Biochimica et Biophysica Acta, 1119 (1992) 97–106

© 1992 Elsevier Science Publishers B.V. All rights reserved 0167-4838/92/$05.00

BBAPRO 34100

The interaction of methanol dehydrogenase and its electron acceptor, cytochrome c_L in methylotrophic bacteria

Jonathan M. Cox, Darren J. Day and Christopher Anthony

SERC Molecular Recognition Centre, Biochemistry Department, University of Southampton, Southampton (U.K.)

(Received 19 June 1991)

Key words: Dehydrogenase mechanism; Electron acceptor; Quinoprotein; (Methylotrophic bacteria)

The interactions of methanol dehydrogenase (MDH, EC1.1.99.8) with its specific electron acceptor cytochrome c_L has been investigated in *Methylobacterium extorquens* and *Methylophilus methylotrophus*. The MDHs of these two very different methylotrophs have the same $\alpha_2\beta_2$ structure; the interaction of these MDHs with their specific electron acceptor, cytochrome c_L, has been studied using a novel assay system. Electrostatic reactions are involved in 'docking' of the two proteins. EDTA inhibits the reaction by a process involving neither metal chelation nor the 'docking' process. Chemical modification studies showed that the two proteins interact by a 'docking' process involving interactions of lysyl residues on MDH and carboxyl residues on cytochrome c_L. When 'zero length', two stage cross-linking was done (with proteins from both bacteria), the α-subunits of MDH cross-linked with cytochrome c_L by way of lysyl groups on MDH and carboxyl groups on the cytochrome. Tuna mitochondrial cytochrome *c* provided a model for cytochrome c_H which is the electron acceptor for cytochrome c_L in the 'methanol oxidase' electron transport chain. Tuna cytochrome *c* was shown to form crosslinked products with carboxyl-modified cytochrome c_L. MDH and tuna cytochrome *c* competed for the same domain on cytochrome c_L. It was concluded that MDH reacts with cytochrome c_L by an electrostatic reaction which involves carboxyl groups on cytochrome c_L and amino groups on the α-subunit of MDH. The same domain on cytochrome c_L is involved in subsequent 'docking' with its electron acceptor.

Introduction

Methanol dehydrogenase (MDH, EC 1.1.99.8) is a quinoprotein that has pyrrolo-quinoline quinone (PQQ) as its prosthetic group and cytochrome c_L as its electron acceptor; both proteins operate in the periplasm of methylotrophic bacteria and constitute the first part of a 'methanol oxidase' electron transport chain which also involves a typical class I *c*-type cytochrome (usually referred to as cytochrome c_H) which is oxidised by a membrane oxidase [1–4]:

Methanol → MDH → Cyt. c_L → Cyt. c_H → Oxidase

It has recently been shown that the MDH of *Methylobacterium extorquens* AM1 is not, as previously thought, a dimer of identical subunits, but a tetramer of two types of subunit in an $\alpha_2\beta_2$ conformation [5]. The α-subunit has a molecular mass of 65 795 Da and the β-subunit has a molecular mass of 8463 Da [5,6]. The subunits are held very tightly together; they are not dissociated by high concentrations of salt but require SDS to separate them. The small subunit is extraordinary in having a very high lysine content (15 of the 74 residues) and it was proposed that these might have a specific function in interaction with the acidic cytochrome c_L [5]. This proposal was based upon the observation that cytochrome c_H is typical of class I *c*-type cytochromes which interact with their substrates (ubiquinol cytochrome *c* oxidoreductase and

Abbreviations: CDTA, trans-1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetracetic acid; DTDP, 2,2'-dithiodipyridine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; DMSO, dimethylsulphoxide, EDDA, ethylenediamine-N,N-diacetic acid; EDTA, ethylenediamine tetracetic acid; EGTA, ethyleneglycol-bis (β-aminoethylether) *N*,*N*,*N'*,*N'*-tetracetic acid; HPG, hydroxyphenylglyoxal; IT, 2-iminothiolane; MBA, mercaptobutyramidate; MDH, methanol dehydrogenase; NEM, *N*-ethylmaleimide; NME, norleucine methyl ester; PDBA, 4-(2-pyridyldithio)butyramidate; PDP, 2-pyridyldithiopropionate; PES, phenazine ethosulphate; PIP, 2,6-dichlorophenolindophenol; PQQ, pyrrolo-quinoline quinone; PG, phenylglyoxal; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; Sulpho-NHS, sulpho-*N*-hydroxysuccinimide; TNBS, 2,4,6-trinitrobenzene sulphonate.

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

cytochrome oxidase) by way of specific lysine residues arranged around the haem cleft [7]. Cytochrome c_H is also likely to react by way of these lysine residues with complementary carboxyl residues on the cytochrome c_L which are also likely to interact with lysine residues on MDH.

The present paper seeks to investigate these proposals using proteins from *Methylobacterium extorquens*, the facultative methylotroph in which the $\alpha_2\beta_2$ structure of MDH was first determined, and also using proteins from an unrelated, obligate, methylotroph, *Methylophilus methylotrophus*. These proposals have been investigated firstly by confirming that the MDH of this second methylotroph also has an $\alpha_2\beta_2$ structure; secondly by demonstrating that 'docking' and electron transfer between MDH and cytochrome c_L does, indeed, involve electrostatic interactions; thirdly by observing the effect on activity of chemically modifying lysine residues on MDH, and carboxyl residues on cytochrome c_L; and finally by investigating which subunits of the MDH are able to form crosslinked complexes with cytochrome c_L.

Materials and Methods

Organism, growth conditions, disruption and fractionation of bacteria

Methylophilus methylotrophus (NCIB 10515) was grown in methanol-limited continuous culture, harvested and cells broken as in [8,9]. *Methylobacterium extorquens* AM1 was grown on methanol in batch culture, harvested and cells broken as described in Ref. 10.

Purification of methanol dehydrogenase and cytochrome c_L

Purification of MDH was as described in Ref. 10. Because of its lability, MDH of *M. methylotrophus* was purified in the presence of 25 mM methanol. After elution from the Mono-S column this MDH was transferred to sodium phosphate buffer (pH 7.0) and frozen rapidly in liquid N_2 to avoid changes in activity and spectral characteristics that occurred during extensive storage in Mes buffer. Cytochrome c_L was purified as described in [11]. The cytochrome of *M. methylotrophus* is often present in two forms (of 17 and 21 kDa). In the present work only the 17 kDa form was used.

Assay of protein, MDH and pyrrolo-quinoline quinone (PQQ)

Protein was determined by the bicinchoninic acid method [12]. The dye-linked assay system, using phenazine ethosulphate as primary electron acceptor and 2,6-dichlorophenolindophenol (PIP) as terminal acceptor, was described in [10]. Two cytochrome-linked assays were used. The first measures reduction of equine cytochrome *c* in the presence of a small amount of bacterial cytochrome c_L [10]. In the second (novel) method PIP (86 μM) replaced the equine cytochrome *c*. The reaction mixture contained, in a 0.5 ml vol. in a spectrophotometer cuvette (1 cm light path), 12 mM Mops buffer (pH 7.0), NH_4Cl (22.5 mM for *M. extorquens* or 10 mM for *M. methylotrophus*), methanol (5 mM), cytochrome c_L (1–5 μM) and PIP (86 μM). Reactions were started by addition of MDH and the reduction of PIP was measured by the decrease in absorbance at 600 nm.

Fluorescence of free PQQ was measured in a Perkin-Elmer LS-3B spectrophotofluorimeter, exciting at 365 nm and measuring emission at 470 nm [13].

SDS-PAGE, measurement of protein and haemoprotein on gels, dissociation of MDH and protein sequencing

SDS-PAGE, and measurement of protein and haemoprotein on gels was done as by [11]. MDH was dissociated into its subunits by incubating MDH at 80 °C for 30 min in 2% SDS followed by gel filtration on a Superose-12 column (Pharmacia) as described in Ref. 5. The fractions containing the large (α) and small (β) subunits were collected separately, concentrated with a Centricon-5 concentrator and precipitated with dry, ice-cold ethanol (90% v/v, final concentration).

Protein sequencing was carried out using an Applied Biosystem 407A 'gas phase' (pulsed liquid) protein sequencer coupled to a model 120 phenylthiohydantoin-derivative analyser. For analysis of the undissociated MDH this was applied direct to the sequencer; this provided the first 13 amino acids of both subunits and permitted an evaluation of the relative amounts of the two subunits [5]. The N-terminal sequences of the dissociated β-subunit was determined by suspension of the ethanol-precipitated subunit in 0.1% trifluoroacetic acid and direct application to the sequencer.

Preparation of antisera and Western blotting

Purified MDH (400 μg) was suspended in 1 ml of Freunds complete adjuvant and used to immunise two New Zealand White rabbits; two boosters of 100 μg were given to each rabbit at 1 month intervals. Blood was collected from the ear and an IgG fraction prepared by ammonium sulphate fractionation as described in Ref. 14. The antisera were then serially diluted and the optimal concentration for use determined by Western blotting of purified and crude preparations of protein followed by probing with the diluted antisera. The highest concentration of antisera which did not give appreciable background staining was used. Samples for immunodetection were separated by SDS PAGE and then Western blotted essentially as described in Ref. 15. After incubation with suitably diluted antisera (12 h at room temperature),

bound antibodies were detected with horseradish peroxidase conjugated to anti-rabbit-IgG using 3,3′-diaminobenzidine tetra-hydrochloride as substrate.

Chemical modification of MDH and cytochrome c_L

Modification of arginine residues of MDH with phenylglyoxal or hydroxyphenylglyoxal. MDH (0.1 mg) in 100 µl of $NaHCO_3$ buffer (pH 8.4) was mixed with phenylglyoxal or hydroxyphenylglyoxal in the same buffer containing 10% methanol so that the final concentration of reagent was 10 mM. The mixture was incubated at room temperature for 45 min and the reaction terminated by removing excess reagent through a P-6 desalting column equilibrated with 20 mM Mes buffer (pH 6.0). The extent of hydroxyphenylglyoxal modification was monitored by the increase in absorbance at 340 nm (extinction coefficient = 18 300 M^{-1} cm^{-1}) [16].

Modification of lysine residues with 2-iminothiolane (IT) in the presence of 2,2′-dithiodipyridine (DTDP) to produce 4-(2-pyridyldithio)butyramidate (PDBA)-derivatives. This method, based upon that of Steenkamp [17], modifies lysine residues; the extent of modification is determined by the amount of IT used. Mercaptobutyramidate (MBA) derivatives of MDH were prepared by reaction with IT and were immediately converted to the PDBA derivative by reaction with DTDP which was also in the reaction mixture. To MDH (26 mg/ml) in 25 mM Hepes/Na^+ buffer (pH 7.5) was added 2,2′-dithiodipyridine (DTDP) to a final concentration of 8 mM from a 200 mM stock solution prepared in dimethylsulphoxide (DMSO). The mixture was stirred vigorously at all times to maintain the DTDP/DMSO as a fine emulsion. Solid 2-iminothiolane was added (final concentration, 25 mM) and the mixture incubated at 4 °C for 30 min before equilibrating with 25 mM-Hepes/Na^+ buffer (pH 7.5) on a PD10 column. The extent of incorporation of the dithione-containing structures was estimated by the increase in absorbance at 343 nm due to release of pyridine-2-thione on adding dithiothreitol to the modified MDH. The sample (1 ml) was divided equally between two spectrophotometer cuvettes. To the reference cuvette was added 50 µl of buffer and to the sample cuvette was added 50 µl of 50 µM DTT, and the increase in absorbance at 343 nm read after 5 min. The concentration of pyridine-2-thione released was calculated using an extinction coefficient of $8.08 \cdot 10^3$ M^{-1} cm^{-1}.

Modification of lysine residues on MDH with N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) to give 2-pyridyldithiopropionate (PDP) derivatives. The method was based upon that described in [18]. MDH (50 µM) in 25 mM Hepes buffer (pH 8.0) was reacted with SPDP at a final concentration of between 50 and 100 µM for 1 h at 4 °C. The reaction was terminated by removing excess reagent, and exchanged into 25 mM Hepes buffer (pH 7.5) by gel filtration on a PD 10 column. The extent of modification was determined by measuring release of pyridine-2-thione as described for production of PDBA-derivatives of MDH (above).

Modification of lysine residues of MDH with trinitrobenzene sulphonate (TNBS). MDH (0.5 mg) was incubated in 100 µl of 25 mM $Na_4P_2O_7$ buffer (pH 9.0) containing TNBS (5 mM). After incubation at 20 °C for 35 min the reaction was terminated by passage down a P-6 column (Bio-Rad) equilibrated with 5 mM sodium phosphate buffer (pH 7.0). The extent of modification was calculated from the absorbance at 346 nm (extinction coefficient = $14.5 \cdot 10^3$ M^{-1} cm^{-1}) [19].

Modification of carboxyl groups of cytochrome c_L by norleucine methyl ester (NME coupled with [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] (EDC). Cytochrome c_L (in 10 mM phosphate buffer, pH 6.5) was concentrated to 25 µM using a Centriprep-10 (Amicon) and added to a large excess of EDC (5 mM) and NME (10 mM) (final concentration). After 2 h the reagents were removed by gel filtration using a P-6 column equilibrated with the same buffer.

Modification of lysine residues of cytochrome c_L with 2-iminothiolane (IT) to give MBA-derivatives. This process yields cytochrome c_L with mercaptobutyramidate (MBA) groups on some lysines [17]. Cytochrome c_L was exchanged into 20 mM Tris buffer (pH 8.0) containing 100 mM NaCl (degassed and purged with O_2-free N_2) using a PD-10 column. The cytochrome was concentrated (to 54 µM) using a centriprep-10 (Amicon) and dithiothreitol (final concentration 1 mM) added. Solid 2-iminothiolane was added (final concentration 25 mM) and the mixture stirred under N_2 at 20 °C, for 10 min, before exchanging into 25 mM Hepes/Na^+ buffer (pH 7.5) that had been degassed with N_2. The sample was stored on ice, in a sealed gas-tight vessel under an atmosphere of N_2.

Crosslinking of PDBA-modified MDH and MBA-modified cytochrome c_L

Crosslinking of the modified proteins occurs by elimination of pyridine-2-thione which was measured at 335 nm. The reaction was initiated by mixing equimolar concentrations of the modified proteins in 15 mM Hepes buffer (pH 7.5). Over a period of 3 min samples were removed and the reaction terminated by addition of ethanolic *N*-ethylmaleimide (NEM) (final concentration, 25 mM).

Two stage cross-linking with EDC, enhanced with sulpho-N-hydroxysuccinimide (Sulpho-NHS)

In this method 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC) reacts with carboxyl groups, generating *O*-acylisourea intermediates. Sulpho-NHS attacks the *O*-acylisourea intermediate, forming an *N*-hydroxysuccinimidyl (NHS-) ester which is resistant to

hydrolysis [20]. The stability of the NHS ester allows the use of a two-stage coupling which is not possible with EDC alone [21]. Nucleophilic attack by an epsilon amino group of lysine results in the release of a substituted urea and generation of an isopeptide bond. EDC is considered to be a zero-length crosslinker as a spacer arm is not introduced during the crosslinking reaction. To achieve crosslinking by activation of carboxyl groups on MDH it was exchanged by gel filtration into 20 mM Mes/Na^+ buffer (pH 6.0) and concentrated to 194 μM using a Centriprep-30 (Amicon). To this was added 5 mM sulpho-NHS and 2 mM EDC (final concentrations). After 30 min excess reagents were removed by gel filtration on a P6 column equilibrated with 5 mM-Hepes buffer (pH 7.5), and an equimolar concentration of unmodified cytochrome c_L was added. After 90 min the reaction was terminated by addition of hydroxylamine to a final concentration of 15 mM. For crosslinking by activation of carboxyl groups on cytochrome c_L or tuna cytochrome *c* (Sigma) the reaction was performed in an analogous manner to that with MDH (above) except that the final concentration of cytochrome was 5–10 μM. After cross-linking the products were separated and identified by SDS-PAGE followed by protein-staining and staining for haem proteins.

Results

The subunit structure of MDH from Methylophilus methylotrophus

As found for the MDH of *Methylobacterium extorquens* [5], it was possible to separate MDH into two subunits by incubating in 2% SDS at 80 °C followed by gel filtration on a Superose-12 column equilibrated in 0.2% SDS. In these conditions the relative proportions of the two types of subunit were identical, and the fluorescent prosthetic group was liberated from the protein during its dissociation. The similarity of the smaller β-subunit of *M. methylotrophus* to that of *M. extorquens* is confirmed by its N-terminal sequence (Fig. 1). The molecular masses of the separated α- and β-subunits, estimated by SDS-PAGE, were about 60 and 9 kDa, respectively; these values compare with values of about 66 and 8.5 kDa for the MDH of *M. extorquens* determined from the gene sequences. All fractions for each of the two subunits after gel filtration in 0.2% SDS were pooled and the total protein measured by the bicinchoninic acid method [12]. The average ratio (α/β) was 1:1.16. During preliminary work on the N-terminal sequence of the holoenzyme it was possible to compare the amounts of PTH-derivatized alanine and glycine in the α subunit with those in the β-subunit as described Ref. 5. The molar ratio (α/β) determined by this method was 1:0.93 using alanine and 1:0.98 using glycine. The total molecular mass by gel filtration was 115 kDa as shown previously [22]. These results suggest that the molar ratio (α/β) is 1:1 and that the MDH of *Methylophilus methylotrophus* also exists in an $\alpha_2\beta_2$ conformation as shown previously in *M. extorquens* [5].

The assay systems for MDH activity

In the dye-linked assay system it is assumed that electrons flow directly from the PQQ prosthetic group to PES and no protein-protein 'docking' is required. When cytochrome c_L is used as primary electron acceptor a terminal electron acceptor (a type I cytochrome *c*) is also essential [23] because the high concentrations of cytochrome c_L required for spectrophotometric measurements would prevent the study of competitive inhibitors. To avoid having two protein–protein interactions in the test system a novel MDH assay system was developed in which cytochrome c_L is used as the primary electron acceptor and PIP is used as the terminal electron acceptor instead of equine cytochrome *c*. In this system the rate of oxidation of ferrocytochrome by oxidised PIP is extremely rapid and always much faster than rates of cytochrome reduction by MDH.

Table I provides a summary of pH optima and kinetic constants for the various assay systems used in this work, using proteins from the two types of bacteria. In the novel cytochrome-linked assay with PIP there was a broad pH optimum of about 7.0 and all subsequent assays were done at this pH value. In this assay system the rate in the absence of activator (NH_4Cl) was about 10% of that in its presence when using the *M. extorquens* proteins, but it was about 35% for the proteins from *M. methylotrophs*. In the PES-linked system the rates were much higher than in the

```
                                10        20        30
                                |         |         |
M. extorquens:          YDGTKCKAAGNCWEPKPGFPEKIAGSKYDPK -
                        ***   * *** ** *** ***********
M. methylotrophus:      YDGQTXKEAGNXWEAKPGYPEKIAGSKYDPK -
```

Fig. 1. The N-terminal sequence of the β-subunit of MDH of *M. methylotrophus* and *M. extorquens*. The sequence of the β-subunit of *M. extorquens* is taken from Ref. 5.

TABLE I

Kinetic parameters in the MDH assay systems

These assay systems link the methanol-dependent reduction of the primary electron acceptor, which is either phenazine ethosulphate (PES) or cytochrome c_L, to reduction of a terminal electron acceptor, which is either the dye 2,6-dichlorophenolindophenol (PIP) or equine cytochrome *c* (cyt. c_{eq}). The V_{max} values and K_m values for cytochrome c_L were measured at the pH optima for the two systems. In the cytochrome-linked systems sufficient NH_4Cl was included to give maximum rates. For the *M. extorquens* system this was 22.5 mM (in 20 mM Mops buffer, pH 7.0); for the *M. methylotrophus* system this was 5 mM when equine cytochrome *c* was the terminal electron acceptor (with 12 mM Tris, pH 8.0) and 10 mM with PIP (with 12 mM MOPS, pH 7.0). V_{max} values are recorded as nmoles of electron acceptor reduced min^{-1} (nmol MDH)$^{-1}$. The K_m values refer to the initial electron acceptor (cytochrome c_L).

	PES/PIP		Cyt. c_L/cyt. c_{eq}			Cyt. c_L/PIP-linked		
	pH	V_{max}	pH	V_{max}	K_m (μM)	pH	V_{max}	K_m (μM)
M. extorquens	9.0	104	7.0	15	3	7.0	40	3.0
M. methylotrophus	9.0	60	8.0	11	1	7.0	4.0	1.0

cytochrome-linked system as has been found previously for MDH from these two bacteria [23].

Electrostatic interactions between MDH and cytochrome c_L

When protein–protein interactions are electrostatic in nature they are affected by the ionic strength of the surrounding medium [7]. As might be expected for electron transport processes involving electrostatic interactions between proteins that occur in the periplasm, the oxidation of methanol by whole bacteria was inhibited by high salt concentrations; for example, oxidation of methanol by *M. extorquens* was inhibited (about 50%) by 100 mM K_2SO_4 or 25 mM sodium phosphate at pH 7.0. No such inhibition occurred with NaCl or potassium phosphate (200 mM) in the PES-linked MDH assay system using crude extracts or pure MDH. By contrast, low concentrations of either salt (50 mM) were able to inhibit MDH in all types of assay in which low concentrations of cytochrome c_L were used. There was an inverse linear relationship between reaction rates and the square root of ionic strength consistent with ionic interactions being involved.

Fig. 2 shows the effect of high ionic strength in decreasing the affinity of MDH for cytrochrome c_L as indicated by the observed increases in K_m values. Inhibition by NaCl was competitive and was relieved by using higher cytochrome concentrations. As expected for proteins whose 'docking' together into an active complex is determined largely by ionic interactions, the inhibitory effects of salts was not specific to any one ionic species (Table II).

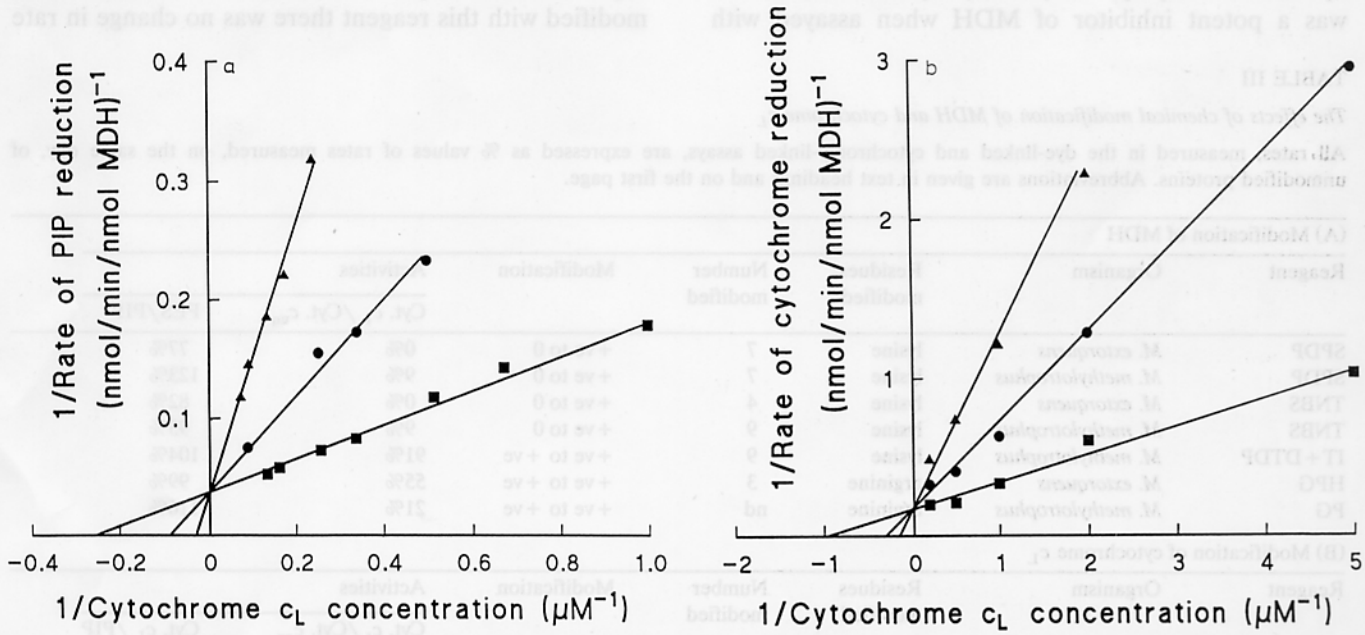


Fig. 2. The effect of NaCl on the affinity of MDH for cytochrome c_L. (a) Proteins from *M. extorquens*. MDH (2 μM) was measured in the PIP-linked system in 20 mM Mops buffer containing 22.5 mM NH_4Cl (pH 7.0). ■, no NaCl, K_m 3 μM. ●, 25 mM NaCl, K_m 8.6 μM. ▲, 50 mM-NaCl, K_m 30 μM. (b) Proteins from *M. methylotrophus*. MDH (2 μM) was measured in the equine cytochrome *c*-linked assay system in 12 mM Tris buffer (pH 8.0) containing 5 mM NH_4Cl. ■, no NaCl, K_m 1.1 μM. ●, 25 mM-NaCl, K_m 3.3 μM. ▲, 50 mM-NaCl, K_m 6.7 μM.

TABLE II

The effect of ionic strength on the MDH of M. methylotrophus

Reaction mixtures contained 2 μM MDH, 2 μM cytochrome c_L and 5 mM methanol. Reduction of equine cytochrome *c* (Cyt. c_{eq}) (50 μM) was measured with 5 mM NH_4Cl in 12 mM Tris buffer (pH 8.0). PIP (86 μM) was measured with 10 mM NH_4 Cl in 12 mM Mops (pH 7.0). The ionic strength due to NH_4Cl has not been included because it is an activator and is required to achieve the maximum rates achieved prior to inhibition by salts. The ionic strengths of added salts that gave 50% inhibition (I_{50} value) were taken from the linear plots of activity against square root of ionic strength for each ion. Abbreviations: EDDA, ethylenediamine-*N*,*N*-diacetic acid; EDTA, ethylenediamine tetracetic acid; EGTA, ethyleneglycol-bis (β-aminoethylether)*N*,*N*,*N'*,*N'*-tetracetic acid; CDTA, trans-1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetracetic acid.

Salt	I_{50} value (ionic strength giving 50% inhibition)	
	Cyt. c_L /Cyt. c_{eq}	Cyt. c_L /PIP
NaCl	0.021	0.019
LiCl	n.d.	0.027
KCl	n.d.	0.016
RbCl	n.d.	0.022
Na_2HPO_4	0.02	0.017
$MgCl_2$	0.044	0.016
$CaCl_2$	0.053	0.036
EDDA	0.04	n.d.
EDTA	0.0012	0.0011
EGTA	0.0012	n.d.
CDTA	0.0014	n.d.

The inhibition of MDH by ethylenediaminetetracetic acid (EDTA)

EDTA (15 mM) inhibits the oxidation of methanol by whole bacteria but not by purified MDH in the dye-linked assay system [24–27]. By contrast, EDTA was a potent inhibitor of MDH when assayed with cytochrome c_L as primary electron acceptor, 50% inhibition occurring at about 120 μM (Table II). The ionic strength at this concentration is only 0.0012, which is about 5% of that required for inhibition by other ions, suggesting that EDTA has an effect that is separate from its activity as a polyanion. There was no evidence to support involvement of divalent cations; the proteins were purified in the absence of divalent cations and these have no activating effect on MDH in any assay system. The I_{50} value recorded for ethylenediamine diacetic acid (EDDA) was similar to that measured with other divalent cations. This suggests that the four carboxyl groups may have a function in inhibition, and the similar results obtained with two other tetracarboxylates, EGTA and CDTA, are consistent with this conclusion. There was no relationship between the inhibitory activities of these compounds and their chelating specificities. The most obvious explanation for this reversible competitive inhibition by EDTA is that it prevents 'docking' of the two proteins by interaction of its carboxyl groups with lysine residues on MDH or cytochrome c_L.

The effect of modification of arginine groups of MDH on its activity

Reaction of MDH with phenylglyoxal, a specific modifier of arginine residues [28], produced a modified MDH having only 21% activity with the cytochrome-linked assay, whereas activity in the dye-linked assay increased slightly (Table III). Modification with hydroxyphenylglyoxal provides a convenient measure of the number of residues modified [17]. When three arginine residues (per mol of tetrameric MDH) were modified with this reagent there was no change in rate

TABLE III

The effects of chemical modification of MDH and cytochrome c_L

All rates, measured in the dye-linked and cytochrome-linked assays, are expressed as % values of rates measured, on the same day, of unmodified proteins. Abbreviations are given in text headings and on the first page.

(A) Modification of MDH

Reagent	Organism	Residues modified	Number modified	Modification	Activities	
					Cyt. c_L /Cyt. c_{eq}	PES/PIP
SPDP	*M. extorquens*	lysine	7	+ve to 0	0%	77%
SPDP	*M. methylotrophus*	lysine	7	+ve to 0	9%	123%
TNBS	*M. extorquens*	lysine	4	+ve to 0	0%	82%
TNBS	*M. methylotrophus*	lysine	9	+ve to 0	9%	93%
IT + DTDP	*M. methylotrophus*	lysine	9	+ve to +ve	91%	104%
HPG	*M. extorquens*	arginine	3	+ve to +ve	55%	99%
PG	*M. methylotrophus*	arginine	nd	+ve to +ve	21%	116%

(B) Modification of cytochrome c_L

Reagent	Organism	Residues modified	Number modified	Modification	Activities	
					Cyt. c_t /Cyt. c_{eq}	Cyt. c_L /PIP
IT	*M. extorquens*	lysine	n.d.	+ve to +ve	77%	n.d.
IT	*M. methylotrophus*	lysine	n.d.	+ve to +ve	96%	n.d.
EDC/NME	*M. methylotrophus*	carboxyl	n.d.	−ve to 0	12%	12%

in the dye-linked assay but a 45% decrease in rate in the assay with cytochrome c_L (Table III). These results are consistent with the suggestion that arginine may play a role in the MDH/cytochrome c_L interaction.

Production of PDBA-derivatives of MDH by modification of lysine residues by reaction with 2-iminothiolane (IT) plus 2,2'-dithiodipyridine (DTDP)

This process modifies lysine residues by formation of 4-(2-pyridyldithio)butyramidate (PDBA) derivatives, which retain a positive charge [17]. When the average number of lysines modified (per mol of tetramer) was fewer than 10, there was little change in dye-linked activity, suggesting that the modified enzyme had suffered no major alteration in its catalytic function. There was also very little change in its cytochrome-linked activity (Table III). This active MBA-derivative of MDH was subsequently used in the crosslinking reaction with cytochrome c_L (see below).

Modification of lysine residues on MDH with N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) to form pyridylthiopropionate (PDP) derivatives

This process modified lysine residues by formation of uncharged PDP derivatives of MDH [18]. When the average number of lysines modified per mol of tetrameric MDH from *M. extorquens* was 7.4 (6% of the total lysines) there was a 23% decrease in dye-linked MDH activity, and complete loss of activity when assayed using cytochrome c_L as electron acceptor (Table III). The absorption spectrum of the modified MDH in the region of the prosthetic group (PQQ) was typical of unmodified enzyme, and the absorption and fluorescence spectra of the dissociated prosthetic group were identical to those of unmodified MDH. Together with the observed retention of activity in the dye-linked assay, this indicates that the prosthetic group had not been modified.

These results are consistent with the suggestion that some of the lysine residues of MDH are involved in recognition and interaction with its electron acceptor, cytochrome c_L. Because all activity was lost when only 6% of the total lysines were modified, it is clear that the lysines involved in 'docking' with cytochrome c_L must have been those that were more available for reaction with the modifying reagent. Similar results were obtained with the MDH from *M. methylotrophus* (Table III).

Modification of lysine residues on MDH with 2,4,6-trinitrobenzene sulphonate (TNBS)

This process modifies lysine residues by formation of uncharged derivatives [29]. When the average number of lysines modified per tetramer was between 4 and 9, the dye-linked activity was not markedly changed. By contrast with the PDBA-modified MDH, that retains the charge (see above), the TNBS-modified MDH lost almost all activity in the cytochrome-linked assay (Table III). This confirms that charged lysine residues are likely to be involved in the MDH/cytochrome interaction. When the modified MDH of *M. methylotrophus* was separated into subunits by SDS-gel filtration about 94% of the TNBS was found to be associated with the α-subunit suggesting that the lysines on the α-subunit are more susceptible to modification than those on the β-subunit, which perhaps argues that the β-lysines do not have a predominant role in 'docking' with cytochrome c_L.

Modification of lysine residues of cytochrome c_L with 2-iminothiolane (IT)

Lysine residues on cytochrome c_L were modified with IT, which gives mercaptobutyramidate (MBA) derivatives that retain the positive charge on lysine. The reaction is the same as the first stage of producing the PDBA derivatives of MDH (above). When cytochrome c_L was modified in this way it was still able to form 'normal' electron transfer complexes with MDH, an important consideration with respect to subsequent cross-linking experiments with this modified cytochrome. The rate for modified cytochrome was more than 75% of that with unmodified cytochrome (Table III). The absorption spectrum of the cytochrome was unchanged.

Modification of carboxyl groups of cytochrome c_L with norleucine methyl ester (NME) in the presence of 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC)

When carboxylates are modified with norleucine methyl ester (NME) in the presence of the carbodiimide EDC, the modified carboxyl carries no change [30]. This modification led to loss of most of the activity when the modified cytochrome c_L was used as the electron acceptor for the MDH of *M. methylotrophus* (Table III).

Crosslinking of MDH and cytochrome c_L using PDBA-modified MDH and MBA-modified cytochrome c_L

Both PDBA- and MBA-derivatives have lysyl residues modified by incorporation of long side arm thiol groups [17]. The proteins, modified as described above, were reacted together to form crosslinks by formation of a disulphide bond, the formation of crosslinked product being complete within 1 min. The products were identified by their relative molecular masses on SDS-PAGE and activity with haem stain. About half the cytochrome was crosslinked. The inclusion in the reaction mixture of NaCl (150 mM), which inhibits interaction and electron transport between MDH and cytochrome c_L (see above) delayed markedly the appearance of the products.

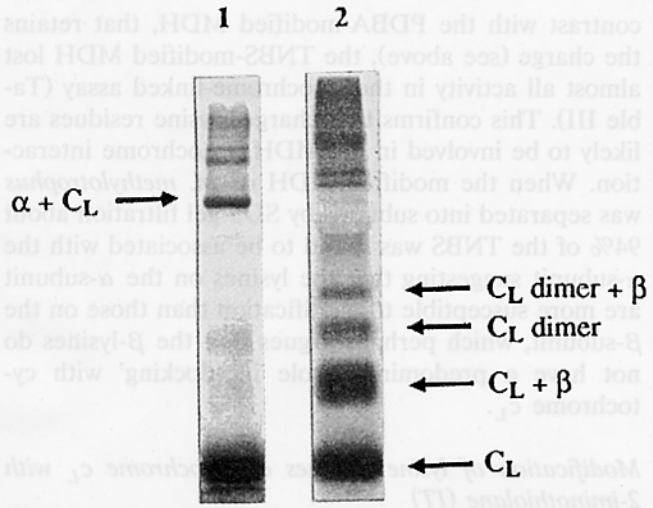


Fig. 3. Crosslinking of PDBA-modified MDH with MBA-modified cytochrome c_L. The proteins were modified as described in Materials and Methods. With proteins from *M. methylotrophus*, 21.5 μM MDH was modified (9 lysines per mol) and reacted with 19 μM modified cytochrome c_L. With proteins from *M. extorquens*, 14.3 μM MDH was modified (3.3 lysines per mol) and reacted with 19 μM modified cytochrome c_L. After 3 min the reaction was terminated by addition of *N*-ethylmaleimide and the reaction mixture separated by SDS-PAGE and stained with haem stain. Lane 1, proteins from *M. methylotrophus*; lane 2, proteins from *M. extorquens*.

TABLE IV

Cross-linking of MDH and cytochrome c_L from M. extorquens and from M. methylotrophus

PDBA-modified MDH was cross-linked with MBA-modified cytochrome c_L and the products separated by SDS-PAGE and stained for haem proteins. The intensity of stain was determined with a Joyce-Loebl gel scanner.

	Intensity of haem-staining (% of total stained protein)	
Product on gel	*M. extorquens*	*M. methylotrophus*
Cytochrome c_L	49.6	44.2
Cytochrome c_L/β-subunit of MDH	31.7	1.5
Cytochrome c_L/α-subunit of MDH	6.0	10.0
Cytochrome c_L dimer	4.5	2.8
Cytochrome c_L dimer/β-subunit	2.0	0
Cytochrome c_L trimer	0.4	0

The results in Fig. 3 and Table IV show that the reactivities of MDH and cytochrome c_L were completely different for the two bacteria used in this work. With *M. methylotrophus* the major cross-linked product was formed between cytochrome c_L and the α-subunit of MDH; there was 6-times more of this product than of the β-subunit crosslinked to cytochrome c_L. By contrast, the major product with the proteins from *M. extorquens* was between cytochrome c_L and the β-subunit of MDH. This result was confirmed by identifying crosslinked products on gels by the use of specific antisera to cytochrome c_L. Only one crosslinked product was detected; this was cytochrome c_L crosslinked to the β-subunit of MDH. When antisera raised against the α-subunit of MDH was used the only crossreactive band was the α-subunit of unmodified MDH.

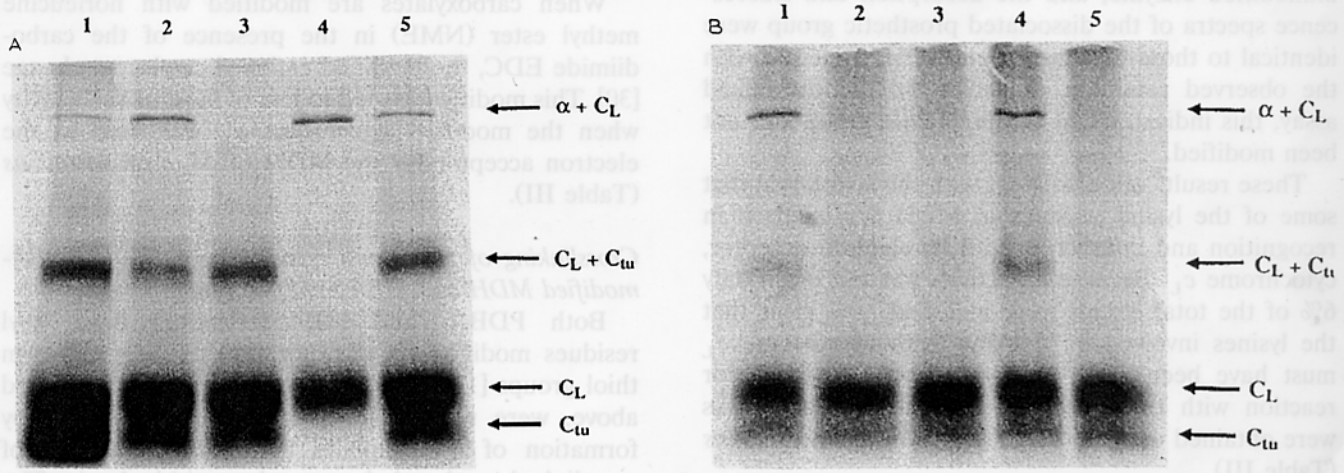


Fig. 4. Two-stage sulpho-NHS-enhanced EDC cross-linking of MDH, cytochrome c_L and a class I c-type cytochrome. These experiments were done with proteins from *M. methylotrophus*. After modification with EDC in the presence of NHS as described in Materials and Methods, cytochrome c_L (4.5 μM) was reacted with either 4.5 μM MDH or 4.5 μM tuna cytochrome *c*, or with both proteins (4.5 or 13.5 μM) for 90 min, the reaction terminated by addition of 15 mM hydroxylamine and the products separated by SDS-PAGE and stained with haem stain. (A) Lane 1, MDH (4.5 μM) + cytochrome c_L (4.5 μM) + tuna cytochrome (13.5 μM); lane 2, MDH (13.5 μM) + cytochrome c_L (4.5 μM) + tuna cytochrome (4.5 μM); lane 3, cytochrome c_L (4.5 μM) + tuna cytochrome (4.5 μM); lane 4, MDH (4.5 μM) + cytochrome c_L (4.5 μM); and lane 5, MDH (4.5 μM) + cytochrome C_L (4.5 μM) + tuna cytochrome (4.5 μM). (B) Lane 1, MDH (4.5 μM) + cytochrome c_L (4.5 μM) + tuna cytochrome (4.5 μM); lane 2 as for lane 1 + 200 mM NaCl; lane 3, as for lane 1 + 35 mM sodium phosphate; lane 4, as for lane 1 + 150 μM EDTA; and lane 5, as for lane 1 + 15 mM EDTA.

Two stage crosslinking of MDH and cytochrome c_L of M. methylotrophus with carbodiimide (EDC) enhanced with sulpho-N-hydroxysuccinimide (Sulpho-NHS)

In this two-stage system the carboxyl group on one protein is modified, and this modified group is attacked by an unmodified lysine on the second protein to give a 'zero-length' crosslink [21]. The use of zero-length crosslinkers is advantageous as it increases the probability that those residues that become linked are located at the site(s) of protein–protein interaction and are involved in salt bridge formation [31]. This method also provides an opportunity to determine the polarity of the interaction at the protein–protein interface [21].

No detectable crosslinking was observed when carboxyls on MDH were first activated and then reacted with cytochrome c_L. By contrast, when carboxyls on cytochrome c_L were modified, and then reacted with MDH, the only product was the MDH α-subunit crosslinked to cytochrome c_L (Fig. 4A). These results are consistent with the suggestion that lysine groups on the α-subunit of MDH are involved in the recognition or 'docking' process with carboxyls on the cytochrome c_L. The same result was obtained using proteins from *M. extorquens* (results not shown). NaCl (200 mM), phosphate (35 mM) and high concentrations of EDTA (15 mM) completely prevented crosslinking, as would be expected if they prevented 'docking' of the two proteins as previously suggested (Fig. 4B). Low concentrations of EDTA were previously shown to inhibit electron transfer between MDH and cytochrome c_L (above). The demonstration that these low concentrations failed to prevent cross-linking of the proteins suggest that EDTA acts as a potent inhibitor of methanol oxidation by inhibiting electron transfer and not by preventing 'docking' of the 2 proteins.

Demonstration that a single binding domain on cytochrome c_L of M. methylotrophus is involved in the reaction with both MDH (α-subunit) and tuna cytochrome c

Much of the rationale for this study was based on the assumption that the lysine residues that constitute the 'docking' site in class I *c*-type cytochromes are also involved in binding to carboxyls of cytochrome c_L which, in turn, are involved in the reaction with MDH. This proposal is supported by the results shown in Fig. 4A. In these experiments cytochrome c_L with modified carboxyl groups was reacted with equimolar amounts of MDH or tuna cytochrome *c*, and cross-linked products were analysed. Tuna cytochrome *c* was used because, although it gave only 75% of the rate of equine cytochrome *c* in the MDH assay, more is known about its 3-D structure than is known for either the cytochrome c_H of *M. methylotrophus* or equine cytochrome *c*. When cytochrome c_L was reacted separately with equimolar amounts of either MDH or tuna cytochrome, cross linked products with the tuna cytochrome and with the α-subunit of MDH were formed (Fig. 4A). No crosslinked products were formed when carboxyls of MDH were modified, or when the carboxyls of tuna cytochrome *c* were modified and then reacted with either MDH or cytochrome c_L. When cytochrome c_L was modified and reacted with equimolar amounts of both MDH and tuna cytochrome at the same time, crosslinked products were formed with either MDH or tuna cytochrome but not with both together, indicating that there is a single site on cytochrome c_L that reacts with both proteins. Further evidence for this is that when the concentration of MDH was increased 3-fold, more crosslinking occurred with MDH (α-subunit), and less occurred with tuna cytochrome *c*. Similarly, when the tuna cytochrome was increased 3-fold, then it formed far more crosslinked product and MDH crosslinking was diminished.

Discussion

It was suggested previously that the interaction of MDH with its electron acceptor, cytochrome c_L, is likely to be electrostatic in nature, involving ionic bonds between lysine or arginine residues on MDH, and carboxyl residues on the cytochrome. It was speculated that the lysine residues that form such a well-defined pattern in predictions of the secondary structure of the β-subunit of MDH of *M. extorquens* might be involved in 'docking' with carboxyls of cytochrome c_L which might also be involved in interaction with lysines of the binding domain of the typical class I *c*-type cytochrome which is the next electron acceptor in the 'methanol oxidase' system [4,5]. The results in the present paper are all consistent with the first of these proposals; as expected for interactions involving primarily electrostatic interaction, the affinity of the two proteins for each other was markedly diminished by low concentrations of salts when using the MDH from *Methylobacterium extorquens*. Similar results were obtained using the MDH from a second, unrelated, methylotroph *Methylophilus methylotrophus*, which was shown to have the same $\alpha_2\beta_2$ structure. The marked inhibitory effect of modification of arginine and lysine residues in such a way that their charges are lost also supports this conclusion, as does the inhibitory effect of modifying the cytochrome c_L in such a way as to diminish its negative charge.

The suggestion that the β-subunit of MDH is specifically involved in 'docking' with cytochrome c_L appeared to be supported by crosslinking experiments using PDBA-modified MDH and MBA-modified cytochrome because the cytochrome c_L of *M. extorquens* formed crosslinks only with the β-subunit. However, when the same experiment was repeated with proteins

from *M. methylotrophus* the α-subunit was the only subunit forming crosslinks with cytochrome c_L. With this sort of crosslinking reagent the length of the crosslinking 'arm' can give rise to misleading results, and results using the two-stage 'zero-length' crosslinking method are more likely to be significant. In these experiments the results with the proteins from the two types of bacteria were the same; crosslinking occurred predominantly with the α-subunit of MDH. This method also provided an opportunity to determine the polarity of the interaction at the protein–protein interface; and all the results were consistent with the conclusion that carboxyl groups on cytochrome c_L interact with lysyl residues on the α-subunit of MDH. Tuna cytochrome *c* provides a model for the typical class I *c*-type cytochrome (cytochrome c_H) which is the electron acceptor for cytochrome c_L in the 'methanol oxidase' electron transport chain. Competition experiments with this cytochrome demonstrated that the same domain on cytochrome c_L is likely to be involved in reaction with both the MDH and with the cytochrome c_H. This result together with the failure to observe formation of any complexes containing MDH together with both cytochromes argues against the possibility that electrons flow between methanol and the membrane-bound oxidase by way of 'wire-like structure' consisting of a complex of MDH, cytochrome c_L and cytochrome c_H.

Acknowledgements

We would like to thank the Molecular Recognition Initiative of the Science and Engineering Research Council (U.K.) for financial support and for the award of a research studentship to J.M. Cox. We also thank Dr. Claude Chan for valuable discussions.

References

1 Anthony, C. (1982) The Biochemistry of Methylotrophs Academic Press, London.
2 Anthony, C. (1986) Adv. Microbial. Physiol. 27, 113–210.
3 Anthony, C. (1988) in Bacterial Energy Transduction (C. Anthony), Academic Press, London, pp. 293–316.
4 Anthony, C. (1990) FEMS Microbiol. Rev. 87, 209–214.
5 Nunn, D.N., Day, D.J. and Anthony, C. (1989) Biochem. J. 260, 857–862.
6 Anderson, D.J., Morris, C.J., Nunn, D.N., Anthony, C. and Lidstrom, M.E. (1990) Gene 90, 173–176.
7 Pettigrew, G.W. and Moore, G.R. (1987) Cytochrome *c*: biological aspects, Springer series in Molecular Biology, Springer-Verlag, New York.
8 Cross, A.R. and Anthony, C. (1980a) Biochem. J. 192, 421–427.
9 Cross, A.R. and Anthony, C. (1980b) Biochem. J. 192, 429–439.
10 Day, D.J. and Anthony, C. (1990a) Methods Enzymol. 188, 210–216.
11 Day, D.J. and Anthony, C. (1990b) Methods Enzymol. 188, 298–303.
12 Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, B.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
13 Anthony, C. and Zatman, L.J. (1967) Biochem. J. 104, 960–969.
14 Hudson, L. and Hay, F.C. (1980) Practical Immunology, 2nd Edn., Blackwell Scientific Publications, pp. 1–3.
15 Gershoni, J.M. and Palade, G.E. (1983) Anal. Biochem. 98, 493–499.
16 Yamasaki, R.B., Vega, A. and Feeney, R.E. (1980) Anal. Biochem. 109, 32–40.
17 Steenkamp, D.J. (1988) Biochem. J. 255, 869–876.
18 Carlson, J., Drevin, H. and Axen, R. (1978) Biochem. J. 13, 723–737.
19 Hollenberg, P.F., Flashner, M. and Coon, M.J. (1971) J. Biol. Chem. 216, 946–953.
20 Lomant, L.J. and Fairbanks, G. (1976) J. Mol. Biol. 104, 243–261.
21 Grabarek, Z. and Gergely, J. (1990) Anal. Biochem. 185, 131–135.
22 Ghosh, R. and Quayle, J.R. (1981) Biochem. J. 199, 245–250.
23 Beardmore-Gray, M., O'Keeffe, D.T. and Anthony, C. (1983) J. Gen. Microbiol. 129, 923–933.
24 Anthony, C. and Zatman, L.J. (1964) Biochem. J. 92, 609–614.
25 Anthony, C. (1975) Biochem. J. 146, 289–298.
26 Carver, M.A., Humphrey, K.M., Patchett, R.A. and Jones, C.W. (1984) Eur. J. Biochem. 138, 611–615.
27 Beardmore-Gray, M. and Anthony, C. (1984) in Microbial growth on C-I compounds (Crawford, R.L. and Hanson, R.S., eds.), American Society of Microbiology, Washington, pp. 97–105.
28 Cheung, S. and Fonda, M.L. (1979) Biochem. Biophys. Res. Commun. 90, 940–947.
29 Johnson, S.C., Bailey, T., Becker, R.R. and Cardenas, J.M. (1979) Biochem. Biophys. Res. Commun. 27, 525–530.
30 Davidson, V.L., Jones, L.H. and Kumar, M.A. (1990) Biochemistry. 29, 10786–10791.
31 Mauk, M.R. and Mauk, A.G. (1989) Eur. J. Biochem. 186, 473–486.