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The Methanol: Cytochrome *c* Oxidoreductase Activity of Methylotrophs

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Both the soluble cytochromes *c* of the obligate methylotroph *Methylophilus methylotrophus* were rapidly autoreducible at high pH. The intramolecular autoreduction mechanism was also involved in the reduction of the cytochrome c_L by methanol dehydrogenase which occurred in the absence of methanol. Pure soluble methanol dehydrogenase was shown to be able to catalyse the methanol-dependent reduction of pure cytochrome *c* from *M. methylotrophus* and from the facultative methylotroph *Pseudomonas* AM1 by coupling oxidation of the bacterial cytochrome to the reduction of a large excess of mammalian cytochrome *c*. Only one of the two cytochromes *c* (cytochrome c_L of each organism) could react with methanol dehydrogenase to give methanol: cytochrome *c* oxidoreductase activity. This activity, using proteins from *M. methylotrophus*, was independent of pH between pH 7·0 and 9·0 and ammonia was not required. By contrast, the pH optimum for the system from *Pseudomonas* AM1 was 9·0 and activity was stimulated about fourfold by NH_4Cl. The product of methanol oxidation was formaldehyde, which was also a substrate for the oxidoreductase system. During formaldehyde oxidation two molecules of cytochrome *c* were reduced for every molecule of formaldehyde oxidized. In a survey of methanol dehydrogenases and cytochromes *c* from *Pseudomonas* AM1, *M. methylotrophus* and the facultative autotroph *Paracoccus denitrificans*, it was shown that, of the two soluble cytochromes *c* found in each methylotroph only one was able to react with methanol dehydrogenase. The cytochrome c_L from *M. methylotrophus* and the cytochrome *c*(2) of *Pa. denitrificans* were specific, only reacting with methanol dehydrogenase from the same organism, whereas the cytochrome c_L of *Pseudomonas* AM1 reacted with all three methanol dehydrogenases tested.

INTRODUCTION

The oxidation of methanol by methylotrophic bacteria involves an unusual quinoprotein methanol dehydrogenase (Duine & Frank, 1981; Anthony, 1982*a*). When purified, this enzyme has a pH optimum of about 9·0; it requires ammonia as activator and an artificial electron acceptor such as phenazine methosulphate. Work with whole organisms and membrane preparations indicates that electrons are donated by methanol dehydrogenase to the electron transport chain at the level of cytochrome *c* (Anthony, 1981, 1982*b*). Methylotrophs usually have at least two soluble cytochromes *c* and some of these have been completely purified and characterized (Cross & Anthony, 1980*a*; O'Keeffe & Anthony, 1980*a, b*; Ohta & Tobari, 1981; Anthony, 1982*b*; Beardmore-Gray *et al.*, 1982). Although indirect evidence suggests that the methanol dehydrogenase may react directly with one of the cytochromes *c* as natural electron acceptor, it has not been possible to demonstrate this unequivocally with the purified proteins. This is because in previous experiments, using completely pure proteins from *Pseudomonas*

Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulphonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulphonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; MOPS, 3-(*N*-morpholino)propanesulphonic acid; PMS, phenazine methosulphate.

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AM1, it was found that addition of methanol dehydrogenase to cytochrome *c* leads to reduction of the cytochrome even in the absence of methanol (O'Keeffe & Anthony, 1980*b*). This appears to be due to an effect of the enzyme on the autoreduction of cytochrome *c*. Autoreduction is the reduction occurring in the absence of added reductant which occurs at high pH due to an intramolecular electron transfer within the cytochrome. It has been proposed that the dehydrogenase (in the absence or presence of methanol) lowers the pK for the necessary initial dissociation of the cytochrome, hence permitting the autoreduction to occur at a relatively low pH (pH 7·0) (O'Keeffe & Anthony, 1980*b*; Anthony, 1982*b*).

The present paper shows that the methanol dehydrogenase and cytochrome *c* of the obligate methylotroph *Methylophilus methylotrophus* interact in a similar way to those from the facultative methylotroph *Pseudomonas* AM1. Using completely pure proteins from both these methylotrophs the problem outlined above has now been overcome and a clear demonstration of methanol-dependent cytochrome *c* reduction catalysed by methanol dehydrogenase has been achieved, this reduction occurring concomitantly with production of formaldehyde from methanol.

A brief description of methanol:cytochrome *c* oxidoreductase activity in *Methylomonas* J, similar to that described below, has recently been published (Ohta & Tobari, 1981).

METHODS

Chemicals. All chemicals were obtained from BDH, except for the following: Sephadex gel filtration media, from Pharmacia; CM-cellulose and DEAE-cellulose, from Whatman; PMS, phenazine ethosulphate, horse heart cytochrome *c* (Type III) and zwitterionic buffers, all from Sigma. The following buffers were used in all the experiments described in this paper: MOPS, pH 6·5–7·9; HEPES, pH 6·8–8·2; CHES, pH 9·0–10·1; CAPS, pH 9·7–11·1. Mammalian cytochrome oxidase (cytochrome a/a_3) was prepared from beef heart by the method of Capaldi & Hayashi (1972) and was a gift from R. J. Froud of this Department. *Pseudomonas aeruginosa* cytochrome c_{551} was a generous gift from Drs C. Greenwood and D. Barber of the University of East Anglia, U.K.

Organisms and growth conditions. Pseudomonas AM1 (NCIB 9133), *Paracoccus denitrificans* (NCIB 8944) and *Methylophilus methylotrophus* (NCIB 10515) were obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, U.K. Growth methods for *Pseudomonas* AM1 and *M. methylotrophus* have been described previously (O'Keeffe & Anthony, 1980*b*; Cross & Anthony, 1980*a*). *Pa. denitrificans* was grown under identical conditions to those described for *Pseudomonas* AM1 growing in batch culture.

Measurement of absorption spectra, autoreduction of cytochrome c and reduction of cytochromes c by methanol dehydrogenase. These methods have been described previously by O'Keeffe & Anthony (1980*b*).

Purification of cytochromes and methanol dehydrogenase from Pseudomonas AM1 and M. methylotrophus. The purification of the cytochromes *c* and methanol dehydrogenase from *Pseudomonas* AM1 and the cytochromes *c* from *M. methylotrophus* were essentially the same as those previously described (Cross & Anthony, 1980*a*; O'Keeffe & Anthony, 1980*a*) except that the cell extracts were not acid-treated. For purification of active methanol dehydrogenase from *M. methylotrophus* it was necessary to include 25 mM-methanol or 25 mM-KCN in all buffers. When using KCN the final step in the purification process was a second step using DEAE-cellulose ion-exchange chromatography. The enzyme was stored at −17 °C. Prior to use the methanol and KCN were removed from the enzyme by dialysis for 12 h against 60 volumes of 25 mM-Tris/HCl, pH 8·0. Protection by methanol or KCN was not essential for the enzyme from *Pseudomonas* AM1. In this case, however, it was necessary to use the dehydrogenase within 48 h of purification and it had to be stored on ice at 0 °C and not frozen at −17 °C. If these precautions were not taken the endogenous reduction of horse heart cytochrome *c* catalysed by the enzyme in the presence of cytochrome c_L increased to a very high level and methanol-dependent reduction of cytochrome could not then be demonstrated.

Purification of methanol dehydrogenase and partial purification of soluble cytochromes c from Pa. denitrificans. Methanol-grown bacteria (20 g wet weight) were suspended in 50 ml 20 mM-Tris/HCl buffer, pH 8·0, disrupted by sonication, and whole cells and membranes removed by centrifugation at 38000 *g* for 2 h. The resulting supernatant was applied to a DEAE-cellulose column (9 × 3 cm) equilibrated with 20 mM-Tris/HCl containing 0·1% methanol. The cytochromes *c* and methanol dehydrogenase were eluted using a linear gradient of 20–500 mM-Tris/HCl, pH 8·0. The methanol dehydrogenase eluted at 250 mM-Tris/HCl and two cytochromes *c* eluted at 300 mM and 350 mM-Tris/HCl, respectively. The first to be eluted is referred to as cytochrome *c*(1) and the second as cytochrome *c*(2). Methanol dehydrogenase was applied to an upward-flow Sephadex G-150 column equilibrated in 100 mM-Tris/HCl, pH 8·0, containing 0·1% methanol. It was then dialysed against 20 mM-Tris/HCl, pH 8·0, containing 0·1% methanol, and applied to a column of DEAE-cellulose equilibrated in the same

buffer. After washing with 200 mM-Tris/HCl the dehydrogenase was eluted with 250 mM-Tris/HCl. The dehydrogenase appeared to be completely pure; it had a typical methanol dehydrogenase absorption spectrum, the ratio of the 280 nm absorbance to the 345 nm absorbance being 11·0. Its specific activity was 70 nmol O_2 reduced min^{-1} (mg protein)$^{-1}$ when measured in the O_2 electrode.

Of the two cytochromes *c* eluted from the DEAE-cellulose column, 70% was cytochrome *c*(1) and 30% was cytochrome *c*(2); the total soluble cytochrome *c* at this stage was 200 nmol. Each cytochrome was purified using the same methods as described above for the methanol dehydrogenase. Cytochrome *c*(1) was eluted from the DEAE-cellulose with 250 mM-Tris/HCl and cytochrome *c*(2) eluted with 400 mM buffer. The cytochromes *c* were not completely pure. The ratio of absorbance at 550 nm to that at 280 nm was 0·5 for cytochrome *c*(1) and 0·3 for cytochrome *c*(2).

Measurement of methanol-dependent reduction of cytochrome c by methanol dehydrogenase from Pseudomonas AM1 and M. methylotrophus. The extent of endogenous reduction of the cytochromes c_L by the methanol dehydrogenases depended on the amount of dehydrogenase present. The endogenous reduction measured with the enzyme from *Pseudomonas* AM1 was always much greater than that with the enzyme from *M. methylotrophus*. In order to measure a high rate of methanol-dependent reduction it would have been necessary to use a high concentration of dehydrogenase with a prohibitively high concentration of cytochrome c_L, which was only available in small quantities. To overcome this problem the cytochrome c_L was recycled by coupling to commercially available horse heart cytochrome *c*. Full details of the concentrations of reactants used are given in the legend to Fig. 2, in which an experiment using proteins from *M. methylotrophus* is described. A similar system was used for reactions using proteins from *Pseudomonas* AM1, except that the buffer used was 25 mM-CAPS, pH 9·0, and 15 mM-NH_4Cl was also included. For determination of the kinetic constants for reduction of cytochrome c_L by methanol dehydrogenase, incubation mixtures were as described for Fig. 2. The concentrations of methanol dehydrogenase used were 0·7 μM, 1·75 μM and 3·5 μM and cytochrome c_L was used in the range 0·4 μM–4·0 μM.

Estimation of formaldehyde. Formaldehyde was assayed by the Hantzsch reaction according to the method of Nash (1953) using acetylacetone in the presence of ammonium salts. Samples to be assayed were treated with 12·5% (w/v) TCA and the resulting protein precipitates removed by centrifugation for 2 min in an Eppendorf Microcentrifuge 5414. The supernatant was added to 2 ml reagent containing 2 M-ammonium acetate, 0·05 M-acetic acid and 0·02 M-acetylacetone. The resulting yellow colour due to the formation of 3:5-diacetyl-1:4-dihydrolutidine was developed fully at 37 °C for 40 min and measured at 412 nm. Formaldehyde standard curves were constructed by adding known quantities of formaldehyde to reaction mixtures parallel to those described above but omitting methanol dehydrogenase and cytochrome c_L. Protein was removed by TCA precipitation and the formaldehyde assayed as described above. Standard curves constructed in this manner were reproducible but there was a 30% loss in sensitivity by measuring formaldehyde in the presence of TCA.

The specificity of cytochromes c as electron acceptors for methanol dehydrogenase. Methanol dehydrogenase (2 μM) and mediating bacterial cytochrome *c* (2 μM) were incubated with horse heart cytochrome *c* (500 μM) in the presence of excess methanol (7·5 mM) in 500 μl reaction mixtures for 12 h at 22 °C; these were then assayed for formaldehyde, and for cytochrome *c* reduction. Reactions with methanol dehydrogenase from *M. methylotrophus* and *Pa. denitrificans* were measured in 25 mM-MOPS buffer at pH 7·0 in the absence of NH_4Cl. Reactions with the methanol dehydrogenase from *Pseudomonas* AM1 were measured in 25 mM-CAPS buffer at pH 9·0 in the presence of NH_4Cl (7·5 mM).

It was necessary to show that any negative results recorded in Table 3 were because the cytochrome and methanol dehydrogenase are unable to react, and not merely because the bacterial cytochrome *c* is unable to react with the mammalian cytochrome *c* used in the experiments. That the cytochrome c_H of *M. methylotrophus* and cytochrome c_{551} from *Pseudomonas aeruginosa* can reduce cytochrome *c* was shown by mixing reduced bacterial cytochrome *c* with a catalytic amount of mammalian cytochrome *c* and cytochrome oxidase (which cannot oxidize the bacterial cytochromes); rapid and complete oxidation of the bacterial cytochromes occurred. That the cytochrome c_H of *Pseudomonas* AM1 does not accept electrons from methanol dehydrogenase was confirmed in a more direct method based on its unusual property of being directly oxidized by mammalian cytochrome oxidase. This property permitted a direct test; no formaldehyde was produced on incubation of *Pseudomonas* AM1 cytochrome c_H with methanol dehydrogenase, methanol and cytochrome oxidase. It is worth noting that the cytochrome c_H of *Pseudomonas* AM1 is also unusual in being unable to transfer electrons to mammalian cytochrome *c*. The observed failure of cytochrome *c*(1) of *Pa. denitrificans* to act as an electron acceptor from methanol dehydrogenase was almost certainly due to failure to react with the methanol dehydrogenase, because it is known that the 'non-methylotrophic' cytochrome *c* of *P. denitrificans* is able to react with mammalian cytochrome *c*.

When negative results were obtained with a particular cytochrome (e.g. cytochrome c_H) the experiments were repeated with larger amounts of material but in no case did this lead to a positive result. The limits of measurement were such that a negative result indicates that the rate of reduction (if it occurred at all) was less than 0·1% of the rate measured with cytochrome c_L.

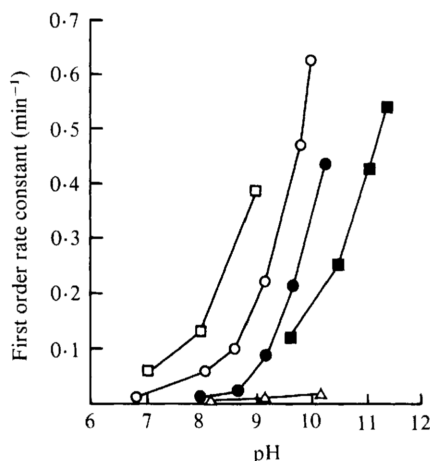

Fig. 1. Autoreduction of the cytochromes *c* of *M. methylotrophus* and *Pseudomonas* AM1. Cytochrome *c* (1·0 µM) was incubated in 30% (v/v) glycerol containing various buffers (25 mM), its reduction was measured at 550 nm, and the first-order rate constants were calculated by conventional methods. The buffers were as described previously and in Methods. ○, Cytochrome c_H of *M. methylotrophus* in the presence or absence of methanol dehydrogenase; ●, cytochrome c_L of *M. methylotrophus*; □, cytochrome c_L of *M. methylotrophus* in the presence of 1·0 µM methanol dehydrogenase; ■, cytochromes c_H and c_L of *Pseudomonas* AM1; △, mammalian cytochrome *c* in the presence or absence of methanol dehydrogenase.

RESULTS

Autoreduction of the cytochromes c of M. methylotrophus

As found for the cytochromes *c* of *Pseudomonas* AM1 (O'Keeffe & Anthony, 1980*b*), cytochromes c_H and c_L from *M. methylotrophus* were both autoreducible at high pH in the absence of added reductant. This autoreduction obeyed first-order kinetics at all pH values tested (pH 7–10). The measured first-order rate constants were independent of the cytochrome concentration as would be expected for a first-order reaction and these rate constants increased with increasing pH (Fig. 1). This Figure also shows that the rates of autoreduction at a given pH of cytochromes *c* from methylotrophs were two orders of magnitude greater than for mammalian cytochrome *c*; and that the rate constants for autoreduction of the cytochromes from *M. methylotrophus* were greater than those from *Pseudomonas* AM1. The autoreduction of cytochromes c_H and c_L from *M. methylotrophus* was not inhibited by *p*-chloromercuribenzoate or iodoacetamide. This result is similar to that found for cytochrome *c* from *Pseudomonas* AM1 (O'Keeffe & Anthony, 1980*b*) but different from the results of Tanaka *et al.* (1978) using autoreducible cytochrome *f* from horse-radish.

Even in the absence of methanol the cytochrome c_L of *M. methylotrophus* became reduced on addition of methanol dehydrogenase at pH 7·0. To avoid the possibility of the presence of enzyme-bound methanol, the dehydrogenase was purified in the absence of methanol; 25 mM-KCN was used as a suitable alternative protecting agent to methanol as it is a competitive inhibitor of the dehydrogenase (Ghosh, 1980). The cytochrome c_L was not reduced by inactive methanol dehydrogenase prepared in the absence of KCN. The reduction of cytochrome c_L by methanol dehydrogenase (prepared in the presence of KCN) obeyed first-order kinetics with respect to oxidized cytochrome c_L. Figure 1 shows the stimulatory effect of the enzyme on the rate constants for autoreduction at various pH values. Methanol had no effect on any of these rate constants.

By contrast with the cytochrome c_H of *Pseudomonas* AM1 (O'Keeffe & Anthony, 1980*b*), the cytochrome c_H of *M. methylotrophus* was not reduced by methanol dehydrogenase, the first-order rate constants for autoreduction of this cytochrome *c* being unaffected by the presence of the enzyme at all pH values.

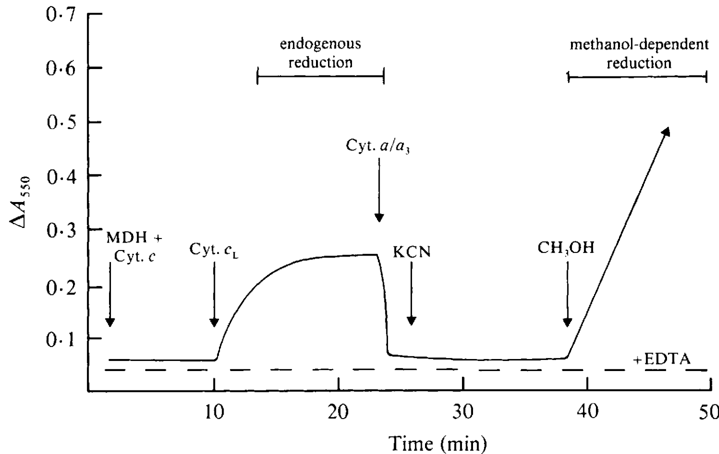

Fig. 2. Methanol-dependent reduction of cytochrome *c* by methanol dehydrogenase. Mammalian cytochrome *c* was incubated with methanol dehydrogenase (MDH) at pH 7·0. Cytochrome c_L was added and the A_{550} recorded until no further increase occurred. The cytochrome was then oxidized by addition of cytochrome oxidase (cyt. a/a_3). The oxidase was then inhibited with KCN and methanol was added as substrate for the methanol-dependent reduction of the cytochrome *c*. The dashed line shows the result of a similar experiment performed in the presence of 5 mM-EDTA. The following concentrations were used: methanol dehydrogenase, 3·5 μM; horse heart cytochrome *c*, 50 μM; cytochrome c_L, 0·8 μM; cytochrome oxidase, 0·24 μM; KCN, 0·5 mM; methanol, 7·5 mM; MOPS buffer, pH 7·0, 25 mM.

All these results with the proteins of *M. methylotrophus* are consistent with the model, previously proposed for autoreduction of the cytochromes *c* of *Pseudomonas* AM1, in which methanol dehydrogenase catalyses the reduction of cytochrome *c* in the absence of methanol by way of the autoreduction mechanism of the cytochrome (O'Keeffe & Anthony, 1980*b*).

Methanol-dependent reduction by methanol dehydrogenases of cytochromes c_L from M. methylotrophus and Pseudomonas AM1

Because addition of pure methanol dehydrogenase to pure cytochrome c_L leads to reduction of the cytochrome even in the absence of methanol, a different approach was necessary to demonstrate electron transfer from methanol by way of the dehydrogenase to cytochrome *c*. This was achieved by devising a system in which the bacterial cytochrome *c* was oxidized by coupling to a large excess of mammalian cytochrome *c*. Figure 2 describes such an experiment using pure methanol dehydrogenase and cytochrome c_L from *M. methylotrophus*. When methanol dehydrogenase and mammalian cytochrome *c* were mixed at pH 7·0 the cytochrome remained oxidized. Addition of a small amount of cytochrome c_L led to some reduction of the mammalian cytochrome *c* (present in 50-fold excess over the cytochrome c_L), presumably due to oxidation of endogenous reductant on the dehydrogenase. Added cytochrome oxidase then rapidly oxidized all the ferrocytochrome *c*, after which KCN was added to inhibit the cytochrome oxidase. Addition of methanol then led to complete reduction of all the cytochrome *c* (mammalian + c_L). The final rate of this methanol-dependent reduction was directly proportional to the concentration of methanol dehydrogenase used, but independent of the concentration of mammalian cytochrome *c* (between 5 μM and 100 μM). The rate of methanol-dependent cytochrome reduction was similar at pH 7·0, 8·0 and 9·0 (Table 1). Ammonia had no effect on the rate of reduction of cytochrome *c* by methanol dehydrogenase, and 5 mM-EDTA completely inhibited its reduction. The rate of reduction of cytochrome *c* measured in the experiment described in Fig. 2 was 0·6 nmol cytochrome *c* reduced min^{-1} (nmol methanol dehydrogenase)$^{-1}$. The methanol dehydrogenase was completely specific for cytochrome c_L; cytochrome c_H could not replace cytochrome c_L in experiments of the sort described in Fig. 2. By using a large excess of cytochrome c_H, instead of mammalian cytochrome *c* plus cytochrome *c* oxidase, it was

Table 1. *Effect of pH on the methanol: cytochrome c oxidoreductase and PMS-linked dehydrogenase activities catalysed by pure methanol dehydrogenases from M. methylotrophus and Pseudomonas AM1*

Methanol dehydrogenase (0·8 μM) was assayed for activity using either PMS or cytochrome c_L (0·7 μM) as primary electron acceptor in the presence or absence of NH_4Cl at various pH values using the zwitterionic buffers described in Methods. The concentration of horse heart cytochrome *c* was 50 μM and the concentration of both NH_4Cl and methanol was 7·5 mM. The method for measuring methanol: cytochrome *c* oxidoreductase activity was as described in Fig. 2, and for measuring the PMS-linked activity in the O_2 electrode as described by Dunstan *et al.* (1972) (except that CAPS buffer, pH 9·0 was used). In the PMS-linked assay there was negligible activity in the absence of NH_4Cl.

Activity measured	pH value	NH_4Cl concn (mM)	Rates measured* *M. methylotrophus*	Rates measured* *Pseudomonas* AM1
Methanol: cytochrome *c* oxidoreductase	7·0	0	0·62	0·25
	7·0	7·5	0·62	1·38
	8·0	0	0·62	0·75
	8·0	7·5	0·62	2·88
	9·0	0	0·58	1·28
	9·0	7·5	0·58	3·90
	9·5	0	—	1·00
	9·5	7·5	—	2·75
	10·0	0	0·52	—
	10·0	7·5	0·52	—
PMS/O_2 reduction	7·0	7·5	10·0	0
	8·0	7·5	24·0	0
	9·0	7·5	504·0	57·0
	9·5	7·5	—	50·0
	10·0	7·5	720·0	—

* The rates of cytochrome *c* reduction and PMS-linked O_2 consumption are both expressed as nmol electron acceptor reduced min^{-1} (nmol methanol dehydrogenase)$^{-1}$; for convenience of comparison both rates are calculated as single electron transfer reactions.

shown that electrons could only pass from methanol dehydrogenase to cytochrome c_H when cytochrome c_L was also present as a mediating cytochrome. Methanol dehydrogenase did not catalyse reduction of mammalian cytochrome *c* and neither cytochrome c_H nor cytochrome c_L was oxidized by mammalian cytochrome oxidase (a/a_3).

A similar system to that described above was used to demonstrate methanol-dependent reduction of cytochrome c_L by methanol dehydrogenase using the pure proteins from *Pseudomonas* AM1. The pH optimum for this reduction was about pH 9·0 and ammonium ions stimulated the rate of reduction about fourfold at all pH values used (Table 1). At pH 9·0 the rate of reduction was 3·9 nmol cytochrome reduced min^{-1} (nmol methanol dehydrogenase)$^{-1}$ and at pH 7·0 the rate was about 20% of this. As found with the proteins from *M. methylotrophus*, the cytochrome c_H of *Pseudomonas* AM1 was unable to catalyse the transfer of electrons from methanol dehydrogenase to mammalian cytochrome *c* and it was able to accept electrons from cytochrome c_L. The cytochrome c_H of *Pseudomonas* AM1 differed from that of *M. methylotrophus* in being oxidized rapidly by mammalian cytochrome oxidase.

Kinetic analysis of cytochrome c reduction by the methanol dehydrogenase of M. methylotrophus

In experiments of the sort described in Fig. 2, the initial rates of reduction of mammalian cytochrome *c* were shown to obey hyperbolic kinetics with respect to the concentration of cytochrome c_L. The K_m value for cytochrome c_L was 1·2 μM and the V_{max} was 1·5 nmol cytochrome *c* reduced min^{-1} (nmol methanol dehydrogenase)$^{-1}$. The initial rate of reduction of mammalian cytochrome *c* was independent of its initial concentration over the range studied (5–100 μM). The design of this experiment was such that the concentrations of reduced methanol dehydrogenase and ferricytochrome c_L remained constant during the measurements and so the concentration of the 'ES' complex remained constant, thus satisfying the essential criterion for

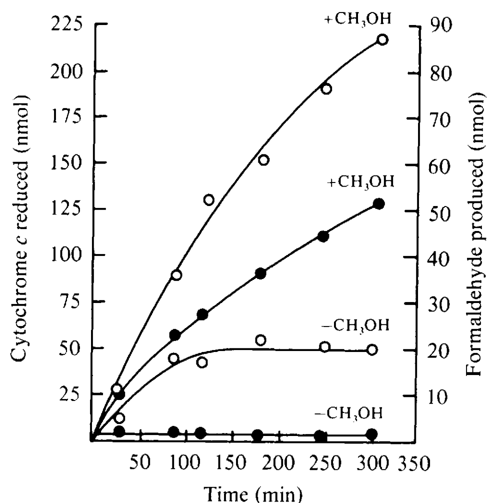

Fig. 3. Production of formaldehyde during methanol oxidation by methanol:cytochrome *c* oxidoreductase. Reaction vials (3 ml) contained mammalian cytochrome *c* (500 μM) and methanol dehydrogenase (2 μM) in the presence of excess methanol (3 mM) in 25 mM-MOPS buffer, pH 7·0. The reaction was initiated by addition of cytochrome c_L (2 μM). At various times aliquots (500 μl) were withdrawn and assayed for the extent of reduction of cytochrome *c* (○) and for formaldehyde production (●).

deriving the Michaelis–Menten equation by the Briggs–Haldane steady-state method (Beardmore-Gray, 1982). This explains why the hyperbolic kinetics were observed although the concentration of enzyme (methanol dehydrogenase) was very similar to that of the substrate (ferricytochrome c_L).

The measured K_m value for cytochrome c_L and the V_{max} for cytochrome reduction were similar during the endogenous reduction observed in the first part of the experiment (Fig. 2) to those observed after addition of methanol. This is what would be expected if the rate-limiting step in the reduction of mammalian cytochrome *c* by methanol is the production of ferrocytochrome c_L. That is, the K_m for cytochrome c_L and the V_{max} values should be independent of the substrate used to reduce the methanol dehydrogenase. It should be noted that the reduction of mammalian cytochrome *c* by endogenous reductant described here is not the same as autoreduction of cytochrome c_L stimulated by methanol dehydrogenase discussed in the first section of this paper.

Production of formaldehyde during methanol oxidation by methanol : cytochrome c oxidoreductase

When methanol dehydrogenase was incubated with methanol, cytochrome c_L and excess mammalian cytochrome *c*, formaldehyde was produced, whereas none was produced in the absence of methanol. The results in Fig. 3 show the time course of formaldehyde production and cytochrome *c* reduction using methanol dehydrogenase and cytochrome c_L from *M. methylotrophus*. Similar results were obtained using the pure proteins from *Pseudomonas* AM1 but in this case a high pH was optimal and NH_4Cl (7·5 mM) was included in the reaction mixture. Similar results to these were obtained when the large amount of mammalian cytochrome was replaced by a large amount of cytochrome c_L.

The ratio of cytochrome *c* reduced to formaldehyde measured in these experiments was between 2 and about 4. Similar stoichiometries (usually between 2 and 3) were measured in many further experiments in which reduction of various limiting amounts of a mammalian cytochrome *c* were allowed to go to completion and the concentration of formaldehyde was determined.

Although no formaldehyde was produced in the absence of methanol, there was considerable reduction of the cytochrome *c* (Fig. 3). As most of the cytochrome present in these experiments was mammalian cytochrome *c* this endogenous reduction cannot be due to stimulation by the

Table 2. *Substrate specificity of methanol: cytochrome c oxidoreductase from M. methylotrophus*

The substrate specificity was determined in the incubation mixture using the system described in Fig. 3. The concentration of potential substrates was 20 mM. The extent of reduction of cytochrome *c* was determined after all reduction in the control vial with no substrate was complete. The data for the PMS-linked assay are from Anthony & Zatman (1965) except for the aldehyde oxidations which were taken from Sperl *et al.* (1974).

Substrate	Cytochrome *c* reduction (% of that with methanol)	Reduction of PMS (% of rate with methanol)
None	36	—
Methanol	100	100
Ethanol	100	100
2-Chloroethanol	100	100
2-Methyl-2-propanol	40	0
Cyclohexanol	36	0
Formaldehyde	100	100
Acetaldehyde	0	0

methanol dehydrogenase of autoreduction of cytochrome *c*; it must be due to oxidation of the endogenous substrates (not methanol or formaldehyde) inferred to be present on the enzyme from previous observations of endogenous dye reduction occurring in the dye-linked assay (Anthony & Zatman, 1964; Ghosh & Quayle, 1981).

Substrate specificity of the methanol: cytochrome c oxidoreductase from M. methylotrophus

Although the oxidation of methanol is the reaction catalysed by methanol dehydrogenase during growth, the enzyme has a wide but well-defined substrate specificity when assayed *in vitro* with PMS as the primary electron acceptor (Anthony & Zatman, 1965; Sperl *et al.*, 1974). The results described in Table 2 show that, as expected, those substrates that are oxidized by methanol dehydrogenase when assayed with PMS were also able to act as substrates in the cytochrome *c* assay system. By contrast, potential substrates that were not oxidized in the dye-linked assay system failed to reduce the cytochrome *c* in excess of the reduction measured in the absence of added substrate. An unexpected result was the unexplained inhibition of this endogenous cytochrome *c* reduction by acetaldehyde. The oxidation of formaldehyde by the methanol: cytochrome *c* oxidoreductase is discussed further below.

The oxidation of formaldehyde by methanol: cytochrome c oxidoreductase

Formaldehyde can also be oxidized by methanol dehydrogenase coupled to cytochrome c_L. This was shown by the method described in Fig. 3, using an initial formaldehyde concentration of 60 μM. The rate of formaldehyde oxidation was similar to that of methanol oxidation. The stoichiometry of the reaction was 2 nmol cytochrome reduced per nmol formaldehyde oxidized, and this stoichiometry was constant throughout the reaction. After oxidation of the formaldehyde the cytochrome was reduced further; the extent of this extra reduction was the same as the extent of endogenous reduction occurring in the absence of any added substrate, suggesting that the endogenous substrate was not oxidized until added substrate had been preferentially oxidized.

These results explain the variable stoichiometry observed during methanol oxidation by the methanol: cytochrome *c* oxidoreductase system. For example, the ratio of nmol cytochrome *c* reduced per nmol formaldehyde measured increased between 2 and 4·3 in the experiment described in Fig. 3. This was presumably because some methanol was oxidized completely to formate during the course of the reaction.

Specificity of cytochromes c as electron acceptors for various methanol dehydrogenases

The specificity of the cytochromes *c* and methanol dehydrogenases from three methylotrophs was investigated directly by measuring methanol-dependent reduction of each cytochrome and also by measuring formaldehyde production occurring concomitantly with cytochrome *c* reduction in the system described in Fig. 3. In control experiments it was shown that no

Table 3. *Specificity of cytochromes c from methylotrophs as electron acceptors for various methanol dehydrogenases*

Full experimental details are given in Methods. The isoelectric points (pI values) of the dehydrogenases from *Pa. denitrificans*, *Pseudomonas* AM1 and *M. methylotrophus* were 3·7, 8·8 and greater than 8, respectively. This information is included to show that there is no clear pattern of reaction between dehydrogenases and cytochromes merely in terms of their isoelectric points. That negative results are not due to lack of the necessary interaction of bacterial and mammalian cytochromes is demonstrated in Methods. + indicates methanol-dependent reduction of cytochrome *c* with concomitant production of formaldehyde; − indicates no methanol-dependent reduction of cytochrome *c* or production of formaldehyde.

	Reduction of cytochromes by methanol dehydrogenase from the following bacteria:		
	M. methylotrophus	*Pa. denitrificans*	*Pseudomonas* AM1
M. methylotrophus			
Cytochrome c_H (pI 8·85)	−	−	−
Cytochrome c_L (pI 4·35)	+	−	−
Pa. denitrificans			
Cytochrome *c*(1) (pI acidic)	−	−	−
Cytochrome *c*(2) (pI acidic)	−	+	−
Pseudomonas AM1			
Cytochrome c_H (pI 8·8)	−	−	−
Cytochrome c_L (pI 4·2)	+	+	+

methanol dehydrogenase was able to reduce mammalian cytochrome *c* (mol. wt 12500; pI 10·4) or cytochrome c_{551} (mol. wt 8100; pI 4·7) from the non-methylotroph *Ps. aeruginosa*. The results in Table 3 show that of the two soluble cytochromes *c* found in each methylotroph only one was able to accept electrons from methanol dehydrogenase. This cytochrome was cytochrome c_L from *M. methylotrophus* and *Pseudomonas* AM1, and one of the cytochromes *c* of *Pa. denitrificans*; this was presumably the extra cytochrome *c* induced during growth on methanol (Van Verseveld & Stouthamer, 1978). The cytochromes *c* from *M. methylotrophus* and *Pa. denitrificans* were specific and thus only able to react with methanol dehydrogenase from the same organism. By contrast, cytochrome c_L from *Pseudomonas* AM1 was able to react with all three methanol dehydrogenases tested. In control experiments, the only systems in which methanol-independent reduction of cytochrome *c* occurred were those in which methanol-dependent cytochrome reduction and concomitant formaldehyde production were also able to occur.

DISCUSSION

The results in the present paper clearly show that one of the cytochromes *c* of methylotrophs is able to act as the primary electron acceptor from methanol dehydrogenase. It is not yet known whether the mechanism of the intramolecular autoreduction of the cytochrome *c* described in the first part of this paper is also involved in the mechanism of the methanol-dependent reduction of cytochrome *c*. Using proteins from *M. methylotrophus* the methanol-dependent reduction of cytochrome c_L was similar to that in whole cells in operating at pH 7·0, in not requiring ammonia as activator, in producing formaldehyde and in being inhibited by EDTA. Although a similar methanol:cytochrome *c* oxidoreductase activity was also demonstrated using pure proteins from *Pseudomonas* AM1 the characteristics of this system were closer to those of the PMS-linked activity in having a high pH optimum and in being stimulated by ammonia. Earlier work by Duine *et al.* (1979), using partially purified extracts of *Hyphomicrobium* X, had indicated that the ammonia requirement in the dye-linked assay was due to an irreversible alteration by O_2 of the methanol dehydrogenase, which also prevented its acting as an electron donor to cytochrome *c*. However, all the work described in the present paper was done in aerobic conditions and successful preparation of proteins catalysing methanol:cytochrome *c* oxidoreductase activity was achieved despite this.

Although no completely satisfactory explanation can be given for the variety of observations described here, one possibility may be that the enigmatic endogenous reductant usually found on methanol dehydrogenase (Anthony & Zatman, 1964; Ghosh & Quayle, 1981) may protect the enzyme against O_2 inactivation to some extent; the extent of protection may depend on the amount of endogenous reductant present on a particular dehydrogenase (Beardmore-Gray, 1982).

The rates of methanol-dependent cytochrome *c* reduction measured using pure proteins in this work were usually lower than the rate of dye reduction using PMS at pH 9·0 in the presence of ammonia. The results in Table 1 illustrate the difference between the systems from *M. methylotrophus* and *Pseudomonas* AM1 that make any general interpretation very difficult. A particularly difficult problem is that the two dehydrogenases have very different specific activities when measured in the PMS-linked assay system; such variations are well known for methanol dehydrogenase (Goldberg, 1976; Bamforth & Quayle, 1978). Whatever the significance of these relative rates of cytochrome and dye reduction, and the effect of pH and ammonia on them, it should be noted that the rates of cytochrome *c* reduction observed [e.g. V_{max}, 1·5 nmol cytochrome reduced min^{-1} (nmol methanol dehydrogenase)$^{-1}$] are far too low to account for the rates of electron transport measured in whole bacteria [150–250 nmol O_2 min^{-1} (mg dry weight)$^{-1}$]. This marked discrepancy between *in vivo* rates of respiration and the activity of the purified and reconstituted system may be related to the very high concentrations of methanol dehydrogenase and cytochrome *c* found in methylotrophs. In *M. methylotrophus*, for example, the concentration of both proteins is about 0·5 mM [if it is assumed that the periplasmic volume is about 20% of the total cell volume and that all the cytochrome c_L and methanol dehydrogenase is periplasmic (Alefounder & Ferguson, 1981; Beardmore-Gray, 1982; Jones *et al.*, 1982)]. Furthermore, all the cytochrome c_L and methanol dehydrogenase will be in the associated form (the K_m of methanol dehydrogenase for cytochrome c_L being very low) and these two proteins may well cover most of the surface of the bacterial membrane. It is possible that the marked change in environment occurring on release of the cytochrome *c* and the dehydrogenase during cell disruption may contribute to the low activity of the proteins as measured in the reconstituted system.

The results in the present paper strongly suggest that cytochrome c_L is the natural electron acceptor for methanol dehydrogenase in both bacteria studied and that electron flow can occur between cytochrome c_L and cytochrome c_H. That this might also occur *in vivo* is indicated by the observation that all of the cytochrome *c* in whole cells is reducible by methanol (Anthony, 1975; Cross & Anthony, 1980*b*). These observations raise a number of questions. Does cytochrome c_L accept electrons from other substrates such as cytochrome *b*? Is cytochrome c_L oxidized directly by terminal oxidases? Are soluble and membrane-bound cytochromes *c* identical and do they have identical functions? Similar questions arise with respect to the function(s) of the other soluble cytochrome *c* (cytochrome c_H) which appears to be specific for methylamine dehydrogenase (S. A. Lawton & C. Anthony, unpublished observations).

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