The interaction of methanol dehydrogenase and its cytochrome electron acceptor

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A fluorescence method is described for direct measurement of the interaction between methanol dehydrogenase (MDH) and its electron acceptor cytochrome c_L. This has permitted a distinction to be made between factors affecting electron transfer and those affecting the initial binding or docking process. It was confirmed that the initial interaction is electrostatic, but previous conclusions with respect to the mechanism of EDTA inhibition have been modified. It is proposed that the initial 'docking' of MDH and cytochrome c_L is by way of ionic interactions between lysyl residues on its surface and carboxylate groups on the surface of cytochrome c_L. This interaction is not inhibited by EDTA, which we suggest acts by binding to nearby lysyl residues, thus preventing movement of the 'docked' cytochrome to its optimal position for electron transfer, which probably involves interaction with the hydrophobic funnel in the surface of MDH.

INTRODUCTION

Methanol dehydrogenase (MDH) in methylotrophic bacteria is a quinoprotein that has pyrrolo-quinoline quinone (PQQ) as its prosthetic group [1] and cytochrome c_L as its electron acceptor [2]. MDH and cytochrome c_L interact in the periplasm, where they are present at high concentrations (about 0.5 mM) [3], and constitute the first part of a 'methanol oxidase' electron transport chain which also involves a typical class I *c*-type cytochrome (cyt.) (usually referred to as cytochrome c_H) which is oxidized by a membrane oxidase:

Methanol → MDH → cyt. c_L → cyt. c_H → oxidase

Evidence from cross-linking experiments indicates that the cytochrome c_L dissociates from MDH before interaction with cytochrome c_H; that is, the same site on cytochrome c_L interacts with the electron donor and electron acceptor, and a three component electron transport complex is not formed [2,4,5].

There is considerable evidence from studies of electron transfer that the interactions between the three proteins from *Methylobacterium extorquens* are ionic in nature [4], including the observation that respiration in whole bacteria is inhibited by high salt concentrations (50% inhibition by 200 mM NaCl or about 25 mM sodium phosphate). Chemical-modification studies have indicated that the interaction is by way of a small number of lysyl residues on MDH and carboxylates on cytochrome c_L; modification of these residues prevents electron transfer between the proteins, without however affecting activity in the phenazine ethosulphate (PES)-linked system [4]. It has been proposed that the well-established inhibition of methanol oxidation by EDTA [6] is a result of its binding to these lysyl residues, thus preventing the electrostatic interaction of MDH with cytochrome c_L. It has recently been suggested by Harris and Davidson, however, that hydrophobic interactions between the two proteins are predominant [7], and this is supported perhaps by the recently reported structure of MDH from *M. extorquens* [at 1.94 Å resolution (1 Å = 0.1 nm)]. This shows that the PQQ is buried within an internal chamber [1,8] which communicates with the exterior of the protein by way of a hydrophobic funnel in the surface, which is perhaps the most likely place for interaction with cytochrome c_L [1,8]. Because the X-ray structures of MDHs from different bacteria are very similar [1,8–10], it seems unlikely that MDH and cytochrome c_L interact exclusively by electrostatic interactions in *M. extorquens* and exclusively by hydrophobic interactions in *Paracoccus denitrificans*.

The present paper describes an investigation of the interaction of MDH and cytochrome c_L from *M. extorquens* by a direct binding method, which has led to a modified description of binding and electron transfer in this system.

MATERIALS AND METHODS

Preparation and assay of proteins

The following methods were as described previously [11,12]: growth, harvesting and breakage of *M. extorquens* (N.C.I.M.B. 9133); purification of MDH and cytochrome c_L; assay of MDH with the dye PES; and assay of MDH with cytochrome c_L using 2,6-dichlorophenol-indophenol (DCPIP) as terminal electron acceptor [4]. Methods for growth of the mutant *mxaA* (previously known as *moxA*), and for work on its MDH, were as described previously [13].

Measurement of binding of MDH and cytochrome c_L

For measurements using the ultrafiltration assay, MDH (7 μM) was mixed with various concentrations of oxidized cytochrome c_L in 5 mM Mops buffer (pH 7.0) in a 1 ml reaction volume in the top compartment of a Centricon-100 concentrator (Amicon). This was centrifuged for 30 s in a bench-top centrifuge at low speed and the filtrate returned to the top compartment. After a second centrifugation, 50 μl samples were removed for analysis by gel filtration on an FPLC Superdex 75 column (Pharmacia) equilibrated in 5 mM potassium phosphate buffer (pH 7.0) con-

Abbreviations used: MDH, methanol dehydrogenase; PES, phenazine ethosulphate; PQQ, pyrrolo-quinoline quinone; DCPIP, 2,6-dichlorophenol-indophenol

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taining 200 mM NaCl at a flow rate of 1 ml/min. Protein was detected at 280 nm by a UV detector linked to a Shimadzu Chromatopak integrator for calculating the area under the peaks for quantification (a linear response was obtained for the range 0.5–50 μM protein).

For binding measurements using the fluorescence assay, fluorescence was recorded with a Hitachi F-2000 spectrophotofluorimeter at 25 °C using a 10 nm bandpass; samples (2 ml) were excited at 295 nm and fluorescence detected at 330 nm. When high concentrations of cytochrome c_L were used it was necessary to correct for the inner and outer filter effects using the following equation [14]:

$$F_{corrected} = F_{observed} + \text{antilog}\ (A_{excitation} + A_{emission})/2$$

RESULTS

High ionic strength prevents effective interaction between MDH and cytochrome c_L

The results in Figure 1 show that electron transfer between MDH and cytochrome c_L from *M. extorquens* was markedly inhibited by high ionic strength, confirming results previously published for *M. extorquens* [4], *Methylophilus methylotrophus* [4], *Acetobacter methanolicus* [5] and *Hyphomicrobium* [15]. The measured K_m value at low ionic strength was 3.5 μM cytochrome c_L, increasing to 31 μM at higher ionic strength (50 mM NaCl; $I = 0.05$). The rate of electron transfer was proportional to the inverse of the square root of the ionic strength, as expected if the salt is acting by decreasing ionic interactions between the two proteins [16]. The reaction between MDH and the artificial dye electron acceptor PES was unaffected by very high ionic strength, suggesting that salt does not inhibit the dehydrogenase reaction, but is affecting the 'docking' of the two proteins or the electron transfer between them [4]. In order to distinguish between these two possibilities it was necessary to devise a method for determination of binding that was not dependent on measurement of electron transfer. This was particularly important because the unexpected suggestion that hydrophobic interactions are predominant in the *P. denitrificans* system was based on experiments in which the binding constant and electron-transfer rates were determined separately [7].

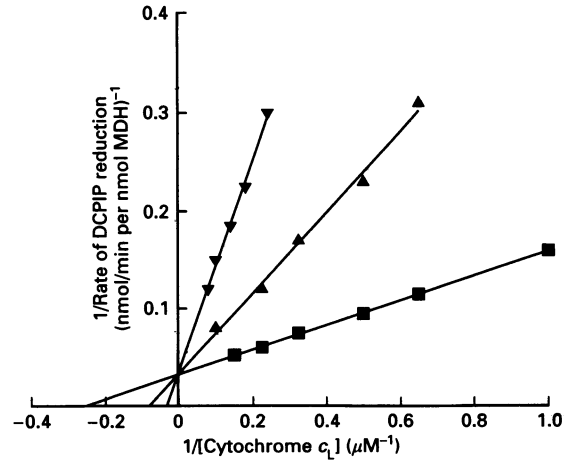

Figure 1 The effect of NaCl on the affinity of MDH for cytochrome c_L

The activity of MDH (2 μM) was measured in the assay system in which electrons pass from MDH to cytochrome c_L and thence to an excess of DCPIP, as described in the Materials and methods section. ■, No NaCl (apparent $K_d = 3.5$ μM); ▲, 25 mM NaCl ($K_d = 13$ μM); ▼, 50 mM NaCl ($K_d = 31$ μM).

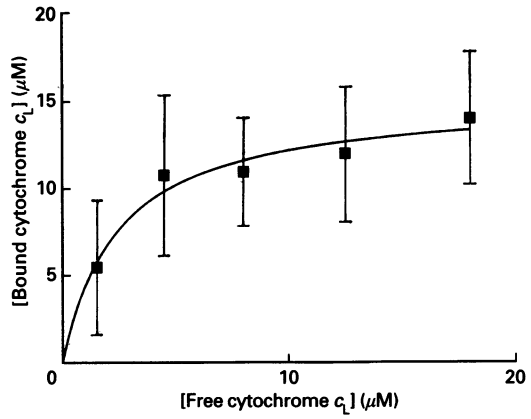

Figure 2 Binding of cytochrome c_L to MDH as measured in an ultrafiltration assay

MDH (7 μM) was mixed with various concentrations of cytochrome c_L and subsequently separated by ultrafiltration on a membrane to determine free and bound cytochrome c_L, as described in the Materials and methods section. The solid line is the fit of the data to the equation:

Bound cytochrome = (capacity of MDH × [free cytochrome])/(K_d + [free cytochrome])

The data correspond to a capacity of 15 μM MDH and a K_d of 2.4 μM cytochrome c_L.

Measurement of binding of MDH and cytochrome c_L by an ultrafiltration method

This assay was first developed [17] to determine the binding constant for the interaction of another quinoprotein dehydrogenase (methylamine dehydrogenase) and its electron acceptor (amicyanin) in the periplasm of *P. denitrificans*. It was used subsequently to study the interaction of methanol dehydrogenase and cytochrome c_L in the same methylotroph [7]. The essence of the method is the separation and measurement of free and bound cytochrome and MDH using a Centricon-100 concentrator (Amicon). The method [7] depends on a very high degree of retention of MDH by the membrane; this was said to be greater than 99% [7,17], which is much more effective than expected from the technical specifications supplied by the manufacturers. In the present work, by contrast, only 85–90% retention was obtained, as expected from the manufacturer's specifications. The relatively poor retention is reflected in the poor quality of the data indicated in Figure 2. It was still possible, however, to obtain an approximate K_d value for interaction in the absence of salt (2.4 μM); this is similar to the K_m value determined by measurement of electron transfer (3.5 μM; see above). In the presence of NaCl ($I = 0.2$) retention of the proteins by the membrane was so poor that no meaningful results were obtained and an alternative method was investigated.

Measurement of binding of MDH and cytochrome c_L by a novel fluorescence method

In order to confirm that salt inhibits binding rather than the electron-transport process itself, an alternative method for measuring binding of the two proteins in *M. extorquens* was developed. This method depends on the decrease in the tryp-

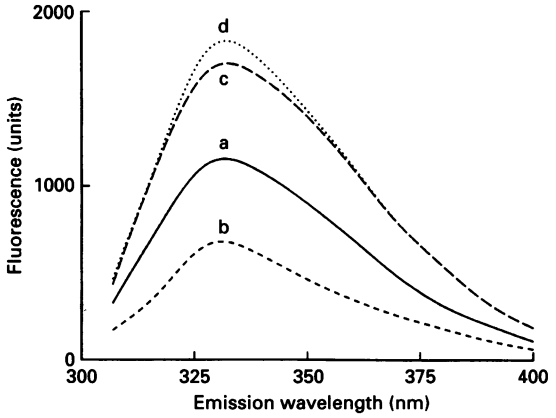

Figure 3 Fluorescence emission spectra of MDH and its complex with cytochrome c_L

Spectra were recorded at 25 °C in a 2 ml reaction mixture containing 5 mM Mops buffer (pH 7.0); excitation was at 295 nm. The concentration of MDH was 1 μM and that of cytochrome c_L was 2 μM. All spectra were corrected for inner and outer filter effects as described in the Materials and methods section. Curve a, MDH alone; curve b, cytochrome c_L alone; curve c, MDH + cytochrome c_L; curve d, theoretical curve obtained by adding curves a + b. The difference between curves c and d is due to quenching of the MDH fluorescence by the haem of cytochrome c_L.

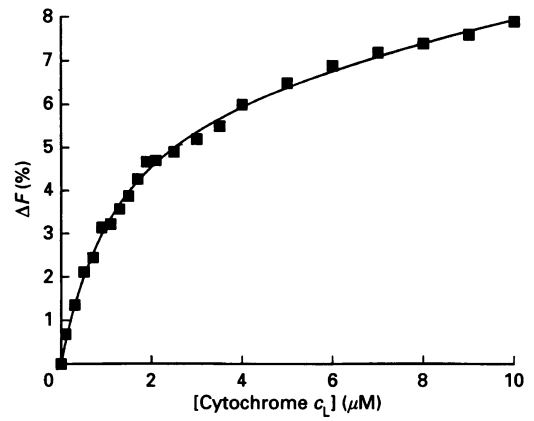

Figure 5 The effect of cytochrome c_L concentration on formation of the MDH–cytochrome c_L complex

The data for this figure are the same as those presented in Figure 4. The change in fluorescence on binding cytochrome c_L is plotted as a percentage of the total fluorescence after subtraction of that due to cytochrome c_L alone. The curve is the fit of the data to the equation: fluorescence change = (maximum fluorescence change × [cytochrome c_L])/(K_d + [cytochrome c_L]).

tophan fluorescence of MDH that occurs on binding to cytochrome c_L due to quenching by its haem prosthetic group (Figure 3); there was no effect when this cytochrome was replaced by equine cytochrome *c*. This specific quenching of MDH fluorescence by cytochrome c_L was exploited to obtain a measure of binding of the two proteins. Figures 4 and 5 show the effect of cytochrome c_L concentration on this fluorescence quenching; the K_d for binding of cytochrome c_L was 1.3 μM. Figure 6 shows the effect of salt on the fluorescence change due to bound cytochrome c_L: increasing the ionic strength to 0.05 increased the K_d about 20-fold. That the change in fluorescence depended upon binding, and not on the activity of MDH, was demonstrated by repeating this experiment with inactive MDH that lacked Ca^{2+}, purified from a mutant (*mxaA*); very similar binding was measured (K_d = 2.5 μM) when using the inactive enzyme, and this was also diminished by high ionic strength (Figure 7).

The effect of EDTA on electron transfer between MDH and cytochrome c_L

It is well established that EDTA inhibits methanol oxidation in whole cells of methylotrophic bacteria, without affecting the dye-linked activity of the enzyme [2,3,6]. Stopped-flow kinetic experiments have shown that inhibition is at the level of re-oxidation of the $PQQH_2$ by cytochrome c_L [15]. It has been suggested that EDTA and EGTA inhibit electron transfer from MDH to

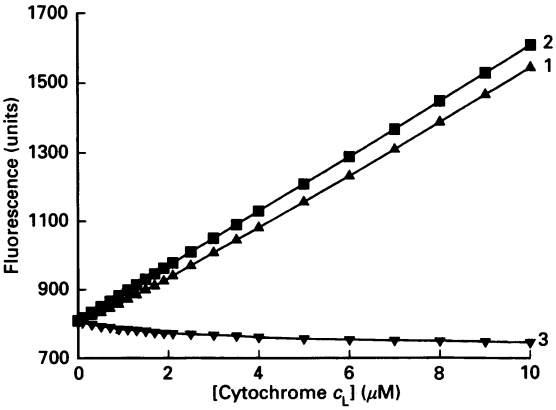

Figure 4 The change in fluorescence of MDH on binding to cytochrome c_L

Fluorescence was recorded in a 2 ml reaction mixture containing 5 mM Mops buffer (pH 7.0) containing MDH (1 μM) (excitation at 295 nm; emission at 330 nm). Cytochrome c_L was added from a stock of 100 μM to give the desired final concentration. Fluorescence was corrected for dilution effects and inner and outer filter effects as described in the Materials and methods section. Curve 1, fluorescence of MDH with added cytochrome c_L; curve 2, linear fluorescence curve calculated from fluorescence of cytochrome c_L plus MDH (1 μM); curve 3 = curve 1 minus curve 2. This difference curve is presented on a larger scale in Figure 5.

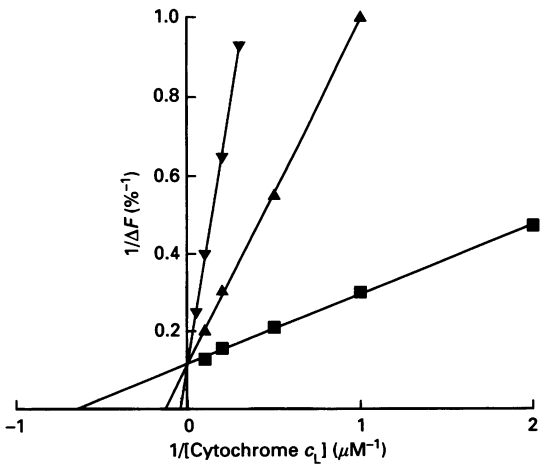

Figure 6 The effect of ionic strength on binding of cytochrome c_L to MDH

Assays were performed as described in Figures 4 and 5, at various concentrations of NaCl: ■, no NaCl (K_d = 1.5 μM); ▲, 25 mM NaCl (K_d = 7.5 μM); ▼, 50 mM NaCl (K_d = 22.5 μM).

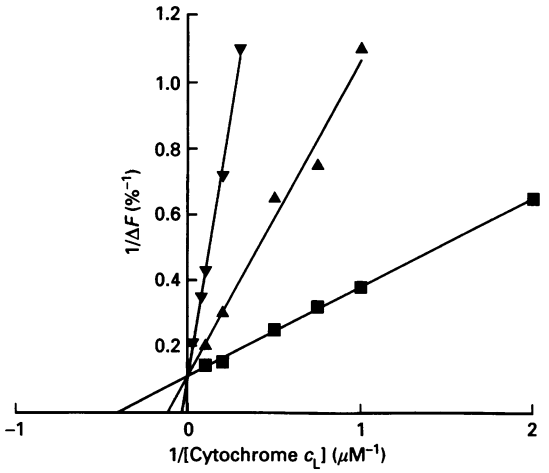

Figure 7 The effect of ionic strength on binding cytochrome c_L to inactive MDH isolated from the mutant *mxaA*

Assays were performed as described in Figures 4 and 5, at various concentrations of NaCl. ■, No NaCl (K_d = 2.5 μM); ▲, 25 mM NaCl (K_d = 8.5 μM); ▼, 50 mM NaCl (K_d = 29 μM).

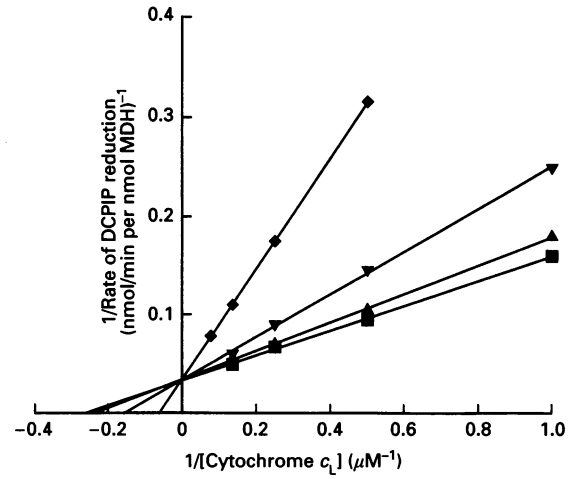

Figure 8 The effect of EDTA on the reduction of cytochrome c_L by methanol catalysed by MDH

The activity of MDH (2 μM) was measured in the assay system in which electrons pass from MDH to cytochrome c_L and thence to an excess of DCPIP, as described in the Materials and methods section. The assays were performed in Ca^{2+}-free Mops buffer (5 mM; pH 7.0) as described previously [13]. ■, No EDTA (apparent K_d = 3.8 μM); ▲, 0.5 μM EDTA (K_d = 4.3 μM); ▼, 1 μM EDTA (K_d = 6.4 μM); ◆, 2 μM EDTA (K_d = 16 μM).

cytochrome c_L by reacting with lysyl residues on MDH, thus preventing the initial docking process [18]. The results in Figure 8 appear to confirm this, EDTA being a competitive inhibitor of the overall process of electron transfer (K_i for EDTA = 1.75 μM). That the mechanism of action is not as straightforward as this, however, is indicated by the unexpected observation that relatively high concentrations of EDTA (1 mM) had no effect on the initial binding of MDH to cytochrome c_L, as shown by the fluorescence assay described above, whereas this concentration of EDTA completely inhibited electron transfer between methanol and the terminal electron acceptor.

DISCUSSION

The results described above confirm the conclusion that the docking between MDH and cytochrome c_L of *M. extorquens* involves ionic interactions, and that salts inhibit this initial binding process and not the subsequent electron transfer between the proteins.

It remains possible, however, that hydrophobic interactions, as suggested by Harris and Davidson [7] are also involved. PQQ is buried within an internal chamber in MDH, communicating with the exterior through a hydrophobic funnel-shaped depression on the surface. The shortest distance for electron flow between PQQ and the outside of the protein is likely to be by way of this funnel, and so hydrophobic interactions may also be essential to hold cytochrome c_L in position for electron transfer.

The failure of EDTA to inhibit binding of MDH to cytochrome c_L in the direct binding assay was unexpected, because binding of fluorescent EDTA analogues (Indo-1 and fura 2) to MDH has previously been demonstrated [18]; this has led to the conclusion that the potent competitive inhibition by EDTA is by competition for the lysyl residues on the surface of MDH that are involved in interaction with cytochrome c_L [18]. The results using the direct binding assays suggest, therefore, that EDTA does not inhibit methanol oxidation by preventing the initial binding of the proteins, but by inhibiting some subsequent interaction before electron transfer.

We suggest that the initial 'docking' of MDH and cytochrome c_L is by way of ionic interactions between lysyl residues on the surface of MDH and carboxylate groups on the surface of cytochrome c_L. This interaction is not inhibited by EDTA, which, we suggest, acts by binding to nearby lysyl residues, thus preventing movement of the 'docked' cytochrome to its optimal position for electron transfer, which probably involves interaction with the hydrophobic funnel in the surface of MDH. Such a separation of initial binding complex (by ionic interactions) and a subsequent electron transfer complex (involving hydrophobic interactions) has been demonstrated previously for the plastocyanin/cytochrome *c* interaction [19].

Until recently, there was no reason to think that the interactions of the electron transport proteins in *P. denitrificans* would differ from those in other methylotrophs. However, MDH and cytochrome c_H (also called cytochrome c_{550}) are unusual in both being acidic in *P. denitrificans* (pI, 3.7 [3] and 4.5 [2] respectively), whereas these proteins are usually basic [2,3]. The cytochrome c_L (also called cytochrome c_{551i} or cytochrome c_{552}) is similar to that in other methylotrophs in having a low pI (3.5) [20]. The interaction between MDH and cytochrome c_{551i} might therefore be unusual in that both proteins are acidic. In the first investigations of the interaction of MDH and its electron acceptor using the proteins from *P. denitrificans* [20,21] however, there was no evidence to suggest that the system is unusual, the rate of electron transfer being similar to that observed with these proteins from other bacteria and their affinity at low ionic strengths being very high (K_m = 0.8 μM) [20].

The conclusion of Harris and Davidson [7] that predominantly hydrophobic interactions are involved was therefore surprising, although inevitable because all their measurements were made at a high ionic strength (about 0.3) at which ionic interactions are likely to be negligible. These authors measured a K_d value of 375 μM at this ionic strength for cytochrome c_{551i} and concluded that this would give a satisfactory rate of electron transfer *in vivo* because the measured concentration of this cytochrome in the periplasm is about 0.5 mM [3]. However, the ionic strength in the periplasm will rarely be more than about 0.02, at which value we have shown that the K_d (or K_m) in all systems (including that of

P. denitrificans is about 5 μM [4,5,20]. It is possible that the apparent discrepancies between the conclusions for *P. denitrificans* and other methylotrophs is related to the fact that our measurement of electron transfer monitored the whole process from methanol to a terminal electron acceptor (either the dye DCPIP or cytochrome c_H) at relatively low protein concentrations, whereas those of Harris and Davidson [7] measured the electron transfer from reduced MDH to cytochrome c_{551i} by stopped-flow techniques using very high protein concentrations. A further investigation using MDH and cytochrome c_{551i} has now confirmed that ionic strength does have some effect on this system, and this has led Davidson and his colleagues to a similar conclusion ourselves: that is, after a non-optimal collision there is a rearrangement of the proteins to produce the most efficient orientation for electron transfer [22].

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