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# The autoreducible cytochromes *c* of the methylotrophs *Methylophilus methylotrophus* and *Pseudomonas* AM1

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The two types of soluble cytochrome *c* (cytochrome $c_H$ and cytochrome $c_L$) found in methylotrophs are completely distinct proteins; one type is not a dimer or degradation product of the other. Free thiol groups are probably not involved in the unusually rapid autoreduction of the cytochromes at high pH. The axial ligands to the haem iron, histidine and methionine, are the same as in other low-spin cytochromes *c*. The methionine ligand is displaced at high pH by an alternative strong-field ligand. This displacement does not occur on reduction of cytochrome $c_L$ by methanol dehydrogenase, but this does not rule out the possibility that the autoreduction mechanism is involved in the interaction of the dehydrogenase and cytochrome *c*.

Methylotrophs usually contain two soluble cytochromes *c*, at least one of which is involved in electron transport from the unusual quinoprotein methanol dehydrogenase (Anthony, 1981, 1982; Ohta & Tobari, 1981). They have been most extensively studied in the obligate methylotroph *Methylophilus methylotrophus* (Cross & Anthony, 1980) and the facultative methylotroph *Pseudomonas* AM1 (O'Keeffe & Anthony, 1980*b*). These studies showed that the two types of cytochrome *c* have markedly different isoelectric points, and these have been used as the basis of a provisional terminology: cytochrome $c_L$ has a low isoelectric point and a high molecular weight (about 20000), whereas cytochrome $c_H$ has a high isoelectric point and a low molecular weight (8500–11000). Because a single mutation leads to loss of both cytochromes *c* (O'Keeffe & Anthony, 1980*b*), it is necessary to consider whether or not one type is a dimer or degradation product of the other. Both types react with CO and both are rapidly reduced at high pH in the absence of added reductant (autoreduction). It has been proposed that this may occur by way of an intramolecular electron transfer to the haem iron from a weakly acidic group (deprotonated) on the protein, and that this group might possibly replace the usual sixth ligand during the autoreduction process (O'Keeffe & Anthony, 1980*a*; Anthony, 1982). Other workers have suggested that cysteine might be the weakly acidic group giving rise to the electron donor during autoreduction of Japanese-radish cytochrome *f* (Tanaka *et al.*, 1978).

Abbreviation used: m.c.d., magnetic circular dichroism.

A key question in relation to the function of the cytochromes *c* of methylotrophs concerns their role in electron transport from methanol dehydrogenase. This enzyme reduces both the cytochromes *c* of *Pseudomonas* AM1 (at pH 7.0) at the same rate in the presence and in the absence of methanol, and it has been suggested that this occurs because the p*K* of the acidic group whose dissociation provides the electron donor in the autoreduction process is lowered on binding methanol dehydrogenase (O'Keeffe & Anthony, 1980*a*). Whether or not the electron transfer from methanol to cytochrome *c* that is catalysed by methanol dehydrogenase also involves the same autoreduction mechanism is not certain.

The present paper addresses the following questions relating to the structure and function of methylotroph cytochromes *c*: are cytochromes $c_H$ and $c_L$ derivatives or dimers of one another; is a thiol group involved in their autoreduction; is methionine the sixth ligand to the iron, as found in other cytochromes *c*; and does the nature of the ligation change during autoreduction by methanol dehydrogenase?

## Materials and methods

All methods for growth of *Pseudomonas* AM1 and *Methylophilus methylotrophus* and of the purification of cytochromes *c* and methanol dehydrogenases from them have been fully described previously (O'Keeffe & Anthony, 1980*b*); Cross & Anthony, 1980). Methods for measurement of



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absorption spectra, autoreduction and reduction of cytochromes by methanol dehydrogenase were as reported by O'Keeffe & Anthony (1980*a*).

*Chemicals*

*Staphylococcus aureus* V8 proteinase was obtained from Miles Laboratories (Stoke Poges, Bucks., U.K.). Trypsin was obtained from Sigma Chemical Co. (Kingston upon Thames, Surrey, U.K.). All other chemicals were obtained from sources previously listed (O'Keeffe & Anthony, 1980*b*).

*Amino acid analysis and proteolysis of cytochromes*

Amino acid analyses were performed with a Rank Hilger Chromaspec amino acid analyser after hydrolysis of the native proteins (5–20nmol) with 6M-HCl at 105°C for 24h and 48h in sealed evacuated ampoules. Cysteine was determined (as cystine) after performic acid oxidation followed by hydrolysis in 6M-HCl for 22h according to the method of Hirs (1967). Tryptophan was determined by titration with *N*-bromosuccinimide by the method of Spande & Witkop (1967). Norleucine was used as standard, and bovine insulin and cytochrome *c* were used to check recovery of amino acids. Enzymic proteolysis and analysis of proteolysis products was based on the method of Cleveland *et al.* (1977) as described for the digestion of proteins in gel slices with *Staphylococcus aureus* V8 proteinase. The method differed slightly from this in that larger amounts of protein could be used (80$\mu$g of cytochrome *c*), and staining was not required to locate the cytochrome after the initial preparative electrophoresis. Molecular-weight calibration during gel electrophoresis was done with horse heart cytochrome *c*, insulin and trypsin with the sodium dodecyl sulphate/Tris buffer system of Weber & Osborn (1975). The *N*-terminal amino acids of cytochrome $c_H$ of *Methylophilus methylotrophus* were determined by Edman degradation (Edman, 1956), with the use of high-pressure liquid chromatography for identification of the modified amino acids.

*M.c.d. measurements of cytochrome $c_L$ from Pseudomonas AM1*

We thank Dr. A. J. Thomson of the University of East Anglia for help with these measurements. They were done at room temperature with a JASCO (Easton, MD, U.S.A.) J500D spectropolarimeter. Spectra were recorded in 10mm-light-path cells in normal and reverse magnetic fields of 4.71T; the spectral bandwidth was 1nm and the scan speed was 0.8nm/s.

Table 1. *Amino acid composition of cytochromes $c_H$ and $c_L$ from Pseudomonas AM1 and Methylophilus methylotrophus*
Details are given in the Materials and methods section. The calculated molecular weights include 1 haem group per molecule of cytochrome *c*. The molecular weights determined by sodium dodecyl sulphate (SDS)/polyacrylamide-gel electrophoresis have been reported previously (O'Keeffe & Anthony, 1980*b*; Cross & Anthony, 1980).

| | *Pseudomonas* AM1 | | *Methylophilus methylotrophus* | |
|---|---|---|---|---|
| Amino acid | Cytochrome $c_H$ | Cytochrome $c_L$ | Cytochrome $c_H$ | Cytochrome $c_L$ |
| Asp | 10 | 24 | 8 | 17 |
| Thr | 4 | 14 | 2 | 4 |
| Ser | 4 | 7 | 4 | 2 |
| Glu | 7 | 26 | 6 | 30 |
| Pro | 4 | 13 | 4 | 16 |
| Gly | 10 | 23 | 7 | 28 |
| Ala | 11 | 14 | 12 | 23 |
| Cys | 2 | 3 | 2 | 3 |
| Val | 5 | 9 | 5 | 5 |
| Met | 1 | 3 | 1 | 3 |
| Ile | 2 | 6 | 5 | 8 |
| Leu | 5 | 15 | 6 | 12 |
| Tyr | 2 | 6 | 2 | 7 |
| Phe | 3 | 5 | 1 | 7 |
| His | 2 | 7 | 2 | 9 |
| Lys | 11 | 13 | 11 | 12 |
| Arg | 1 | 4 | 0 | 4 |
| Trp | 1 | 2 | 1 | 3 |
| Calc. mol.wt. | 9859 | 19053 | 8919 | 21200 |
| Mol.wt. estimated by SDS/polyacrylamide-gel electrophoresis | 11000 | 20900 | 8500 | 21000 |

## Results and discussion

### *Amino acid composition and proteolytic digestion of the cytochromes*

The amino acid compositions of both cytochromes $c_H$ and $c_L$ are shown in Table 1. The *N*-terminal sequence of amino acids of cytochrome $c_H$ from *M. methylotrophus* was shown to be Ala-Asp-. The cytochromes $c_H$ contained only the two cysteine residues involved in thioether linkages to the haem, but the cytochromes $c_L$ contained an additional cysteine residue. Whereas this cysteine might be involved in the autoreduction mechanism, as proposed for Japanese-radish cytochrome *f* (Tanaka *et al.*, 1978), the lack of an extra cysteine residue rules this out for the cytochromes $c_H$. As the characteristics of autoreduction are very similar in the two types of cytochrome ($c_H$ and $c_L$), it is probable that the cysteine residue is not involved in autoreduction of either of the cytochromes *c* of these methylotrophs.

The amino acid composition of the cytochrome $c_H$ from *M. methylotrophus* is similar to that of the cytochrome $c_H$ of *Pseudomonas* AM1, and the two cytochromes $c_L$ are also similar to each other. There is, however, little similarity between cytochrome $c_H$ and cytochrome $c_L$. Comparison of the relative proportions of amino acids in the two types shows that cytochrome $c_L$ is not a dimeric form of cytochrome $c_H$. However, because cytochrome $c_L$

Table 2. *Proteolytic digestion products from cytochrome $c_H$ and $c_L$ from Pseudomonas AM1 and Methylophilus methylotrophus*

Experimental details are given in the Materials and methods section. The proteinase concentration was 15–125pg of V8 proteinase/μg of cytochrome *c*. Raising the proteinase concentration 20-fold led to complete digestion of the cytochrome $c_L$ (no detectable digestion products), but it led to no further digestion of cytochrome $c_H$. The molecular weights given below are averages taken from four separate digestions, each of which were analysed in eight different gels; all values fell within ±3% of the average value. Band 1 is the undigested cytochrome. The cytochrome $c_L$ of *M. methylotrophus* is 21000 when isolated, but readily loses a 4000-dalton fragment on storage (Cross & Anthony, 1980); a sample that had lost this fragment was used for this digestion work.

| | Mol.wts. of digestion products of cytochromes | |
|---|---|---|
| | *Pseudomonas* AM1 | *M. methylotrophus* |
| Cytochrome $c_H$ | | |
| Band 1 | 11000 | 8500 |
| Band 2 | 9300 | — |
| Cytochrome $c_L$ | | |
| Band 1 | 21000 | — |
| Band 2 | 17800 | 17000 |
| Band 3 | 14600 | 14450 |
| Band 4 | 11600 | 11900 |
| Band 5 | 9300 | 11000 |

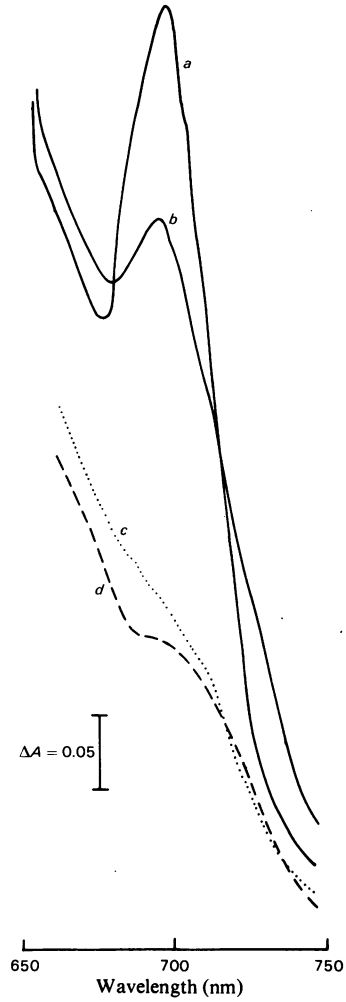


Fig. 1. *Near-i.r.-absorption spectra of the cytochromes c from Pseudomonas AM1*

The spectra were run at 20°C in 20mM-Mops (4-morpholinepropanesulphonic acid) buffer, pH 7.0, or in 20mM-Caps (cyclohexylaminopropanesulphonic acid) buffer, pH 10.0. The concentration of cytochromes was 135μM. Spectrum *a*, ferricytochrome $c_L$ at pH 7.0; spectrum *b*, ferricytochrome $c_H$ at pH 7.0; spectrum *c*, cytochrome $c_L$ at pH 10.0; spectrum *d*, cytochrome $c_L$ after incubation with 2μM-methanol dehydrogenase at pH 7.0 in the absence or in the presence of methanol.

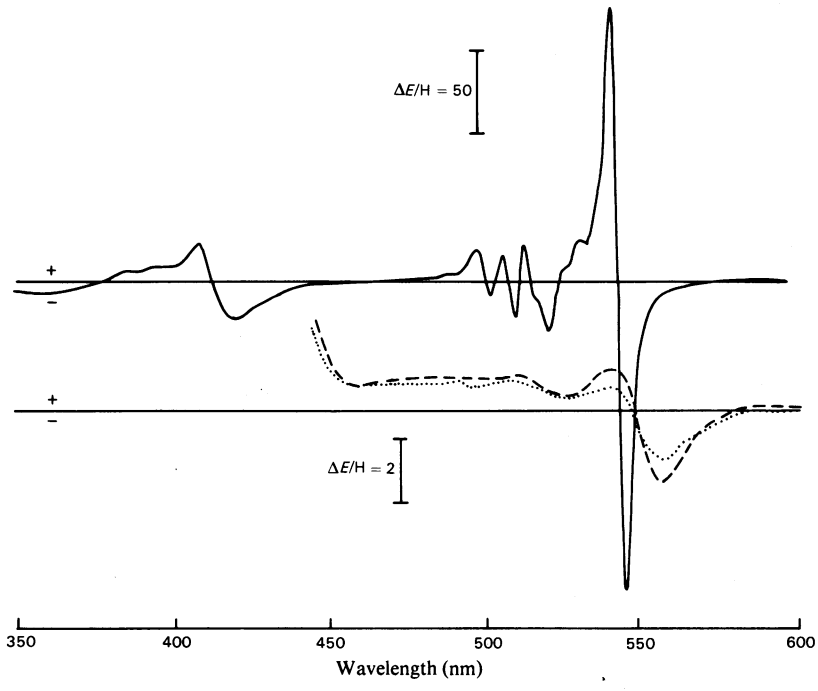


Fig. 2. *M.c.d. spectra of cytochrome $c_L$ from Pseudomonas AM1*

Experimental details are given in the Materials and methods section. The concentration of cytochrome was 93 μM. Spectra were run at 20°C in 50mM-Mops buffer, pH 7.0, or in 50mM-Caps buffer, pH 10.0. The horizontal lines indicate the zero-field positions and the $\Delta E/H$ units are $(M \cdot cm \cdot T)^{-1}$. ——, Dithionite-reduced cytochrome $c_L$ at pH 7.0; – – – –, ferricyanide-oxidized cytochrome $c_L$ at pH 7.0; ·····, ferricytochrome $c_L$ after autoreduction at pH 10.0 followed by oxidation at this pH with ferricyanide.

contained more of most amino acids than cytochrome $c_H$, further evidence was necessary to show that cytochrome $c_H$ is not derived from cytochrome $c_L$. This was done by analysing the products of digestion of the two types of cytochrome *c* by trypsin and by *Staphylococcus aureus* V8 proteinase. The results shown in Table 2 confirm that cytochromes $c_H$ and $c_L$ are completely distinct entities, and that the two cytochromes $c_H$ are similar to each other, as are the two cytochromes $c_L$. Thus the cytochromes $c_H$ are relatively insensitive to proteinase, whereas the cytochromes $c_L$ gave rise to at least four bands during electrophoresis of the digestion products. These were of similar molecular weights for the cytochromes $c_L$ from *Pseudomonas* AM1 and *M. methylotrophus*. Conditions that led to complete digestion of the larger cytochromes $c_L$ resulted in very little digestion of the smaller cytochromes $c_H$. This indicates that cytochrome $c_H$ is not a 'fragment' of cytochrome $c_L$. This conclusion was confirmed by using trypsin instead of V8 proteinase. Cytochrome $c_L$ from both organisms was completely digested, whereas the smaller cytochromes $c_H$ were completely unaffected by trypsin and showed only the native cytochrome *c* on subsequent electrophoresis.

*Near-i.r. and m.c.d. spectra of cytochromes c of methylotrophs*

These studies depend on the fact that cytochromes having methionine as the sixth ligand to the haem iron have an i.r.-absorption band at 695nm in the oxidized form but not in the reduced form (Dickerson & Timkovitch, 1975; Pettigrew *et al.*, 1978), and on the use of m.c.d. spectra to indicate the spin state and the nature of ligation of the haem iron (Vickery *et al.*, 1976).

The ferricytochromes $c_H$ and $c_L$ from both bacteria had well-defined absorption bands at 695nm, the absorption coefficients being 0.5 and $0.35\,mM^{-1} \cdot cm^{-1}$ respectively (Fig. 1). The 695nm-absorption bands of all the cytochromes disappeared on reduction with dithionite at pH 7.0, and were restored by subsequent oxidation with ferricyanide. Reduction of the cytochrome $c_L$ of *Pseudomonas* AM1, occurring on addition of methanol dehydro-

genase in the presence or in the absence of methanol, also led to disappearance of the 695 nm band, which was again restored by oxidation with ferricyanide. When the pH of this cytochrome was raised to 10.0 (leading to autoreduction), the 695 nm band immediately disappeared (Fig. 1); it could not be restored by oxidation with ferricyanide, although lowering the pH to 7.0 led to its re-appearance.

The rate of disappearance of the 695 nm band on reduction by methanol dehydrogenase (in the presence or in the absence of methanol) was identical with the rate of appearance of the 550 nm band. By contrast, the rate of autoreduction at pH 10.0 as measured by the appearance of the 550 nm band was markedly lower than the rate of disappearance of the 695 nm band, which was extremely rapid.

Fig. 2 shows the visible m.c.d. spectrum of cytochrome $c_L$ of *Pseudomonas* AM1 after reduction with dithionite at pH 7.0; it was the same as that obtained after reduction with methanol dehydrogenase at pH 7.0 and after autoreduction at pH 10.0. Fig. 2 shows that the m.c.d. spectra of the oxidized cytochromes were similar at pH 7.0 and pH 10.0. All the spectra in Fig. 2 were similar to those of mammalian cytochrome *c* (Vickery *et al.*, 1976).

These results show that cytochrome $c_L$ from *Pseudomonas* AM1 is a low-spin cytochrome *c* having (at pH 7.0) the usual axial ligands, to the haem iron, of histidine and methionine. As found with mammalian cytochrome *c*, the methionine ligand is displaced at high pH (pH 10.0) by an alternative strong-field ligand. By contrast, no displacement occurs on reduction of cytochrome $c_L$ by methanol dehydrogenase at pH 7.0. At high pH the displacement of the methionine ligand occurs far more rapidly than the intramolecular autoreduction of the cytochrome, thus suggesting that the two processes occur independently. If this is the case, then the reduction of cytochrome $c_L$ by methanol dehydrogenase might possibly involve the autoreduction mechanism, as previously suggested (O'Keeffe & Anthony, 1980*a*), even though the displacement of the methionine ligand does not occur during the reduction by methanol dehydrogenase.

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