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Methanol:Cytochrome *c* Oxidoreductase Activity of Methylophs

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It has only recently been unequivocally demonstrated that pure methanol dehydrogenase (MDH) can catalyze the methanol-dependent reduction of pure cytochrome *c* (12, 33). At first glance this is surprising because MDH and its reaction with the electron transfer chain at the level of cytochrome *c* have been known for a considerable time (2, 7, 44). The importance of acquiring an understanding of the mechanism of electron transfer from the dehydrogenase is related to the fact that it contains a novel prosthetic group (pyrroloquinoline quinone [PQQ]) (21, 37, 43) and that it is the first enzyme to be described with the ability to catalyze the oxidation of an organic compound with the concomitant reduction of cytochrome *c*. Further work on this system is necessary, however, because the rates measured in vitro were much lower than those required to account for the in vivo respiration rates. This article attempts to review the relevant work on the unusual properties of MDH and cytochrome *c* which need to be taken into account in any such future investigations.

OXIDATION OF METHANOL BY WHOLE BACTERIA

Suspensions of methanol-grown bacteria rapidly oxidize methanol; this oxidation is inhibited by EDTA, *p*-nitrophenylhydrazine, and high concentrations of phosphate (2, 6). The point of entry of electrons from methanol into the electron transfer chain is at the level of cytochrome *c*, which is oxidized by cytochrome *o* or cytochrome *a/a₃*; the lower potential part of the chain, which consists of flavoproteins, quinones, and *b*-type cytochromes, is not involved (3-5). The oxidation of methanol is coupled to proton "translocation," generation of a proton motive force, and ATP synthesis (P/O ratio = 1) (16-19, 31, 32, 41). To achieve this the MDH and cytochrome *c* appear to react on the outer side of the periplasmic membrane, with oxygen being consumed by the oxidase on the inner side of the membrane (1, 30, 34).

It is relevant to mention at this point the system for oxidation of ethanol by *Acinetobacter calcoaceticus*. It has been said that in this organism ethanol is oxidized by a membrane-bound, PQQ-containing alcohol dehydrogenase which, except for its poor capacity for methanol

oxidation, is very similar to the MDHs of methylophs (22). The presence of this ethanol dehydrogenase in *A. calcoaceticus* appeared to argue against the possibility that all PQQ-containing alcohol dehydrogenases might necessarily react at the level of cytochrome *c*, because an essential characteristic of *Acinetobacter* species is their lack of this cytochrome. This conflict has now been resolved, however, because we have shown that this interesting alcohol dehydrogenase was not in fact from *A. calcoaceticus*, which has instead a conventional NAD⁺-linked alcohol dehydrogenase responsible for oxidation of ethanol. It transpired that the PQQ-containing alcohol dehydrogenase was isolated from an unidentified organism which contained cytochrome *c* and which has now been lost (10).

DYE-LINKED MDH

As mentioned above, MDH contains the novel prosthetic group PQQ which during the catalytic cycle also occurs as the reduced PQQH₂ and the free radical species PQQ· (or PQQH·) (21).

After extraction from the bacteria, the MDH is assayed by coupling the oxidation of methanol to the reduction of an artificial electron acceptor which is usually phenazine methosulfate, phenazine ethosulfate, or Würster blue. It has been suggested that the free radicals present in alkaline solutions of these acceptors are the true electron acceptors in the assay system (24, 26), a suggestion that is compatible with other evidence that oxidation of the enzyme occurs by way of one-electron steps (21). The pH optimum of the enzyme is always high (pH 9 or above), and ammonia is required as an activator, although some enzymes can use alternatives such as methylated amines or glycine esters (7, 9; J. A. Duine and J. Frank, Proc. Sheffield Symposium, 1980, p. 42). The dehydrogenase is not inhibited by EDTA, *p*-nitrophenylhydrazine, or high concentrations of phosphate (8, 23). A comparison of the pattern of inhibition of formaldehyde oxidation with that for methanol oxidation indicates that formaldehyde is not oxidized exclusively by way of MDH in whole bacteria despite the fact that this enzyme does oxidize formaldehyde (6, 38). This conclusion is supported by the properties of mutants lacking MDH (25, 29).

A remarkable property of MDH is that even after purification and extensive dialysis the enzyme catalyzes a rapid and unexpectedly large reduction of added artificial electron acceptors (7, 9, 24, 28). That this is not bound methanol has been shown by using the enzyme from *Methylophilus methylotrophus* (27); each dimeric molecule was shown to have 2 bound molecules of methanol (or formaldehyde) and, in addition, a further 90 molecules of an unidentified endogenous reductant. Incubation of the enzyme with phenazine methosulfate and ammonia in the absence of a substrate or alternative protecting molecules such as cyanide leads to oxidation of the endogenous reductant but always yields an inactive enzyme (20).

"FUNCTIONAL COUPLING" OF MDH TO CYTOCHROME *c*

Although it has been clear for some time from work with whole cells and membrane preparations that MDH is coupled to the electron transfer chain at the level of cytochrome *c* (4), it was impossible to demonstrate catalysis by pure MDH of electron transfer from methanol to the pure cytochrome. This was because when MDH and cytochrome *c* were purified together or, after separation, incubated together, cytochrome *c* was always found in the reduced state, although methanol was absent (2). When cytochrome *c* was then oxidized with ferricyanide, methanol-dependent cytochrome reduction could no longer be demonstrated (35). This result, observed with all MDHs studied, suggested that either some extra component is required for activity or that MDH or cytochrome *c* became damaged during extraction and thus are unable to react. This latter suggestion appeared to have been confirmed by the work of Duine and Frank (23), who used enzyme purified from *Hyphomicrobium* sp. strain X. Extracts prepared anaerobically and partially purified by passage through DEAE-Sephacel under anaerobic conditions contained both MDH and cytochrome *c*. After oxidation with ferricyanide, reduction of the cytochrome by methanol could be demonstrated by measuring difference spectra. The reaction occurred at pH 6.5 and was similar to the process of methanol oxidation by whole cells in that it did not require ammonia and was inhibited by EDTA or high phosphate concentrations. Reduction of the artificial electron acceptor (Würster blue) by the anaerobically prepared MDH at pH 9.0 did not require ammonia as an activator. After exposure of the "anaerobic preparation" to air for 1 h, ammonia became essential for dye reduction, and the cytochrome *c* in the preparation became oxidized and no longer reducible by the addition of methanol. This oxidation of cytochrome *c* was

unexpected because the same isolation procedure, when done under aerobic conditions, always yielded a preparation containing reduced cytochrome *c*.

We have obtained results very similar to these with *Hyphomicrobium* sp. strain X and some preparations of *Pseudomonas* sp. strain AM1 (D. T. O'Keeffe, Ph.D. thesis, University of Southampton, Southampton, U.K., 1980). Most preparations of *Pseudomonas* sp. strain AM1, however, differed in some important respects and behaved more like those that we have always obtained with preparations of *M. methylotrophus*; anaerobic preparations of this organism always required ammonia for activity in the dye-linked assay, and aeration did not lead to oxidation of cytochrome *c* (M. Beardmore-Gray, Ph.D. thesis, University of Southampton, Southampton, United Kingdom, 1982). To investigate this further, the reduction of cytochrome *c* was measured by using anaerobically prepared crude extracts containing MDH and cytochrome *c*. The rate of cytochrome reduction was greater in the presence of methanol than in its absence (Fig. 1). After aeration, the failure to demonstrate methanol-dependent reduction of cytochrome *c* was due to a stimulation in the rate of endogenous reduction up to the level occurring in the presence of methanol. Ammonia was not required for this reaction (either before or after aeration), and EDTA always inhibited the cytochrome *c* reduction (in the presence or absence of methanol).

In summary, the following can be stated. (i) Using anaerobic preparations, we demonstrated methanol-dependent cytochrome *c* reduction at pH 6.5 to 7.0 in all of the systems studied; this activity never required ammonia and was always inhibited by EDTA. (ii) Anaerobic preparations catalyzed methanol-dependent dye reduction, which did not always require ammonia. (iii) Aeration led to a changed MDH, but the nature of the change was not the same in all preparations. Thus, in extracts of *Hyphomicrobium* sp. strain X and in some preparations of *Pseudomonas* sp. strain AM1, ammonia-independent dye-linked activity was destroyed, and the cytochrome *c* became oxidized and no longer susceptible to reduction by methanol or endogenous reductant. By contrast, in aerated extracts of *M. methylotrophus* the cytochrome *c* remained in the reduced state and, after oxidation with ferricyanide, it was still reducible by MDH, with the endogenous rate being increased to that measured in the presence of methanol.

Perhaps the most important conclusion from these studies is that there is not always a clear relationship between the loss of functional coupling of MDH to cytochrome *c* and production of "classical," ammonia-requiring MDH. No

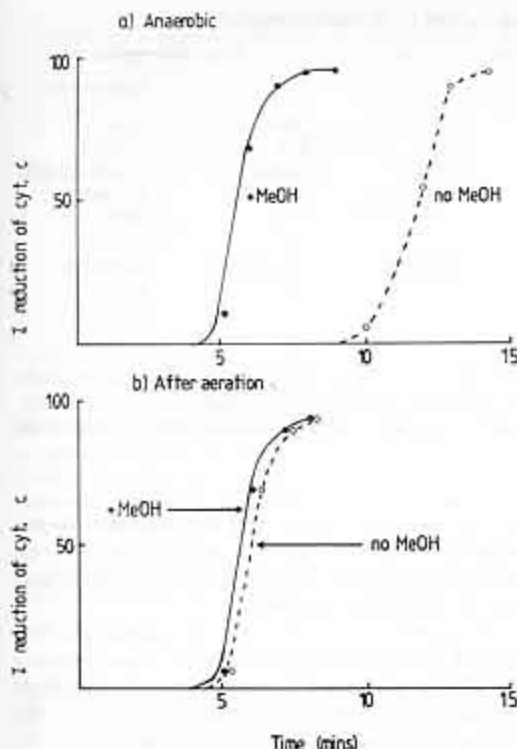


FIG. 1. Reduction of cytochrome *c* by MDH in anaerobically prepared extracts of *M. methylotrophus*. The reduction of cytochrome *c* was measured anaerobically (at 550 nm) by using crude extracts from *M. methylotrophus* containing MDH and cytochrome *c* (2.1 mg total protein) after oxidation with 0.5 μ mol of ferricyanide. The rate of reduction was 70 μ mol of cytochrome *c* reduced (μ mol of MDH) $^{-1}$ in the presence of methanol; this was twice the rate measured in its absence. After exposure to air for 20 min, the rate in the absence of methanol increased to the rate measured in its presence. It should be noted that what is actually measured in this type of experiment is the rate of ferricyanide reduction by the MDH-cytochrome "complex"; it is only after the ferricyanide has been completely reduced that the reduced cytochrome *c* starts to accumulate. The rate of reduction was directly proportional to the concentration of MDH present. When larger amounts of ferricyanide were used, the rates of the endogenous reaction were markedly diminished (to zero when sufficient was used), and the rates with added methanol also decreased to some extent. It appeared that the ferricyanide was destroying the MDH or cytochrome or both in the absence of partially protecting methanol. Similar experiments were done with *Hyphomicrobium* sp. strain X and *Pseudomonas* sp. strain AM1, but after aeration of *Hyphomicrobium* extracts and of some extracts of *Pseudomonas* sp. strain AM1, the cytochromes could no longer be reduced with methanol.

completely satisfactory explanation can be given, however, for the variety of observations described here. One possibility is that the enig-

matic endogenous reductant usually found on MDH may protect the enzyme from O_2 inactivation, with the extent of protection perhaps depending on the amount of reductant present on a particular dehydrogenase. It is probable that more complete conclusions will have to await further information on the various states of the PQQ prosthetic group occurring during enzyme catalysis and the effects of oxygen, phenazine methosulfate, ferricyanide, and ammonia on them.

CYTOCHROMES *c* OF METHYLOTROPHS

Having shown that MDH is able to catalyze the methanol-dependent reduction of cytochrome *c* in crude extracts, it is necessary to digress on the subject of the cytochromes themselves. The first point of importance to note is that all methylotrophs studied have at least two soluble cytochromes *c*. This has been shown in *Pseudomonas* sp. strain AM1 (36), *M. methylotrophus* (14), *Methylomonas* sp. strain J (33), *Paracoccus denitrificans* (40), strain 4025 (42), and *Hyphomicrobium* sp. strain X (Beardmore-Gray, thesis). Those described in most detail (from *Pseudomonas* sp. strain AM1 and *M. methylotrophus*) are similar in many important respects (Table 1): they have a single polypeptide chain, a single heme with histidine and methionine as the two axial ligands, a high midpoint redox potential (E_m greater than 250 mV), and characteristic reduced α -bands at about 550 nm, and they are low spin in both oxidized and reduced states.

For convenience we labeled the two cytochromes according to their isoelectric points, cytochrome c_H having the higher isoelectric point and cytochrome c_L the lower. It should be noted, however, that although obviously applicable to the *c* cytochromes from *Pseudomonas* sp. strain AM1, *M. methylotrophus*, and *Hyphomicrobium* sp. strain X, because their isoelectric points differ by about 4 pH units, this distinction is not so marked for the cytochromes from *Methylomonas* sp. strain J and *Paracoccus denitrificans*. Although their cytochromes *c* with the lower isoelectric points appear to correspond to the cytochromes c_L of the other methylotrophs in function, the differences in their isoelectric points are not so great, and both are below pH 7.0.

The difference in isoelectric points between cytochromes c_H and c_L is not due to differences in lysine content but probably due to differences in the extent of amidation of aspartate and glutamate (Table 1). The division of the two types of cytochrome *c* according to isoelectric points correlates with differences in many other characteristics (Table 1); cytochrome c_L is larger, has a lower isoelectric point and a lower

conversion of aspartic acid
to amide.

TABLE 1. Properties of cytochromes c_H and c_L of methylotrophs^a

Property	<i>Pseudomonas</i> sp. strain AM1		<i>M. methylotrophus</i>	
	Cytochrome c_H	Cytochrome c_L	Cytochrome c_H	Cytochrome c_L
Relative proportions in crude extracts	72%	28%	50%	42%
Isoelectric point	8.8	4.2	8.9	4.0-4.4
Mol wt	11,000	20,900	8,500	17,000-21,000
Redox potential [Em ₇]	294 mV	256 mV	373 mV	310 mV
%CO binding	36%	72%	7%	60%
No. of cysteine residues per molecule	2	3	2	3
Sensitivity to proteolytic digestion	Insensitive	Sensitive	Insensitive	Sensitive
No. of lysine residues per molecule	11	13	11	12
Stimulation of autoreduction by MDH	+	+	-	+
Electron acceptor for MDH	-	+	-	+

^a *M. methylotrophus* also contains a third soluble cytochrome c constituting 8% of the total (14). The material in this table is taken from references 11, 12, 14, and 36. After purification, cytochrome c_L from *M. methylotrophus* is in two forms, one of which may arise by the loss of a 4,000-dalton fragment from the other (14).

midpoint redox potential, reacts more completely with CO, has an extra cysteine residue, and is the most sensitive to digestion by proteinases. Cytochrome c_H , by contrast, has a higher isoelectric point, a higher midpoint redox potential, a lower molecular weight, and only two cysteine residues, binds less well to CO, and is relatively insensitive to proteolytic digestion.

An analysis of the amino acid composition and the products of partial proteolytic hydrolysis showed that cytochrome c_H from *Pseudomonas* sp. strain AM1 is very similar to that from *M. methylotrophus* and that the two cytochromes c_L are also similar to each other (11). There is, however, little similarity between the cytochromes c_H and cytochromes c_L . These studies also showed that one type of cytochrome c is not a dimer or degradation product of the other.

Having described the two types of cytochrome c found in methylotrophs, we must now consider whether or not they have any unusual features that might relate to special functions. Two such features might be their reactivity with CO and their autoreducibility at high pH values. The reaction of the methylotroph cytochromes c with CO (albeit slow and incomplete) has led to their being referred to as cytochrome c_{CO} . We have avoided this terminology because it sometimes tends to be associated with an assumed physiological oxidase or oxygenase function, for which there is no evidence (4, 15, 44). It is probable that the reaction with CO merely reflects the structure around the heme pocket that allows a more readily dissociable iron-methionine bond. That the heme environment might be slightly unusual is perhaps indicated by the unusual response of the midpoint redox potential of the cytochromes c of *Pseudomonas* sp. strain AM1 to changing pH (36). Both cytochromes have two ionizing groups affecting the redox potentials, with pK values of 3.5 and 5.5

in the oxidized forms and 4.5 and 6.5 in the reduced forms; the two propionate residues on the heme itself are the most obvious candidates for the dissociating groups.

A second, more unusual, characteristic feature of the cytochromes c of both *Pseudomonas* sp. strain AM1 and *M. methylotrophus* is their capacity for rapid autoreduction (12, 35). Although this characteristic is responsible for some of the problems in studying MDH-cytochrome c interactions it may also be a clue to the mechanism of these interactions. Autoreduction is the reduction of the heme iron of ferricytochrome c occurring in the absence of an added reducing agent. This phenomenon occurs in horse heart cytochrome c (13), but we have shown that it occurs at about 100 times the rate in the methylotroph cytochromes c (12). The autoreduction process is a first-order intramolecular reaction occurring at high pH values; the pK for this process is >10. It has been proposed (35) that a weakly acidic group (XH) dissociates at high pH to give a negatively charged species capable of donating an electron to the heme, with the free radical produced by this process being stabilized by sharing an electron with the heme iron (Fig. 2).

AUTOREDUCTION OF CYTOCHROME c INDUCED BY MDH

When pure MDH is mixed with pure oxidized cytochrome c , the cytochrome becomes rapidly reduced, the presence of substrate methanol being irrelevant to this reaction (12, 35). From what has been stated above, one might think that we are once again observing electron transfer from the mysterious endogenous reductant to cytochrome c , but this does not appear to be the case. The "reduction" by MDH is a first-order reaction with respect to ferricytochrome c , indi-

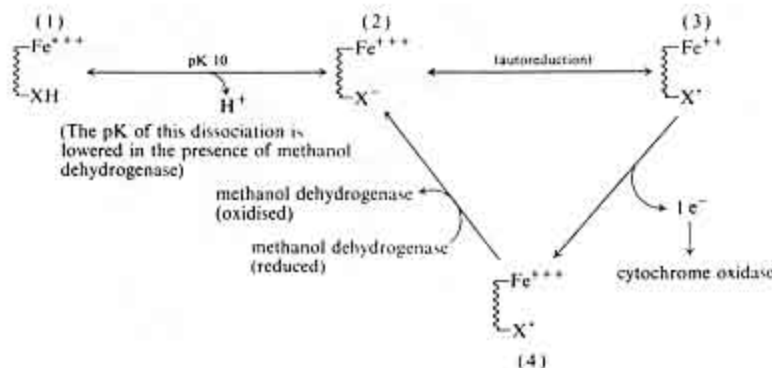


FIG. 2. A speculative mechanism for the reduction of cytochrome *c* by MDH (12, 35). In this scheme the electron donor for autoreduction is a weakly acidic group (XH) on the cytochrome that dissociates to give a negatively charged species able to donate an electron to the heme. Species 1 is the undissociated ferricytochrome *c* which becomes dissociated in the presence of MDH (or at high pH) to species 2. Species 3 is the radical complex of ferrous iron isoelectronic with the ferric species 2. Species 4 is the ferric form of the radical and can only be formed in the presence of an added electron acceptor; this would be cytochrome oxidase (or perhaps cytochromes c_{11}) in physiological conditions, ferricyanide (Fig. 1), or mammalian cytochrome *c* (Fig. 3). Species 4 can be reduced to species 2 to complete the cycle by electron transfer from MDH, the electrons arising from methanol or from the endogenous reductant.

cating an intramolecular oxidation-reduction reaction exactly as proposed for the autoreduction of this cytochrome. Autoreduction at high pH is initiated by dissociation of a proton from a group on the oxidized cytochrome (Fig. 2); all of our data are consistent with the conclusion that the addition of MDH to ferricytochrome c_L lowers the pK for this initial dissociation, thus allowing the intramolecular autoreduction to occur at a lower pH (pH 7.0) than it otherwise would (12, 35).

METHANOL:CYTOCHROME *c* OXIDOREDUCTASE ACTIVITY OF METHYLOTROPHS

It is clear from the above discussion that there are at least three independent problems to be overcome in demonstrating methanol-dependent cytochrome reduction by MDH. The first is the instability of MDH during purification (possibly related to the oxygen sensitivity discussed earlier); the second is the presence of the endogenous reductant on the MDH, and the third is the MDH-induced autoreduction of cytochrome *c*. The first of these problems was avoided by using MDH from *M. methylotrophus* which was protected during aerobic preparation by using KCN, a substrate analog. The other problems have been overcome by devising a system in which the bacterial cytochrome *c* is oxidized by coupling to a large excess of mammalian cytochrome *c*.

When MDH and mammalian cytochrome *c* were mixed, the cytochrome remained oxidized (Fig. 3). Addition of a small amount of cyto-

chrome c_L led to reduction of the mammalian cytochrome *c* (present in 50-fold excess over the cytochrome c_L), presumably due to the oxidation of endogenous reductant on the MDH. Added mammalian cytochrome oxidase then rapidly oxidized all of the ferrocyclochrome *c*, after which KCN was added to inhibit the oxidase. Addition of methanol then led to the complete reduction of all of the cytochrome *c* present; the final rate of this methanol-dependent reduction was directly proportional to the concentration of MDH. The rate of reaction was independent of pH between pH 7.0 and 9.0; as in whole cells, ammonia had no effect on the rate of reduction, and EDTA completely inhibited it. Cytochrome c_L was essential for the reaction and could not be replaced with cytochrome c_H . Using a large excess of cytochrome c_H instead of mammalian cytochrome *c*, we showed that electrons could only pass from MDH to cytochrome c_H when cytochrome c_L was also present as a mediator.

The initial rates of reduction were shown to obey hyperbolic kinetics with respect to the concentration of cytochrome c_L . The K_m was 1.2 μM , and the $V_{\max}$ was 1.5 nmol of cytochrome reduced min^{-1} nmol of MDH $^{-1}$. These values were the same during the endogenous reduction observed in the first part of the experiment as those observed after the addition of methanol (Fig. 3). That is, the $V_{\max}$ and K_m values for cytochrome c_L were independent of the substrate responsible for the initial reduction of MDH. It should be emphasized that the reduction of mammalian cytochrome *c* by en-

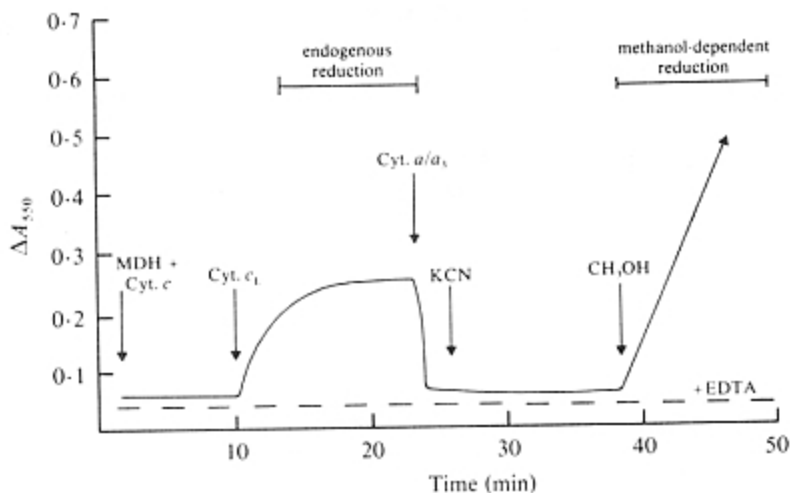


FIG. 3. Demonstration of methanol-dependent reduction of cytochrome *c* by MDH (reproduced with permission, from reference 12). This experiment is fully described in the text. The following concentrations were used: MDH, 3.5 μ M; horse heart cytochrome *c* (Cyt. *c*), 50 μ M; cytochrome *c*_L (Cyt. *c*_L), 0.8 μ M; cytochrome oxidase (Cyt. *a/a*₃), 0.24 μ M; KCN, 0.5 mM; methanol, 7.5 mM; morpholinepropanesulfonic acid buffer (pH 7.0), 25 mM.

dogenous reductant described here does involve electron transfer from MDH; it is not the same as the autoreduction of cytochrome *c*_L stimulated by MDH described in the previous section of this review. No endogenous reduction occurred when cytochrome *c*_H was used instead of cytochrome *c*_L.

After growth on methanol, *M. methylotrophus* contained a blue copper protein probably similar to that found in *Methylomonas* sp. strain J (39; Beardmore-Gray, thesis). This protein (partially purified) could not replace cytochrome *c*_L in the system described in Fig. 3, and it had no effect on methanol:cytochrome *c*_L oxidoreductase activity.

Table 2 summarizes the results of an investigation of the specificity of the cytochromes *c* as electron acceptors for various MDHs. No cytochrome *c* from a non-methylotroph was active, and of the two soluble *c* cytochromes found in each methylotroph, only one was able to accept electrons from MDH with concomitant production of formaldehyde. This was cytochrome *c*_L from *M. methylotrophus* and *Pseudomonas* sp. strain AM1 and one of the cytochromes *c* from *Paracoccus denitrificans* which was presumably the extra cytochrome *c* induced during growth on methanol (40).

It is of particular importance to note that the methanol:cytochrome *c* oxidoreductase activity demonstrated here with completely pure proteins was inhibited by EDTA, as is the case in whole cells. The obvious target to consider for EDTA action is a divalent cation, perhaps involved in binding MDH and cytochrome together.

er, but we have no evidence to support such a mechanism; the proteins were purified in the absence of divalent cations, and these had no effect when added to the oxidoreductase assay system. Preliminary experiments have shown that the reversible competitive inhibition occurs at very low concentrations of EDTA (K_i of <20 μ M), which acts by increasing the K_m of MDH for cytochrome *c*_L. These results suggest that the EDTA reversibly binds to a site on either MDH or cytochrome *c*_L crucial for formation of an active complex. If the carboxyl groups of the PQQ prosthetic group are involved in binding MDH to cytochrome *c*_L, then it is possible that EDTA, whose four carboxyl groups can take up many configurations, may bind to the same site on the cytochrome.

PRODUCTS OF METHANOL:CYTOCHROME *c* OXIDOREDUCTASE ACTIVITY

Because of the problems arising from the endogenous reductant on MDH, and the MDH-induced autoreduction of cytochrome *c*, it was of interest to determine the stoichiometry of the oxidation of methanol to product(s) during cytochrome *c*_L reduction. To achieve this the MDH from *M. methylotrophus* was purified, using KCN instead of methanol as a protective agent during purification, thus ensuring that bound methanol was absent or kept to a minimum. When MDH was incubated with methanol, cytochrome *c*_L, and excess mammalian cytochrome *c*, formaldehyde was immediately produced, indicating that the added methanol was used in preference to any endogenous reductant (Fig. 4).

Upd. the Ca²⁺ in cytochrome c

TABLE 2. The specificity of cytochromes *c* from methylotrophs as electron acceptors for various MDHs (12)^a

Source and cytochrome	Reduction of cytochromes by MDH from the following bacteria:		
	<i>M. methylotrophus</i>	<i>Paracoccus denitrificans</i>	<i>Pseudomonas</i> sp. strain AM1
<i>M. methylotrophus</i>			
Cytochrome <i>c</i> _H (pI 8.9)	—	—	—
Cytochrome <i>c</i> _L (pI 4.4)	+	—	—
<i>Paracoccus denitrificans</i>			
Cytochrome <i>c</i> (pI, acidic)	—	—	—
Cytochrome <i>c</i> (pI, more acidic)	—	+	—
<i>Pseudomonas</i> sp. strain AM1			
Cytochrome <i>c</i> _H (pI 8.8)	—	—	—
Cytochrome <i>c</i> _L (pI 4.2)	+	+	+

^a MDH (2 μ M) and mediating cytochrome *c* (2 μ M) were incubated with horse heart cytochrome *c* (500 μ M) in the presence of excess methanol (7.5 mM). The reaction mixture was incubated for 12 h and assayed for reduction of cytochrome *c* and production of formaldehyde. Reactions with MDH from *M. methylotrophus* and *Paracoccus denitrificans* were measured at pH 7.0 in the absence of NH_4Cl , whereas reactions with the MDH from *Pseudomonas* sp. strain AM1 were measured at its pH optimum (pH 9.0) in the presence of NH_4Cl , which stimulated the rate fourfold. The isoelectric points (pI values) of the dehydrogenases from *Paracoccus denitrificans*, *Pseudomonas* sp. strain AM1, and *M. methylotrophus* were 3.7, 8.8, and >8.0, respectively. This information is provided to show that there is no clear pattern of reaction between the cytochromes and dehydrogenases merely in terms of their isoelectric points. No MDH was able to reduce horse heart cytochrome *c* (pI 10.4) or the cytochrome *c*₅₅₁ (pI 4.7) from the non-methylotroph *Pseudomonas aeruginosa*. Symbols: +, methanol-dependent reduction of cytochrome *c* with concomitant production of formaldehyde; —, no methanol-dependent reduction of cytochrome *c* or production of formaldehyde.

In the absence of methanol, no formaldehyde was produced, although there was considerable reduction of the cytochrome *c* (Fig. 4). As most of the cytochrome present in these experiments was mammalian cytochrome *c*, this endogenous reduction cannot have been due to MDH-induced autoreduction of cytochrome *c*_L; it must

have been due to oxidation of an endogenous reductant which could not have been methanol or formaldehyde.

Formaldehyde was also oxidized by MDH coupled to cytochrome *c*_L, the rate of oxidation of the two substrates being similar (Fig. 5). The stoichiometry was two molecules of cytochrome reduced per molecule of formaldehyde oxidized, and this value was constant throughout the reaction. After the complete disappearance of formaldehyde, the cytochrome became further reduced. The extent of this extra reduction was the same as the reduction occurring in the absence of added substrate, confirming that added substrate is oxidized in preference to endogenous substrate. These results with formaldehyde explain the variable stoichiometries observed during methanol oxidation. The ratio of molecules of cytochrome reduced per molecule of formaldehyde measured increased throughout the experiment, from an initial value of 2, because some methanol was oxidized completely to formate during the reaction (Fig. 4).

It has been known for some time that MDH can catalyze the oxidation of methanol to formate in the dye-linked assay (24, 38). That it can do so in the cytochrome-coupled system is of some physiological importance. As formalde-

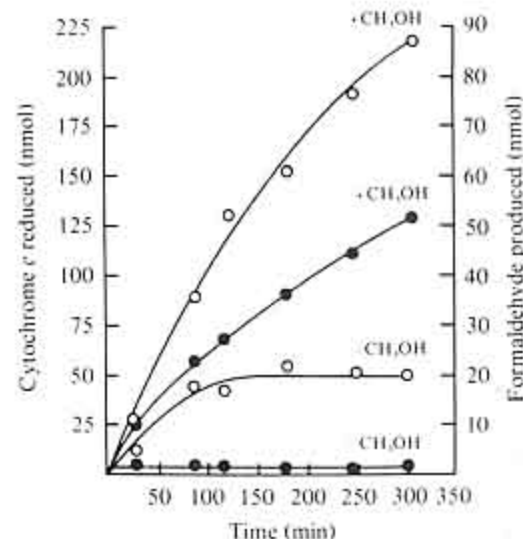


FIG. 4. Production of formaldehyde during methanol oxidation by methanol:cytochrome *c* oxidoreductase (reproduced with permission, from reference 12). Reaction vials (3 ml) contained mammalian cytochrome *c* (500 μ M) and MDH (2 μ M) in the presence of excess methanol (3 mM) in 25 mM morpholinepropanesulfonic acid buffer, pH 7.0. The reaction was initiated by the addition of cytochrome *c*_L (2 μ M). At various times samples (500 μ l) were withdrawn and assayed for the extent of reduction of cytochrome *c* (○) and for formaldehyde production (●).

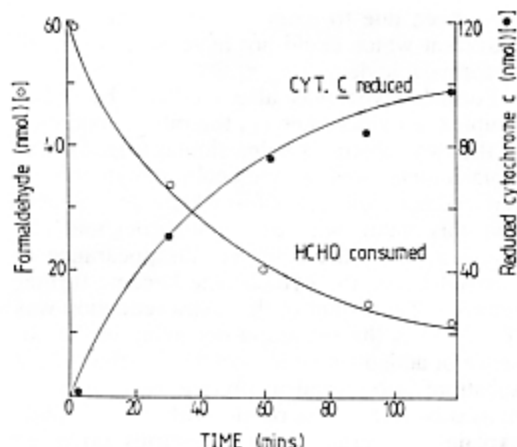


FIG. 5. The formaldehyde:cytochrome c oxidoreductase activity of MDH (12). The reaction mixture was as described in the legend to Fig. 4, except that methanol was replaced by formaldehyde (60 μ M).

hyde is required for assimilation, there must be some regulation of the methanol:cytochrome c oxidoreductase activity with respect to formaldehyde oxidation. In addition to this, there is the further problem of transport of the formaldehyde into the cell from the periplasmic space.

DISCUSSION

Having demonstrated that pure MDH can catalyze methanol-dependent cytochrome c_L reduction with concomitant product formation, it is necessary to consider how this finding relates to the oxidation of methanol *in vivo*. In this context the most important point that must be stressed is that the rates of *in vitro* electron transfer are far too low (less than 1%) to account for the rates of electron transfer in whole bacteria. That the *in vitro* oxidoreductase activity is not completely spurious is indicated by the specificity with respect to both cytochrome c and MDH and by its inhibition by EDTA, the well-established inhibitor of methanol oxidation in whole bacteria. We consider that the marked discrepancy between the *in vivo* rates of respiration and the activity of the purified and reconstituted system may be related to the high concentrations of MDH and cytochrome c found in methylotrophs. In *M. methylotrophus*, for example, the concentration of both of these proteins is ca. 0.5 mM, assuming that the periplasmic volume is ca. 20% of the cell volume and that all of the cytochrome c_L and MDH is periplasmic (30; Beardmore-Gray, thesis). Furthermore, all of the cytochrome c_L and MDH will be in the associated form most of the time because the K_m of MDH for cytochrome c_L is

very low. It is possible that the two proteins completely cover most of the outer surface of the bacterial membrane, and the marked change in environment occurring on their release during cell disruption may contribute to their low activity when measured in the reconstituted system.

A second point to consider is the relationship between the MDH-induced autoreduction of cytochrome c_L and the methanol:cytochrome c_L oxidoreductase activity of MDH. In this context, the scheme in Fig. 2 provides a mechanistic model that incorporates three key observations. (i) Cytochrome c_L is autoreducible, obeying first-order kinetics. (ii) In the absence of an added electron acceptor, the reduction of cytochrome c_L by MDH obeys first-order kinetics with respect to the oxidized cytochrome c_L and does not require added methanol. (iii) In the presence of an added electron acceptor (mammalian cytochrome c , ferricyanide, or oxidase) the rate-limiting step in the reaction is the reduction of cytochrome c_L by MDH. The simplest interpretation of these observations is that in the absence of an added electron acceptor no electron transfer occurs between MDH and cytochrome c_L , the sole effect of the MDH being to stimulate autoreduction by lowering the pK of the dissociating group on the cytochrome. In the presence of an added electron acceptor, the autoreduced cytochrome species is able to donate an electron to the acceptor, thus generating an oxidized form of the cytochrome which can be subsequently reduced to the original species with electrons derived from the oxidation of either methanol or the endogenous reductant.

This model allows us to make the following predictions which must be tested to raise the model from speculation to mechanism. (i) A free radical, due to the cytochrome c , should be detectable during the reaction with MDH. (ii) The rate of reduction of the external electron acceptor is unlikely to be greater than the rate of MDH-induced autoreduction. (iii) In the presence of methanol, but in the absence of electron acceptor, no formaldehyde should be produced during the reduction of cytochrome c_L by MDH.

Finally, it is worth considering the following questions, now amenable to investigation, that must be answered to obtain a more complete picture of methanol oxidation by methylotrophs. What is the function of cytochrome c_H , and is the demonstrated electron flow between this cytochrome and cytochrome c_L physiologically significant? Does cytochrome c_L accept electrons from other substrates such as cytochrome b , and are soluble and membrane-bound cytochromes c_L identical, with identical functions? Is cytochrome c_L oxidized directly by terminal oxidases, and if so, is cytochrome c_L released from MDH before reacting with the oxidase?

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