The purification and properties of the soluble cytochromes c of the obligate methylotroph *Methylophilus methylotrophus*

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The obligate methylotroph *Methylophilus methylotrophus* contains three distinct soluble cytochromes c. The major cytochromes, cytochrome c_H (about 50% of the total) and cytochrome c_L (about 42%), were similar in most respects to the cytochromes c_H and c_L of the facultative methylotroph *Pseudomonas* AM1 [O'Keeffe & Anthony (1980) *Biochem. J.* **192**, 411–419]. Cytochrome c_H had a high isoelectric point, a midpoint redox potential at pH 7.0 of 373 mV and a low molecular weight (8500). The cytochrome c_L had a low isoelectric point, a midpoint potential of 310 mV and a molecular weight of 21000. The third cytochrome, cytochrome c_{LM}, was clearly distinct from cytochromes c_H and c_L. Like the cytochrome c_L of *Pseudomonas* AM1, the cytochrome c_L of *M. methylotrophus* had the lowest midpoint potential, it reacted most rapidly with methanol dehydrogenase and it combined to the greatest extent with CO. Cytochromes c_H and $_L$ of *M. methylotrophus* differed from those from *Pseudomonas* AM1 in having unusually high midpoint redox potentials for non-photosynthetic bacteria and in exhibiting a split α-band at low temperatures.

Bacterial cytochromes c are of interest in a general sense in relation to growth efficiency because it has been shown that the presence of a high-potential cytochrome c is a prerequisite for a high P/O ratio in bacteria (Jones *et al.*, 1975), and the properties of the cytochromes c of methylotrophs are of special interest in view of their involvement with the unusual methanol dehydrogenase found in these bacteria (Anthony, 1975*a,b*; Widdowson & Anthony, 1975; Keevil & Anthony, 1979; Bamforth & Quayle, 1978; Duine *et al.*, 1979; Higgins, 1979). It has recently been shown that the facultative methylotroph *Pseudomonas* AM1 has two distinct soluble cytochromes c and that these undergo the unusual process of autoreduction at high pH values (O'Keeffe & Anthony, 1980*a,b*). *Methylophilus methylotrophus* is an obligate methylotroph that grows by way of the alternative ribulose monophosphate pathway instead of the serine pathway of carbon assimilation (Anthony, 1975*a*). It was thus of interest to determine whether or not multiple cytochromes c were also present in this totally different sort of methylotroph.

The present paper describes the purification and characterization of three distinct soluble cyto-

Abbreviation used: SDS, sodium dodecyl sulphate.
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chromes c from *M. methylotrophus* that are unusual in having high redox potentials; these are called cytochromes c_H, c_{LM} and c_L. Numerical subscripts have been avoided in order to prevent confusion with mitochondrial cytochrome c_1, cytochrome c_2 of photosynthetic bacteria etc. The subscripts H and L are used to denote cytochromes c with high and low isoelectric points respectively; the minor component cytochrome c_{LM} also has a low isoelectric point.

Materials and methods

Chemicals

These were obtained from sources previously described (O'Keeffe & Anthony, 1980*b*).

Growth medium

The growth medium was based on that of MacLennan *et al.* (1971). It contained 3.6 g of $(NH_4)_2SO_4$/litre; to this was added H_3PO_4 (13.3 M) to a final concentration of 10 mM, $MgSO_4,7H_2O$ (40%) to a final concentration of 0.4 g/litre and 10 ml of trace-element solution/litre. The trace-element solution contained (per litre): $CaCl_2,2H_2O$, 1.06 g; $MnSO_4,5H_2O$, 50 mg; $CuSO_4,5H_2O$, 8 mg; $ZnSO_4,7H_2O$, 40 mg; $FeSO_4,7H_2O$, 800 mg; $NaMoO_4,2H_2O$, 8 mg; $CoCl_2,6H_2O$, 8 mg; H_3BO_4, 6 mg; 98% H_2SO_4, 0.3 ml. The medium was auto-

claved at 121°C for 2h in 20-litre glass containers, and methanol, previously autoclaved separately in sealed glass bottles, was added to a final concentration of 1% (v/v). During continuous culture the pH in the culture vessel was maintained at pH 6.8 by automatic addition of sterile 0.5 M-KOH/NaOH.

Organism and maintainance of stock cultures and growths in continuous culture

Methylophilus methylotrophus (N.C.I.B. 10515) was a gift from Dr. I. J. Taylor of ICI Ltd. Stock cultures were maintained in 30% (v/v) glycerol at −15°C. They were prepared by the addition of 2.5 ml of a exponential-phase culture to a Bijou bottle containing 1.5 ml of sterile 80% (v/v) glycerol. Growth in continuous culture was as described for *Pseudomonas* AM1 by Keevil & Anthony (1979).

Culture purity

Culture purity was checked by streaking out cultures on nutrient agar, nutrient agar plus methanol and minimal medium plus methanol. *M. methylotrophus* forms only pinhead colonies on nutrient agar alone. Samples were also checked for purity by microscopy after Gram staining.

Purification of the soluble cytochromes c of Methylophilus methylotrophus

These methods were based on those described previously (O'Keeffe & Anthony, 1980b). A 500g (wet wt.) batch of frozen bacteria (grown in continuous culture conditions of methanol limitation) was homogenized in 500 ml of 20 mM-sodium/potassium phosphate buffer, pH 7.0. Then 50 mg of deoxyribonuclease was added, the cells were passed twice through a French pressure cell at 135 MPa (20 000 lb/in²), and the resulting deep-red solution was centrifuged at 23 000 g for 1h to remove whole bacteria and cell debris. The resulting cell-free extract was acidified with 1 M-HCl to pH 4.2, and the precipitated protein and nucleic acid were removed by centrifugation. The pH of the extract was again adjusted to pH 4.2, and the centrifugation was repeated. The deep-red supernatant was adjusted to pH 8.0 with 1 M-NaOH and concentrated in a Chemlab 400 ml concentrator (Chemlab Instruments, Hornchurch, Essex, U.K.) with a G-05-T membrane (5000-mol.wt. cut-off) under an atmosphere of N_2 to a volume of 300 ml. This was dialysed against 30 litres of 10 mM-Tris/HCl buffer, pH 8.0 in a beaker dialyser (Bio-Rad Laboratories) and applied to a column (4 cm × 23 cm) of DEAE-cellulose (Whatman DE-52) equilibrated in the same buffer. Some of the cytochrome came straight through the column (designated cytochrome c_H), and two other cytochrome bands were left bound to the column. The unbound cytochrome was pooled, concentrated

and dialysed against 5 litres of 5 mM-sodium acetate buffer pH 5.6. The lower band on the column was eluted with 50 mM-Tris/HCl buffer, pH 8.0, in 80 ml and was designated cytochrome c_{LM}. The upper band on the column was eluted in 100 mM-NaCl/ 100 mM-Tris/HCl buffer, pH 8.0, and was designated cytochrome c_L.

The cytochrome c_H was applied to an upward-flow Sephadex G-150 column (90 cm × 2 cm), and two coloured fractions were obtained; the first was found to be methanol dehydrogenase and the second was the cytochrome. The cytochrome c_H was applied to a 8.5 cm × 3 cm column of CM-cellulose (Whatman CM-52) in 5 mM-sodium acetate buffer, pH 5.6, and it bound as a dense band at the top of the column. The cytochrome was eluted with 100 mM buffer, and two cytochrome bands were seen; the major band (more than 90%) was in the reduced form and was eluted first, and was used for further purification. On the basis of its absorption spectrum the minor band appeared to be the oxidized form of the same cytochrome and, being of low specific activity, was discarded. The cytochrome was re-applied to a CM-cellulose column (8 cm × 3 cm) in 5 mM-acetate buffer, pH 5.6, and was eluted with a linear gradient of 5–120 mM-sodium acetate buffer, pH 5.6, containing 5 mM-ascorbic acid; the cytochrome was eluted in about 60 mM buffer. The u.v. absorption of the eluate was monitored with a Uvicord recorder (LKB), and the cytochrome peak corresponded exactly with the peak of u.v. absorbance. The cytochrome was dialysed against 10 litres of distilled water in a beaker dialyser and was stored frozen at −20°C. The cytochrome appeared as one band on SDS/polyacrylamide gels after electrophoresis.

Cytochrome c_{LM} was concentrated to 25 ml in a concentration cell and applied to an upward-flow Sephadex G-150 column (90 cm × 2 cm) in 100 mM-Tris/HCl buffer, pH 8.0. The purest fractions were pooled and applied to a DEAE-cellulose column (10 cm × 3 cm) in 5 mM-Tris/HCl buffer, pH 8.0, and eluted with a linear gradient of 5–100 mM buffer. The cytochrome was eluted in 40 mM buffer, and was dialysed against 6 litres of distilled water in a beaker dialyser and stored frozen at −20°C. The cytochrome appeared as one band on SDS/polyacrylamide gels after electrophoresis.

Cytochrome c_L was concentrated to 30 ml in a concentration cell and applied to an upward-flow Sephadex G-150 column (90 cm × 2 cm) in 100 mM-Tris/HCl buffer, pH 8.0. It was eluted before a yellow fraction of unknown composition. The cytochrome fraction was applied to a DEAE-cellulose column (9 cm × 3 cm) in 10 mM-Tris/HCl buffer, pH 8.0, and was eluted with a linear gradient of 10 mM-Tris/HCl buffer, pH 8.0, to 125 mM-NaCl/ 125 mM-Tris/HCl buffer, pH 8.0. The cytochrome was eluted in approx. 90 mM-NaCl/90 mM-Tris/HCl

buffer, pH 8.0. The cytochrome was concentrated to 15 ml in a concentration cell and applied to a Sephadex G-75 (superfine grade) column (100 cm × 2 cm) in 100 mM-Tris/HCl buffer, pH 8.0. Two overlapping cytochromes were eluted; fractions were pooled from the earliest part of the first band, the mixed part of the bands and the latest part of the second band. The early and late fractions both gave single bands on polyacrylamide gels after electrophoresis, but of differing molecular weight; the mixed fraction contained most of the cytochrome and gave rise to both bands on SDS/polyacrylamide-gel electrophoresis. The mixed fraction was used for subsequent purification by isoelectric focusing. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Measurement of absorption spectra, electrophoretic methods, determination of haem and redox potentiometry.

These were as described previously (O'Keeffe & Anthony, 1980*b*). The redox titrations at pH 5.0 and 9.0 were done in 25 mM-Tris/succinate buffer and 25 mM-2-(N-cyclohexylamine)ethanesulphonic acid buffer respectively.

Results

Purification of the cytochrome c

Three soluble cytochromes c were purified from *M. methylotrophus* (Table 1). If it is assumed that the proportion of each cytochrome after the ion-exchange step was the same as that in the cell-free extract, then the initial proportion of the cytochromes were 50% cytochrome c_H, 8% cytochrome c_{LM} and 42% cytochrome c_L. Because it is impossible to distinguish between the three cytochromes in mixtures and because the overall yield in the first two purification steps was only 60%, the accurate proportions in crude extracts before separation could not be determined.

Purity of the cytochromes, their molecular weights and isoelectric points

The purified cytochrome c_H gave a single band on SDS/polyacrylamide gels corresponding to a

Table 1. *Purification of the soluble cytochromes c from Methylophilus methylotrophus*

Details are given in the Materials and methods section. Cytochrome c_H is the cytochrome having a high isoelectric point (thus not binding to DEAE-cellulose); cytochrome c_{LM} and cytochrome c_L have low isoelectric points and thus bind to DEAE-cellulose. The cytochrome concentrations were calculated by using a molar absorption coefficient of 19.1 mM^{-1}·cm^{-1} for the reduced-minus-oxidized difference spectra; this was very close to the values found for the difference spectra of the individual purified cytochromes. Because accurate determinations of cytochrome in crude extracts were difficult, the yield and purification factors for the later purification stages were calculated from the supernatant after acid treatment. This initial treatment gave a purification of about 3-fold in about 90% yield. The purification table assumes that the proportion of each cytochrome (c_H, c_{LM} and c_L) recovered after the DEAE-cellulose step was the same as the proportion of each cytochrome in the initial cell-free extract, i.e. cytochrome c_H constituted 50%, cytochrome c_{LM} 8% and cytochrome c_L 42%. The results of gel filtration and isoelectric focusing of cytochrome c_L led to complex results that are discussed more extensively in the text.

Purification fraction	Cytochrome content (nmol/mg of protein)	Volume (ml)	Total cytochrome (nmol)	Yield (%)	Purification
Cytochrome c_H					
Acid treatment	1.79	300	5372	89.5	1
DEAE-cellulose	2.53	190	3669	61	1.4
Sephadex G-150	23.0	150	2463	41	12.8
CM-cellulose (I)	103.3	42	2304	38	57.4
CM-cellulose (II)	115.8	170	1864	31	64.4
Cytochrome c_{LM}					
Acid treatment	0.28	300	845	89.5	1.
DEAE-cellulose	0.55	80	577	61	2.1
Sephadex G-150	17.5	84	521	55	62
DEAE-cellulose	47.9	130	206	22	170
Cytochrome c_L					
Acid treatment	1.53	300	4603	89.5	1
DEAE-cellulose	11.46	490	3140	61	7.4
Sephadex G-150	21.2	180	3108	60.4	13.8
DEAE-cellulose	29.33	295	2423	47	19.0
Sephadex G-75	22.3	10	72	1.4	14.5
(21 000 mol.wt.)	41.5	14	285	5.5	27.0
(17 000 mol.wt.) (mixed fractions)	25.8	80	905	17.6	16.7
Electrofocusing of mixed fractions	43.7		581	11.2	28.4

mol.wt. of 8500 (two determinations), and it gave one band after analytical isoelectric focusing; the isoelectric point of the protein was pH 8.85 (two determinations).

Cytochrome c_{LM} gave one band on SDS/polyacrylamide gels corresponding to a mol.wt. of 16 800 (two determinations), and it gave one band after analytical isoelectric focusing; the isoelectric point was pH 4.55 (two determinations).

The properties of cytochrome c_L were more complex; three distinct components were obtained by gel filtration on Sephadex G-75. The cytochrome fraction that was eluted first (less than 10% of the total) gave one band on SDS/polyacrylamide gels corresponding to a mol.wt. of 21 000; the cytochrome fraction that was eluted last (about 30% of the total) also gave one band but of 17 000 mol.wt. On SDS/polyacrylamide-gel electrophoresis the intermediate cytochrome fractions gave two major cytochrome bands of approximately equal intensity (which could be seen in the unstained gel), and a number of other minor protein bands were detected by staining. The cytochrome bands from this intermediate fraction corresponded exactly to the bands on the gels of the first and last fractions, and thus appeared to be a mixture of two cytochrome species of 17 000 and 21 000 mol.wts. When the three cytochrome fractions were subjected to analytical isoelectric focusing they all gave a number of cytochrome bands of similar isoelectric point (pH 4–4.35). The cytochromes from the first and last fractions had identical spectra and midpoint redox potentials (see below). The mixed fraction (containing both species) was further purified by preparative

isoelectric focusing; this resulted in seven cytochrome bands of similar isoelectric points (pH 4.0–4.35). Because of the proximity of the bands, they were collected as five fractions. Samples of the individual fractions from the isoelectric focusing were run on SDS/polyacrylamide gels as before. Two cytochrome species had mol.wts. of 17 000 and 21 000 (as found in the original fractions from the Sephadex G-75 column); they were spectrally indistinguishable and had the same midpoint redox potentials. The proportion of the 21 000-mol.wt. species was less than in the original mixed fraction, indicating that the lower-molecular-weight cytochrome may be a breakdown product of the higher-molecular-weight cytochrome. This proposal is substantiated by the similarity of the midpoint redox potentials and of the spectral properties of the two species. Furthermore, the samples after isoelectric focusing gave an additional uncoloured minor protein band on SDS/polyacrylamide-gel electrophoresis corresponding to a protein of mol.wt. of about 4000 that could be the fragment formed in the conversion of the higher-molecular-weight cytochrome into the lower-molecular-weight cytochrome. The larger cytochrome c_L (mol.wt. 17 000–21 000) is unlikely to be a dimer of cytochrome c_H (mol.wt. 8500) because the molecular weights were determined by SDS/polyacrylamide-gel electrophoresis, which dissociates dimers (Swank & Burris, 1969).

The simplest interpretation of this analysis of the soluble cytochromes c of M. methylotrophus is that the organism produces three different soluble cytochromes c (cytochromes c_H, c_{LM} and c_L) and that

Table 2. *Properties of the three pure soluble cytochromes c of Methylophilus methylotrophus*

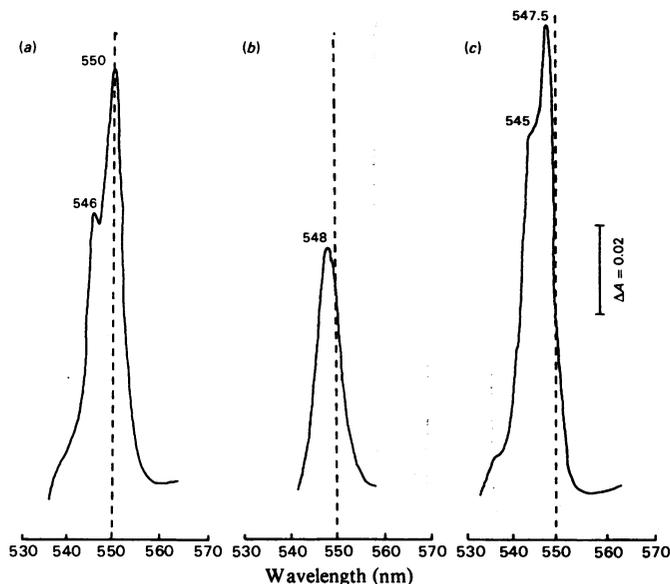|	Cytochrome c_H	Cytochrome c_{LM}	Cytochrome c_L
Relative proportions in crude extracts (estimated)	50%	8%	42%
Isoelectric point	8.85	4.55	4.0–4.35
Molecular weight	8500	16 800	17 000–21 000
Midpoint redox potentials at pH 5.0, 7.0 and 9.0	385 mV, 373 mV, 375 mV	349 mV, 336 mV, 325 mV	312 mV, 310 mV, 297 mV
Absorption maxima of ferrocytochrome (α, γ)	551.25 nm, 416.25 nm	550.75 nm, 416.5 nm	549.75 nm, 416 nm
Absorption maxima of ferricytochrome (γ)	408 nm	408 nm	410 nm
Absorption coefficients ($mM^{-1} \cdot cm^{-1}$) (α, γ)	27.1, 128	25.2, 116	26.9, 139
Ratio of γ-absorption/α-absorption (ferrocytochrome)	4.72	4.27	5.16
Absorption maxima at 77 K	Split; 550 nm and 546 nm	Not split; 548 nm	Split; 547.5 nm and 545 nm
Ratio of $(A_\alpha - A_{575})/A_{280}$ at 20°C	0.83	0.95	1.05
Haem groups/molecule of protein	1	Not determined	1
% CO-binding	7%	24%	60% (both forms)
Autoxidizability	Slow	Slow	Slow

Fig. 1. *Spectra of ferrocytochromes c measured at 77 K*

(a) Cytochrome c_H (2.8 μM); (b) cytochrome c_{LM} (1.7 μM); (c) cytochrome c_L (2.5 μM). Details are given in the Materials and methods section. The light-path was 2 mm and the reference cuvette contained water.
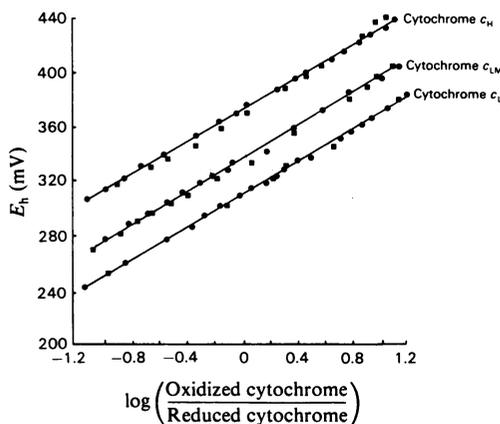

Fig. 2. *Redox titrations of cytochromes c_H, c_{LM} and c_L at pH 7.0*

●, Reductive titration with ascorbate; ■, oxidative titration with ferricyanide. The lines on the Figure are theoretical curves for single-electron transfer reactions ($n = 1$) described in the equation relating E'_0 (the midpoint potential) to E_h (the measured redox potential):

$$E_h = E'_0 + 59 \log \left(\frac{\text{oxidized cytochrome}}{\text{reduced cytochrome}} \right)$$

cytochrome c_L is in at least two forms, one of which may arise by the 'loss' of a 4000-mol.wt. component from the other. There is no evidence that the

cytochromes c_H, c_{LM} and c_L arise by proteolytic digestion of a single cytochrome, and the demonstration of three similar cytochromes on membranes of these bacteria argues strongly against this possibility (Cross & Anthony, 1980).

Spectral characteristics of the three cytochromes c

The absorption spectra of the cytochromes had absorption maxima at about 550 nm and were typical of monohaem cytochromes c (absorption maxima and molar absorption coefficients are listed in Table 2). At low temperature (77 K) the absorption-wavelength maxima were slightly lower and, for the two major components, cytochromes c_H and c_L (both 17 000-mol.wt. and 21 000-mol.wt forms), the peak was split (Fig. 1), in common with many other cytochromes c (Estabrook, 1961) but in contrast with those of the facultative methylotroph *Pseudomonas* AM1 (O'Keeffe & Anthony, 1980b). The three soluble cytochromes appear to be the same as those bound to membranes of *M. methylotrophus* (Cross & Anthony, 1980).

The measured haem/protein ratios were 1.2 and 1.21 for cytochrome c_H and cytochrome c_L respectively; this indicates that the cytochromes are typical in having only one haem group per molecule of cytochrome. The molar absorption coefficients of the three cytochromes were very similar. These were determined by measuring the absorbance of a known molarity of pure cytochrome, the molarity being calculated from measurement of protein

(Lowry *et al.*, 1951) and molecular weights as described above.

Midpoint redox potentials of the three cytochromes c

Potentiometric titrations of the purified cytochromes are described in Fig. 2, and the midpoint potentials from these titrations and from similar titrations at pH 5.0, pH 7.0 and pH 9.5 are listed in Table 2. The midpoint potentials of the cytochromes were relatively independent of pH over the range tested, but they differed from each other in their slight responses to changing pH. Insufficient results were obtained to conclude that these cytochromes are markedly different from those of *Pseudomonas* AM1 in this respect (see O'Keeffe & Anthony, 1980b).

Reaction with CO and autoxidation

None of the cytochromes was rapidly autoxidizable, but all bound CO to some extent. Assuming the absorption coefficient of $55\,\text{mM}^{-1}\cdot\text{cm}^{-1}$ for the peak-to-trough in the Soret region, 7% of the cytochrome c_H combined with CO after bubbling the gas for 3 min followed by 20 min incubation in the dark; 24% of the cytochrome c_{LM} and 60% of the cytochrome c_L combined with CO after similar treatment. This CO-binding is unlikely to be due to denaturation of the cytochromes during purification, since CO-binding cytochrome c constituted about 30% of the cytochrome c in whole bacteria and cell extracts. CO-binding cytochromes c seem to be a common feature of methylotrophic bacteria (see Higgins, 1979; O'Keeffe & Anthony, 1980b). The cytochromes almost certainly do not function as physiological oxidases, and no aerobic methylotrophic bacterium has been reported without a functional a- or o-type terminal oxidase in addition to the CO-binding cytochrome c.

Discussion

The results given in the present paper are summarized in Table 2. They clearly show that there are at least three soluble cytochromes c in *M. methylotrophus*. These differ in isoelectric points, molecular weights, midpoint redox potentials, extent of CO-binding, reaction with methanol dehydrogenase and their absorption spectra. They are similar in having one haem group per molecule and in all having unusually high midpoint redox potentials for non-photosynthetic bacteria. Redox potential measurements of cytochrome c on membranes (Cross & Anthony, 1980) indicated that these same cytochromes are all able to become firmly bound to the membrane. The α-bands of the two major soluble cytochromes c (c_H and c_L) both split at 77 K, but no α-band splitting was observed in the minor cytochrome, cytochrome c_{LM}.

There are few full descriptions of pure cytochromes c from methylotrophs, and these have been discussed in detail in the preceding paper (O'Keeffe & Anthony, 1980b). Two soluble cytochromes c have been purified from the facultative methylotroph *Pseudomonas* AM1, and these have been well characterized (O'Keeffe & Anthony, 1980b). The two major cytochromes c (cytochrome c_H and cytochrome c_L) from *M. methylotrophus* are similar in most respects to the corresponding cytochromes c_H and c_L from *Pseudomonas* AM1. The main differences between them are that the midpoint redox potentials of the *M. methylotrophus* cytochromes are higher than those of the *Pseudomonas* AM1 cytochromes, although in both bacteria the cytochrome c_H midpoint redox potential is higher than the cytochrome c_L midpoint redox potential. Another difference is that no α-band splitting of either of the *Pseudomonas* AM1 cytochromes c occurred at low temperature. Perhaps of particular importance is the observation that the cytochromes c_L of both bacteria were similar in being most readily autoxidized, in combining with CO to the greatest extent and in reacting most rapidly with methanol dehydrogenase.

In view of the differences in the properties of the three soluble cytochromes c from *M. methylotrophus* it is unlikely that they are performing identical functions in the cell. Although it is not possible to identify a particular role for a particular cytochrome, it is possible that only one of the cytochromes may be involved in reacting with methanol dehydrogenase. It is also possible that each of the two alternative oxidases (o and $a + a_3$) accepts electrons from a specific cytochrome c (see Cross & Anthony, 1980).

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