formation. One of these is between adjacent cysteine residues which form a novel ring structure in the active site. It is readily reduced, the reduced enzyme being inactive in electron transfer to cytochrome c_L. The inactivation is not a result of major structural change or to modification of the prosthetic group pyrrolo-quinoline quinone (PQQ). The reduced enzyme appears to remain active with the artificial electron acceptor phenazine ethosulphate but this is because the dye re-oxidizes the adjacent thiols back to the original disulphide bridge. No free thiols were detected during the reaction cycle with cytochrome c_L. Carboxymethylation of the thiols produced by reduction of the novel disulphide ring led to formation of active enzyme. Reconstitution of inactive Ca^{2+}-free MDH with Ca^{2+} led to active enzyme containing the oxidized bridge and reduced quinol, $PQQH_2$, consistent with the conclusion that no hydrogen transfer occurs between these groups in the active site. It is concluded that the disulphide ring in the active site of MDH does not function as a redox component of the reaction. The disulphide ring has no special function in the process of Ca^{2+} incorporation into the active site. It is suggested that this novel structure might function in the stabilization or protection of the free radical semiquinone form of the prosthetic group ($PQQH^{\cdot}$) from solvent at the entrance to the active site.

INTRODUCTION

Methanol dehydrogenase (MDH) of *Methylobacterium extorquens* is a periplasmic quinoprotein, oxidizing methanol to formaldehyde; its prosthetic group is pyrrolo-quinoline quinone (PQQ), from which electrons are passed to a specific *c*-type cytochrome (cytochrome c_L), or to phenazine ethosulphate (PES) in the dye-linked assay system [1–3]. Its active site chamber contains PQQ bonded to a Ca^{2+} ion, the floor of the chamber being formed from a tryptophan (Trp^{243}) whose indole group is parallel to, and in contact with, the planar ring system of PQQ [4–6]. The ceiling of the chamber is formed by a novel ring structure arising from a disulphide bridge between adjacent cysteine residues ($Cys^{103-104}$), both S atoms being within 4 Å of the plane of the PQQ (Figure 1). The adjacent cysteines are bonded by an unusual non-planar *trans* peptide bond within a strained 8-membered ring in which the S–S distance is considerably less than in a typical disulphide bond.

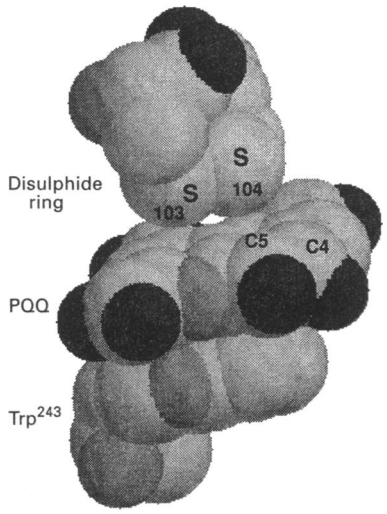


Figure 1 The active site of MDH

This shows the relative locations of the tryptophan (Trp^{243}) that forms the base of the active site chamber, the Cys^{103}–Cys^{104} disulphide ring and the PQQ prosthetic group, which is in the semiquinone form having the oxygen of the C4 carbonyl displaced out of the plane of the ring. This structure is presented in detail in [6].

In our first description of this structure in the MDH of *M. extorquens* we presented a brief summary of evidence (presented in full in the first part of this paper) suggesting that the novel disulphide structure might mediate electron transfer from the quinol ($PQQH_2$) to the electron acceptor cytochrome c_L [4]. Alternative functions to be considered include a redox role in the mechanism of MDH, a role in the incorporation of Ca^{2+} or PQQ into the active site of the enzyme, or a regulatory role.

The present paper characterizes in more detail the novel disulphide and describes an investigation into its function.

MATERIALS AND METHODS

Preparation and assay of proteins and prosthetic groups

The following methods were as described previously [7,8]: growth, harvesting and breakage of *M. extorquens* AM1 (N.C.I.M.B. 9133); purification of MDH and cytochrome c_L from *M. extorquens* AM1; assay of MDH with the dye PES; assay of MDH with cytochrome c_L, using equine cytochrome *c* as terminal electron acceptor; and SDS/PAGE. Methods for

Abbreviations used: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; MDH, methanol dehydrogenase; PES, phenazine ethosulphate; PQQ, pyrrolo-quinoline quinone; TFA, trifluoroacetic acid.

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growth of the mutant *mxaA* and for work on its MDH were as described previously [9]. Protein was assayed with the bicinchoninic acid method adapted for microtitre plates [10].

Unless otherwise stated all experiments on the disulphide bridges in MDH were done under anaerobic conditions in degassed Tris buffer (50 mM, pH 8.0) containing 1 mM EDTA. Proteins were separated from reducing agents etc. by rapid gel filtration on a Pharmacia G25 HR10/10 column, separated protein being collected within 3 min of application of the sample.

To determine the redox state of the prosthetic group, MDH was concentrated to 10 mg ml^{-1} in a Uniscience Univap vacuum centrifuge and the prosthetic group was determined by acid extraction in phosphoric acid at pH 1 followed by reverse-phase HPLC as described previously [9] using standard PQQ, $PQQH_2$ [11] and $PQQH_4$ [12]; A Waters Novapak C_{18} column was used with a Shimadzu LC-GA liquid chromatograph. Absorbance was monitored at 313 nm using a Biosystems model 178A detector and fluorescence was monitored at wavelengths above 418 nm (excitation at 365 nm) using an Applied Biosystems Model 980 detector.

Determination of thiol groups on native, denatured or reduced MDH

Thiols were determined by reaction with Ellman's reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described in [13], or by reaction with [^{3}H]- or [^{14}C]iodoacetate at pH 8.5 in 50 mM Tris buffer containing 1 mM EDTA for 3 h at 37 °C in the dark. After reaction, MDH was separated from reagent by rapid gel filtration and the protein concentration and radioactivity determined. When relevant, the enzyme was first denatured in 2% SDS and disulphide bridges reduced by incubating for 45 min with 5 mM dithiothreitol (DTT); similar results were obtained using 6 M guanidinium chloride and 3 mM mercaptoethanol.

Formation of carboxymethylated MDH, and identity of the carboxymethylated cysteine residues

MDH (5 nmol) was reduced by reaction with 5 mM DTT at room temperature for 1 h. An excess (20 mM) of [^{14}C]iodoacetate (specific radioactivity, 1.08×10^6 d.p.m./μmol) was added and the incubation continued for 3 h. To identify the carboxymethylated subunit, reagents were removed by rapid gel filtration, carboxymethylated MDH was dissociated with SDS into subunits as described in [14] and protein peaks were collected and radioactivity determined in a scintillation counter.

To identify the carboxymethylated cysteine residues, carboxymethylated MDH (60 nmol) was prepared as described above and then digested with trypsin (1:25, w/w) in 50 mM Tris buffer (pH 8) at 37 °C for 16 h. The peptides were separated at room temperature on a reverse-phase C_{18} column using a solvent gradient (0–51% acetonitrile); solvent A was 0.05% trifluoroacetic acid (TFA) in water, and solvent B was 0.05% TFA in acetonitrile. Of the 51 fractions (1 ml) collected, only one contained significant radioactivity. This fraction was purified on a second similar reverse-phase C_{18} column; solvent A was 10 mM ammonium acetate at pH 7; solvent B was 90% acetonitrile + 10% solvent A. The two radioactive fractions detected were dried *in vacuo* and sequenced using an Applied Biosystem 477A gas-phase (pulsed liquid) protein sequencer coupled to a model 120A phenylthiohydantoin-derivative analyser.

Spectroscopy

Absorption spectra were recorded at 20 °C on a SLM-AMINCO DW-2000 UV/VIS spectrophotometer using a scan speed of 100 nm min^{-1} with a spectral band-width of 2 nm and 10 mm light path.

CD spectra were recorded at 20 °C on a JASCO 720 spectropolarimeter using a scan speed of 50 nm min^{-1} in a 3 ml cylindrical quartz cuvette with a 10 mm light path. Molar ellipticity values were calculated using the equation $[\theta]_M = 330 \times \Delta_\epsilon$, where Δ_ϵ is the molar CD absorption coefficient.

EPR spectra were recorded on a Bruker ER series spectrometer in 5 mM potassium phosphate buffer (pH 7.0) at 20 °C between 333 and 343 mT.

RESULTS

The active site of MDH contains a novel disulphide bridge, formed from adjacent cysteines, which appears to be essential for enzyme activity

All cysteines in MDH are in the form of intra-chain disulphide bridges

No free thiols could be detected in native MDH before or after denaturation in 2% SDS or 6 M guanidinium chloride. After reduction of MDH under denaturing conditions, values of 10.53, 11.54, 11.88, 12.08 and 12.20 free thiols per $\alpha_2\beta_2$ tetramer were measured (five separate experiments). This result is as expected from the gene sequence which indicated the presence of four cysteines in the 66 kDa α-subunit [15,16] and two cysteines in the 8.5 kDa β-subunit [14,16]. PAGE or gel filtration on a Superose-12 column in the presence of 2% SDS led to the appearance of two protein bands (66 and 8.5 kDa), whether or not mercaptoethanol was included to break any disulphide bridges between subunits.

These results confirm that all the cysteines in MDH are present in the form of intra-chain disulphide bridges. This conclusion is consistent with the position of the cysteine residues seen in the X-ray structure which indicates bridges between Cys^6 and Cys^{12} in the β subunit and between Cys^{386} and Cys^{415} in the α-subunit [4,5]. They also confirm that the adjacent cysteines (Cys^{103} and Cys^{104}) in the α-subunit do form a disulphide bridge in the active site.

One disulphide bridge on the α-subunit is highly susceptible to reduction in the native enzyme

When native MDH was reduced with 5 mM DTT for 1 h, about four free thiol groups per $\alpha_2\beta_2$ tetramer were detected by reaction with DTNB or iodoacetate (^{3}H or ^{14}C) (values of 3.55, 3.97, 3.8, 3.62 were recorded in four separate experiments); incubation for up to 16 h led to no further reduction. To confirm that the reaction measured with [^{14}C]iodoacetate was complete, and entirely due to carboxymethylation of thiols on the reduced MDH, a second sample of reduced enzyme was reacted with non-radioactive iodoacetate (20 mM) for 3 h followed by incubation with [^{14}C]iodoacetate (20 mM) and incubation for a further 3 h before rapid gel filtration. The MDH treated in this way showed no more than the background level of radioactivity. That the readily-reducible disulphide was on the α-subunit was shown by separating the carboxymethylated enzyme (5 nmol) by gel filtration in SDS on a Superose 12 column; 96% of the radioactive label was in the α-subunit and 4% in the β-subunit (895 and 53 d.p.m. per nmol protein respectively).

The readily reduced disulphide bond is formed between cysteines 103 and 104

After reduction for 1 h with 5 mM DTT followed by reaction with [^{14}C]iodoacetate, the carboxymethylated MDH (60 nmol) was digested with trypsin and the products separated by HPLC

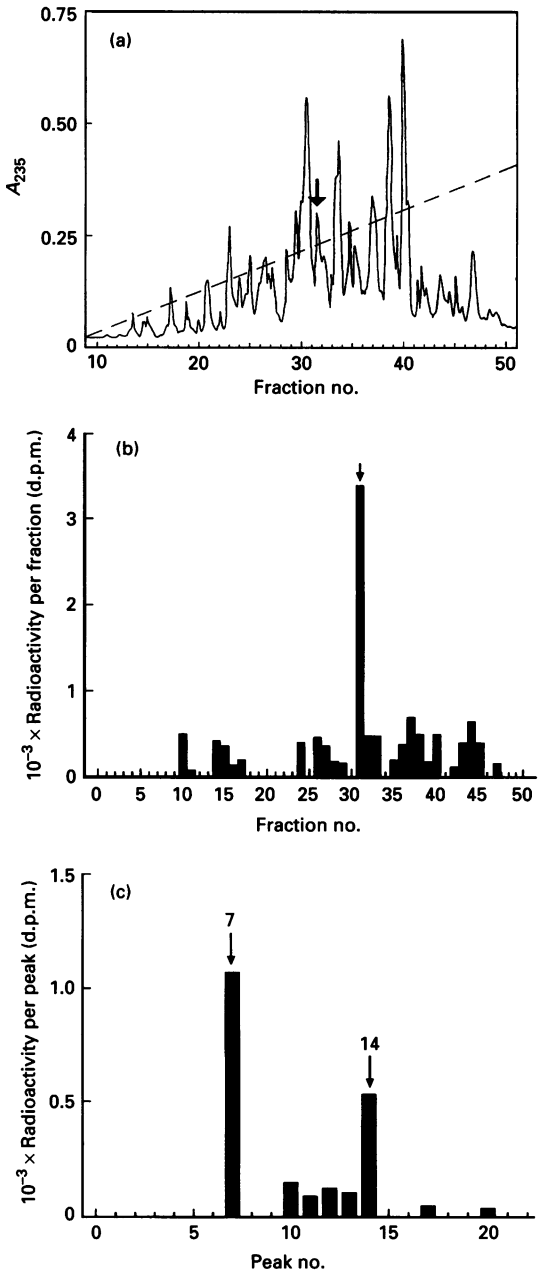


Figure 2 Separation of peptides produced by trypsin digestion of carboxymethylated MDH

[^{14}C]carboxymethylated MDH was prepared, digested with trypsin and peptides were separated by HPLC as described in the Materials and methods section. (**a**) HPLC of the tryptic digest; the broken line indicates the acetonitrile gradient (0–51% over 51 fractions); the solid line indicates absorbance at 235 nm. (**b**) Radioactivity profile of the peptides separated in (**a**); the baseline (85 d.p.m.) has been subtracted. (**c**) Radioactive profile of the column fractions after further chromatography of the fraction indicated in (**a**); the baseline (74 d.p.m.) has been subtracted. Arrows indicate the radioactive fractions selected for further purification or sequencing.

as shown in Figures 2(a) and 2(b). The only fraction containing significant radioactivity was further fractionated by a second HPLC run. Of the 22 peak fractions, only two were labelled significantly above the background level; these fractions contained 75% of the total radioactivity applied to the column (Figure 2c). One fraction (14) contained a peptide having the

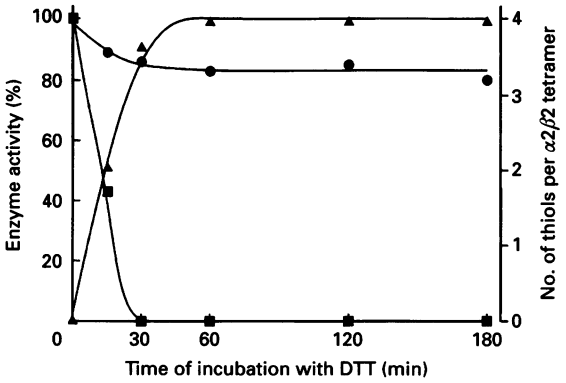


Figure 3 Loss of MDH activity on reduction with DTT

MDH (2.6 nmol) was incubated with 5 mM DTT; samples were removed at various times and passed through a rapid gel filtration column to remove reductant. The activity of the reduced enzyme was then determined and the number of thiols produced was measured by reaction with [^{14}C]iodoacetate as described in the Materials and methods section. Enzyme activity is expressed as a percentage of that measured in a sample of untreated MDH after passage down the same rapid gel filtration column. The initial rates were 28 nmol cytochrome *c* reduced per min per mg protein and 150 nmol PES reduced per min per mg protein. ■, Activity in the cytochrome-linked assay; ●, activity in the dye-linked assay; ▲, number of thiols per $\alpha_2\beta_2$ tetramer.

sequence TGEQVWR, corresponding to a sequence starting with Thr^{191}; it is not obvious why this peptide was radioactive (30% of the recovered radioactivity) and this was not pursued further. The other radioactive fraction (fraction 7), containing 70% of the recovered activity, was very well separated (to the baseline) from all other peaks, showed no absorbance at 280 nm and had the sequence AVAC*C*DLVNR in which C* represents carboxymethylated cysteine. This corresponds to a peptide starting with Ala^{100}, containing the adjacent cysteines Cys^{103} and Cys^{104}. As both cysteines were labelled, and as the total label corresponded to production of four thiol groups per $\alpha_2\beta_2$ tetramer, it can be concluded that both of the adjacent cysteines in each α-subunit had been carboxymethylated.

Reduction of MDH leads to loss of activity with the cytochrome electron acceptor

When MDH was reduced with excess DTT (5 mM) and the reductant removed by rapid gel filtration, its specific activity was diminished by only about 15% in the dye-linked assay system (Figure 3). By contrast, all activity was rapidly lost when using cytochrome c_L as electron acceptor (about 50% of activity was lost within 15 min), and this loss of activity was correlated with the extent of reduction of disulphide to free thiols. This suggests that the disulphide bridge might play a specific role in electron transfer from the quinol form of the prosthetic group ($PQQH_2$) to cytochrome c_L. The fact that activity with PES was unaffected by reduction of the active site disulphide suggested that there is a route for direct electron transfer between $PQQH_2$ and the dye, with no involvement of the disulphide structure.

Loss of activity on reduction of the novel disulphide ring in the active site of MDH is not due to major structural changes in the enzyme or prosthetic group

The results described above show that the novel disulphide bridge in the active site of MDH is particularly susceptible to reduction, the reduced enzyme being unable to transfer electrons

Table 1 The activity of MDH after reduction, followed by carboxymethylation or by oxidation with air or PES

MDH was reduced with DTT as described previously. It was subsequently oxidized in air for 1 h or carboxymethylated with iodoacetate as described above. A sample (3.3 nmol) was incubated in 50 mM Tris buffer (pH 8.0), with or without 5 mM methanol, PES (27.5 mM) was added and MDH immediately separated from the incubation mixture by rapid gel filtration. The total time between addition of PES and separation from PES was between 1 and 1.5 min. Enzyme activity was measured as the rate of reduction of PES or cytochrome c_L; 1 unit is the amount required to reduce 1 nmol min^{-1}. The number of free thiols (per $\alpha_2\beta_2$ tetramer) and the redox state of the extracted prosthetic group were determined as described in the Materials and methods section. Results were identical (±5%) in the presence or absence of methanol.

Treatment of MDH	Specific activity (units/mg)		Redox state		
	PES	Cytochrome c_L	PQQ (%)	$PQQH_2$ (%)	Thiols per tetramer
None	356	32.8	42	58	0.02
Incubation with iodoacetate	342	32.3	nd	nd	0.05
Reduction with DTT (to $MDH_{red.}$)	352	3.7	17	83	3.97
Carboxymethylation of $MDH_{red.}$ (to MDH_{cm})	339	30.1	43	57	0.05
Incubation of MDH_{cm} with DTT	354	33.0	nd	nd	0.07
Oxidation of $MDH_{red.}$ in air	350	30.7	36	64	0.39
Oxidation of $MDH_{red.}$ with PES (to MDH_{PES})	349	16.7	65	35	0.26
Incubation of MDH_{PES} with iodoacetate	310	17.3	54	46	0.37

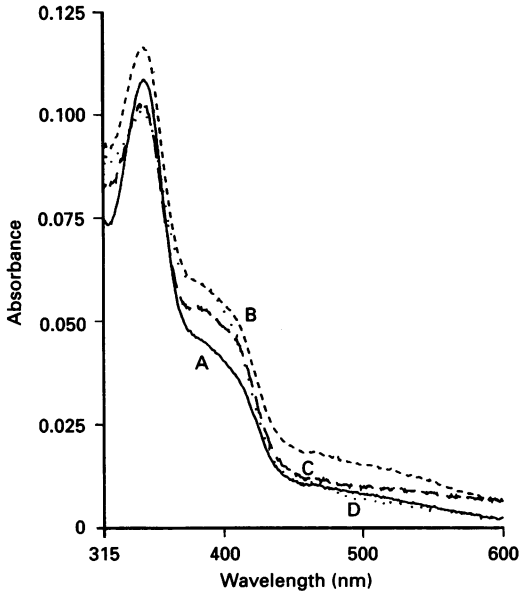


Figure 4 Absorption spectra of MDH

A, untreated MDH (1 mg ml^{-1}); B, MDH reduced with DTT; C, carboxymethylated MDH; D, MDH after reduction with DTT followed by incubation in air for 1 h.

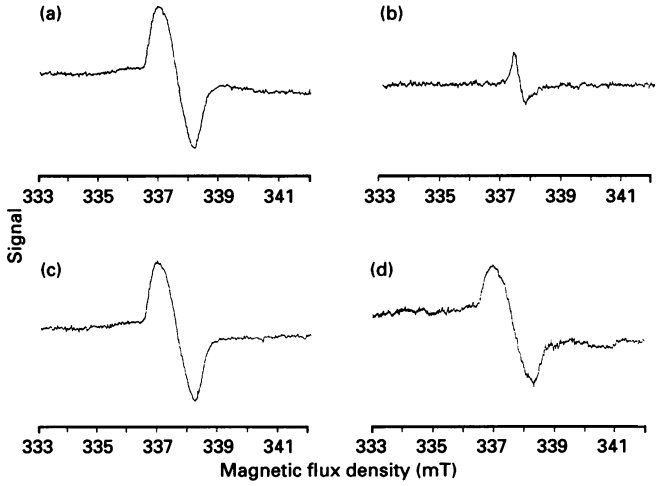


Figure 5 EPR spectra of MDH

The EPR spectrum of untreated MDH had a line-width of 1.25 mT and a g value of 2.0056 (as previously described [9]). (**a**) Untreated MDH (34 mg ml^{-1}). (**b**) MDH (30 mg ml^{-1}) reduced with DTT; because of the procedures used, some re-oxidation had occurred, giving MDH having 33% of the activity of untreated enzyme and 29% of the peak height of its EPR signal. (**c**) DTT-reduced MDH (34 mg ml^{-1}) after oxidation for 1 h in air. (**d**) Carboxymethylated MDH (36 mg ml^{-1}) (3.6 thiols were carboxymethylated per $\alpha_2\beta_2$ tetramer).

to cytochrome c_L. That a major change in structure did not occur on reduction of the novel disulphide ring structure is indicated by the retention of activity in the PES-linked assay system (Figure 3) and by the reversibility of the inactivation; when reduced inactive MDH was incubated in air for 1 h at 30 °C, 94% of activity with cytochrome c_L was recovered; in this re-activated enzyme fewer than 0.4 free thiols per $\alpha_2\beta_2$ tetramer could be detected with DTNB or [^{14}C]iodoacetate (Table 1).

It was necessary to demonstrate that the loss of activity on reduction of MDH was due to reduction of the disulphide bridge alone and not due to production of an altered inactive prosthetic group. The absorption spectrum (Figure 4) indicated that reduction of MDH led to no major change in the environment of the prosthetic group in the active site; the change in spectrum was consistent with spectral changes previously observed [1,2,17,18] on reduction of the normal semiquinone (free radical) form of PQQ to the quinol ($PQQH_2$). This was confirmed by the loss of the EPR signal on reduction (Figure 5) and by the demonstration that, when extracted, most of the prosthetic group was in the reduced, quinol form (Figure 6, Table 1). This contrasts with the extracted products from untreated enzyme; similar amounts of the quinone and quinol forms of PQQ were present due to disproportionation of the semiquinone, which is the major form of the prosthetic group, as previously described [9,17,18] (Figure 6, Table 1). Figure 6 also confirms that when the prosthetic group was extracted from the enzyme (native or

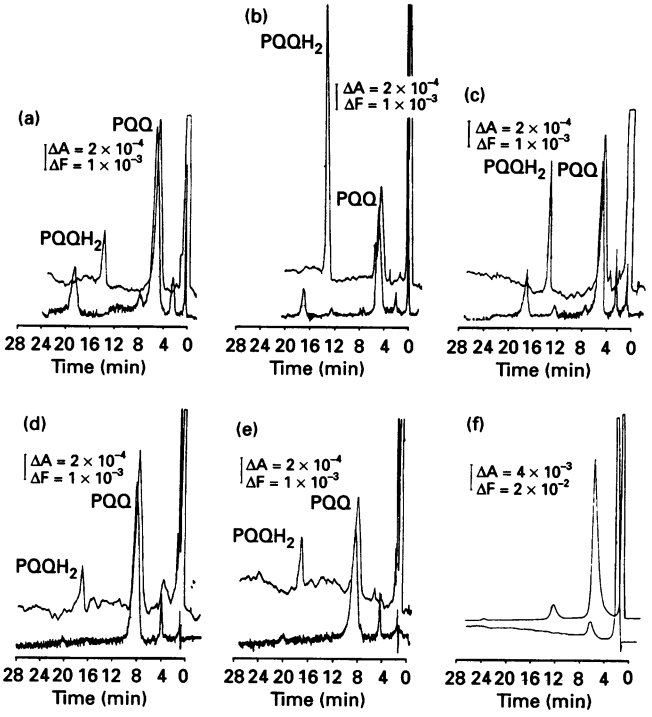


Figure 6 HPLC of the prosthetic group of MDH

Methods for extraction and separation are given in the Materials and methods section: (**a**) from untreated MDH; (**b**) from MDH reduced with DTT; (**c**) MDH after DTT-reduction and incubation in air for 1 h; (**d**) carboxymethylated MDH; (**e**) carboxymethylated MDH after incubation in air for 1 h; (**f**) $PQQH_4$ (3 nmol) prepared as described in the Materials and methods section. The upper trace in each case represents absorption, and the lower trace represents fluorescence.

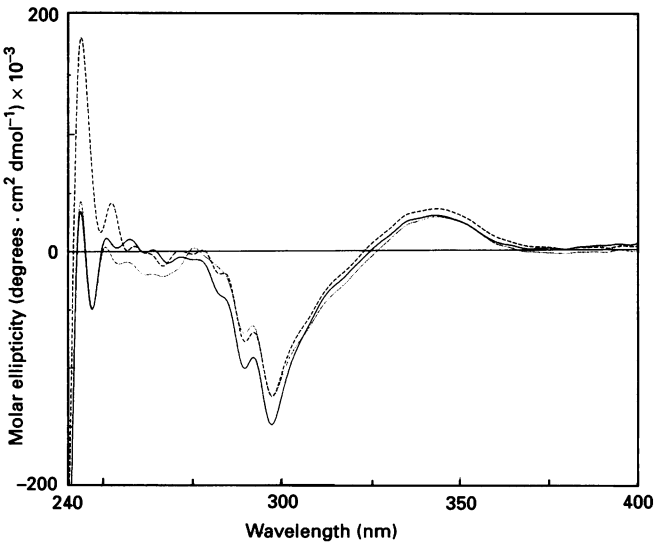


Figure 7 CD spectra of MDH

Native enzyme (6.7 nmol) in 50 mM Tris/HCl (pH 8.0) containing 1 mM EDTA was incubated for 1 h with 5 mM DTT, separated from the reaction mixture and oxidized in air at 20 °C for 48 h. CD spectra were recorded as described in the Materials and methods section. (——), native MDH; (– – –), reduced MDH; (· · · · ·), reduced MDH after aeration.

reduced), the only forms ever observed were the quinone or quinol; there was no reduction of the prosthetic group to the fully reduced form $PQQH_4$ which would be inactive in the dehydrogenase.

The slight changes in the CD spectrum on reduction are also consistent with little structural change, the altered signal at 345 nm being due to the change in oxidation state of the prosthetic group (Figure 7).

Evidence that the disulphide ring does not have a specific role in electron transfer to cytochrome c_L

Oxidation by PES of inactive, reduced MDH to active oxidized enzyme

The retention of activity with PES when MDH was reduced to enzyme that is inactive with cytochrome c_L, suggested initially that the disulphide bridge plays a specific role in electron transfer from $PQQH_2$ to cytochrome c_L. However, the results summarized in Table 1 show that this conclusion is untenable. Incubation of reduced enzyme with PES (in anaerobic conditions) led to rapid production of active enzyme which no longer contained any free thiols, as indicated by its failure to react with [^{14}C]iodoacetate (Table 1).

Thus, it is impossible to determine whether or not electron transfer to PES can occur in the enzyme in which adjacent cysteines are reduced; we cannot therefore conclude from these initial studies that the bridge plays a specific role in the path of electron transfer to cytochrome c_L from $PQQH_2$ in the enzyme.

Free thiols are not detectable during the catalytic cycle

If the disulphide bridge is acting as a typical electron transfer component then it should be possible to detect free thiols during the catalytic cycle, and the reaction rate should be diminished by iodoacetate acting as a thiol reagent. This was tested by incubation of MDH (1 nmol) with 5 nmol of cytochrome c_L and 300 nmol of equine cytochrome *c* as terminal electron acceptor in 50 mM Tris buffer (pH 8.5). The equine cytochrome *c* was added in increments of 50 nmol. Inclusion of 20 mM [^{14}C]iodoacetate in this reaction mixture decreased the rate by about 40 %. However, this could be explained by the usual effect of increasing the ionic strength of the medium on the interaction between MDH and cytochrome c_L [19]. After separation of the MDH from reactants by rapid gel filtration it was shown that no carboxymethylation had occurred. Although this negative result might merely reflect a slow rate of carboxymethylation compared with the rate of turnover of the disulphide, it is consistent with the suggestion that there is no redox change in the active-site disulphide during the reaction cycle.

Carboxymethylated enzyme is active in electron transfer

When ^{14}C-carboxymethylated MDH, prepared from inactive reduced enzyme as described above, was assayed anaerobically in the usual systems it was shown to have recovered almost all of its activity with cytochrome c_L (Table 1). Similar results were obtained when iodoacetamide or DTNB replaced iodoacetate. When the carboxymethylated enzyme was recovered by gel filtration from the reaction mixture it was shown to have retained the [^{14}C]carboxymethyl label, and the prosthetic group was shown to be mainly in the semiquinone form as shown by its EPR spectrum and by HPLC analysis (Table 1, Figures 5 and 6). Subsequent treatment with 5 mM DTT for 1 h led to no loss of activity with cytochrome c_L, and no further reaction with

Table 2 Characterization of reconstituted MDH from mutant *mxaA*

Reduced MDH ($MDH_{red.}$) and carboxymethylated MDH (MDH_{cm}) were prepared from mutant *mxaA* as described for MDH from wild-type bacteria. The modified enzymes (3.5 nmol) were incubated in 1 ml of 50 mM Tris buffer (pH 9.0) containing 10 mM $CaCl_2$ for 16 h at 20 °C; they were then separated from the reaction mixture by rapid gel filtration in the same buffer. Activities in the PES- and cytochrome-linked assays, determination of free thiols and determination of the redox state of the prosthetic group were as described in the Materials and methods section. Specific activities are expressed as nmol electron acceptor reduced · min^{-1} · mg $protein^{-1}$.

	Before reconstitution with Ca^{2+}					After reconstitution with Ca^{2+}				
	Specific activity (units/mg protein)		Thiols per tetramer	Redox state of prosthetic group		Specific activity (units/mg protein)		Thiols per teramer	Redox state of prosthetic group	
	PES	Cytochrome c_L	(Mol/mol)	PQQ (%)	$PQQH_2$ (%)	PES	Cytochrome c_L	(Mol/mol)	PQQ (%)	$PQQH_2$ (%)
MDH	14	0	0.04	86	14	468	39.0	0.00	2	98
$MDH_{red.}$	12	0	3.29	16	84	432	0.6	3.95	5	95
MDH_{cm}	17	0	0.07	92	8	476	29.0	0.04	52	48

[^{14}C]iodoacetate could be measured, confirming that the disulphide bridge had not re-formed (Table 1).

It is unlikely that there is any hydrogen transfer from the quinol form of the prosthetic group to the disulphide bridge

It was not possible, using enzyme isolated from wild-type bacteria, to determine directly whether or not hydrogen atoms are transferred during the reaction cycle to the disulphide bridge from the prosthetic group ($PQQH_2$) because this is always in the semiquinone form. This free radical form cannot be reduced by methanol, which only reacts with the oxidized quinone form (PQQ) [17,18], and other reductants that might be used also reduce the disulphide bridge to free thiols. However, we were able to produce reduced enzyme while retaining the disulphide bridge by using MDH isolated from the mutant *mxaA*. MDH from this mutant lacks the Ca^{2+} ion which has a marked effect on the configuration of PQQ in the active site; after incubation at pH 9.0 in the presence of methanol and 10 mM $CaCl_2$, active enzyme containing Ca^{2+} and with a normal absorption spectrum is produced [9]. Using the techniques described above, we demonstrated that this active, reconstituted enzyme contained the usual disulphide ring, as demonstrated by reduction to inactive enzyme with DTT followed by carboxymethylation with [^{14}C]iodoacetate to give active enzyme; the reconstituted enzyme had no EPR spectrum and its prosthetic group was almost exclusively in the reduced form (Table 2).

This demonstration that active MDH can exist in a form containing both quinol and disulphide bridge is consistent with the conclusion that hydrogen does not transfer during the catalytic cycle from the quinol form of the prosthetic group to the disulphide bridge.

The disulphide bridge plays no particular role in the process of Ca^{2+} insertion

The possibility that the role of the novel disulphide ring might not be in electron transport but might be to aid incorporation of the Ca^{2+} ion into the active site was tested by using the MDH from *mxaA* described above. This Ca^{2+}-free inactive enzyme was similar to MDH from the wild-type organism in having no free thiols. Its prosthetic group could be rapidly reduced with DTT to the quinol and the disulphide ring in the active site was rapidly reduced as indicated by carboxymethylation with [^{14}C]iodoacetate (Table 2). Incubation of the reduced enzyme from *mxaA* with 10 mM Ca^{2+} at pH 9.0 produced MDH having the same absorption spectrum as the normal reduced MDH. This reconstituted, reduced enzyme was inactive and contained the quinol ($PQQH_2$) and free thiol groups as shown by carboxylation with iodoacetate (Table 2). Oxidation in air, or carboxymethylation, led to the formation of active enzyme as shown for the enzyme from wild-type bacteria.

When the inactive Ca^{2+}-free MDH from the *mxaA* mutant was carboxymethylated and incubated with Ca^{2+}, the rate of Ca^{2+} incorporation to produce an active enzyme was similar to that when normal enzyme was used.

These results show that neither the quinone form of PQQ, nor the disulphide ring or its reduced form are absolutely essential for calcium incorporation into the active site.

DISCUSSION

The results presented in this paper show that the adjacent pair of cysteines ($Cys^{103-104}$) in the α-subunit of MDH are particularly susceptible to reduction, as previously demonstrated for the disulphide bridge between adjacent cysteines in the agonist-binding site of the acetylcholine receptor [20]. In that case, however, the reduction had only a mild effect on the function of the receptor. The only enzyme in which a disulphide bridge has been observed between adjacent cysteines is mercuric ion reductase; however, in this case it is a preparation artefact and for activity a reduction step is first necessary to provide two thiols which are essential for coordination of the mercuric ion [21]. The rarity of such disulphide bridges in proteins suggests that the novel disulphide ring structure in the active site of MDH must serve an important biological function, and the loss of activity on its reductive cleavage is consistent with this conclusion. It is evident from the results presented here that its function is unlikely to be as a redox component of the enzyme, and a role in Ca^{2+} incorporation was also ruled out. An alternative is that the disulphide ring merely acts to hold the prosthetic group in place in the active site; cleavage of the ring by reduction might thus displace the PQQ into an inactive configuration, which is reversed by carboxymethylation. Although it is difficult to discount this interpretation, the lack of a major change in the spectrum, as seen previously with Ca^{2+}-free enzyme, due to an altered configuration of PQQ, suggests there might be an alternative interpretation.

That the disulphide ring is not an absolute requirement for PQQ-containing enzymes is indicated by the sequences of these enzymes (Figure 8). There is considerable sequence similarity between MDH and the alcohol dehydrogenase of acetic acid

Methanol dehydrogenase	
Methylobacterium extorquens [15,16]:	AAR--AVACCDLVNRGLAYWPGDG
Methylobacterium organophilum [16,26]:	AAR--AVACCDLVNRGLAYWPGDG
Paracoccus denitrificans [16,27]:	WAR--WVACCDVVNRGLAYWPGDD
Alcohol dehydrogenase	
Acetobacter aceti [16,28,29]:	NIA--DKGCCDTVNRGAGYWNG--
Acetobacter polyoxygenes [30]:	NIA--DKGCCDTVNRGAAYWNG--
Glucose dehydrogenase	
Acinetobacter calcoaceticus [16,31]:	DKSFQHLTC-----RGVMYYDANN
Escherichia coli [16,32]:	NESFQHLTC-----RGVSYHEAKE
Gluconobacter oxydans [33]:	NPGFQHLTC-----RGVSFHETPA

Figure 8 Sequence similarities between quinoproteins in the region of the Cys^{103}–Cys^{104} disulphide bond of MDH from *M. extorquens*

Conserved cysteines are in bold type.

bacteria; this is similar to MDH in having cytochrome *c* as electron acceptor, although in this case it is a domain on the dehydrogenase rather than a separate protein. The adjacent cysteines are not present in the membrane glucose dehydrogenase, whose electron acceptor is membrane ubiquinone. This would seem to imply that the disulphide does have a direct role in electron transfer, but there is an alternative explanation. In native MDH the PQQ is always found in the semiquinone form, consistent with its role in mediating between the two-electron oxidation of methanol and the single-electron reduction of cytochrome c_L [17,22]. By contrast, the process in glucose dehydrogenase is a two-electron transfer from glucose to ubiquinone [23]. Although the semiquinone might be involved as an intermediate, it has never been observed during the reaction. It is possible therefore that the disulphide ring in the active site of MDH functions in the stabilization (or protection) of the free radical form of PQQ. In this respect the ring would be acting in a manner analogous to the tryptophans in the active sites of galactose oxidase [24] and methylamine dehydrogenase [25] which stabilize a tyrosine free radical and a tryptophan quinone free radical respectively. Reduction of the disulphide ring in MDH clearly diminishes the protection it provides; subsequent carboxymethylation presumably restores the protection. It is worth noting that in these three enzymes the proposed protecting groups are clearly visible in surface views of the active site, limiting access to the prosthetic group from the surface.

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