

The two cytochromes *c* in the facultative methylotroph *Pseudomonas* AM1

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It was previously suggested that there is only one soluble cytochrome *c* in *Pseudomonas* AM1, having a molecular weight of 20000, a redox midpoint potential of about +260 mV and a low isoelectric point [Anthony (1975) *Biochem. J.* **146**, 289–298; Widdowson & Anthony (1975) *Biochem. J.* **152**, 349–356]. A more thorough examination of the soluble fraction of methanol-grown *Pseudomonas* AM1 has now revealed the presence of two different cytochromes *c*. These were both purified to homogeneity by acid treatment, ion-exchange chromatography, gel filtration, chromatography on hydroxyapatite and preparative isoelectric focusing. Molecular weights were determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; midpoint redox potentials were determined directly by using platinum and calomel electrodes; isoelectric points were estimated by electrophoresis and by the behaviour of the two cytochromes on ion-exchange celluloses. The more abundant cytochrome c_H (λ_{\max} , 550.5 nm) had a low molecular weight (11000), a midpoint potential of about +294 mV and a high isoelectric point, not being adsorbed on DEAE-cellulose in 20 mM-Tris/HCl buffer, pH 8.0. The less abundant cytochrome c_L (λ_{\max} , 549 nm) was about 30% of the total; it had a high molecular weight (20900), a midpoint potential of about +256 mV and a low isoelectric point, binding strongly to DEAE-cellulose in 20 mM-Tris/HCl buffer, pH 8.0. The pH-dependence of the midpoint redox potentials of the two cytochromes *c* were very similar. There were four ionizations affecting the redox potentials in the pH range studied (pH 4.0–9.5), two in the oxidized form (p*K* values about 3.5 and 5.5) and two in the reduced form (p*K* values about 4.5 and 6.5), suggesting that the ionizing groups involved may be the two propionate side chains of the haem. Neither of the cytochromes *c* was present in mutant PCT76, which was unable to oxidize or grow on C_1 compounds, although still able to grow well on multicarbon compounds such as succinate. Whether or not these two cytochromes *c* have separate physiological functions is not yet certain.

Pseudomonas AM1 is a facultative methylotroph unable to grow on methane but able to grow on other C_1 compounds and on a wide range of multicarbon compounds such as succinate. Electron transport from methanol requires an unusual methanol dehydrogenase and cytochrome *c*, but not cytochrome *b* (Anthony, 1975; Widdowson & Anthony, 1975; Netrusov & Anthony, 1979). Cytochrome *c* is also involved in methanol oxidation in methanotrophs (Higgins, 1979) and in *Paracoccus denitrificans* (van Verseveld & Stouthamer, 1978; Bamforth & Quayle, 1978). Whether or not methanol dehydrogenase and cytochrome *c* interact directly is not yet certain (see Anthony, 1975; Duine *et al.*, 1979). In order to investigate the possibility of

such a direct reaction we set out to purify and characterize the cytochrome *c* of *Pseudomonas* AM1. During this work it was found that this methylotroph produces two species of cytochrome *c*, and that the description of cytochrome *c* previously published from our laboratory (Anthony, 1975) was in fact a composite, and thus erroneous, description of two different cytochromes *c*. The present paper describes the complete purification and characterization of these two different species of cytochrome *c* from *Pseudomonas* AM1.

It should be noted that the cytochromes *c* from methylotrophs are unusual in binding CO to some extent; they are therefore sometimes referred to as cytochromes c_{CO} . Because we consider that this phenomenon has not been sufficiently investigated and because its physiological significance (if any)

Abbreviation used: SDS, sodium dodecyl sulphate.

has not yet been elucidated, we have avoided this terminology.

In the present paper the subscripts H and L are used to denote cytochrome *c* with high and low isoelectric points respectively. Numerical subscripts have been avoided in order to prevent confusion with mitochondrial cytochrome *c*₁, cytochrome *c*₂ of photosynthetic bacteria, cytochromes *c*₄ and *c*₅ of *Azotobacter* etc.

A preliminary report of this work has been published (O'Keeffe & Anthony, 1979).

Materials and methods

Chemicals

All chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K., except the following: 2,3,5,6-tetramethyl-*p*-phenylenediamine (from Aldrich Chemical Co., Gillingham, Dorset, U.K.); acrylamide (from Koch-Light Laboratories, Colnbrook, Bucks., U.K.); Sephadex G-150, G-75, G-50 and G-25 (from Pharmacia Fine Chemicals, Uppsala, Sweden); 4-morpholinepropanesulphonic acid, Tris, insulin, myoglobin, ovalbumin and trypsin [all from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.]; DEAE-cellulose and CM-cellulose (from Whatman, Maidstone, Kent, U.K.); hydroxyapatite (from Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Organisms and growth media

Pseudomonas AM1 (N.C.I.B. 9133) was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, U.K. Stock cultures were maintained on methylamine/agar. The defined liquid growth medium of MacLennan *et al.* (1971) was used, and methanol (0.4%, w/v) was used as carbon source.

Growth and harvesting of bacteria

Bacteria were grown as stirred aerated 18-litre batch cultures in 20-litre glass pots at 30°C. The bacterial cultures were harvested at 4°C by using a Sharples Super centrifuge. They were washed twice in 20 mM-Tris/HCl buffer pH 8.0, at 4°C before disruption.

Measurement of absorption spectra

All spectra were recorded with a Cary 118C double-beam spectrophotometer (Varian Associates, Walton on Thames, Surrey, U.K.). Unless otherwise stated, cuvettes of 10 mm light-path were used. Spectra were recorded at 20–25°C unless otherwise stated. The scan speed was usually 1 nm/s and the pen period was 1 s. The slit-width was 0.03 mm, which corresponds to a spectral bandwidth at 550 nm of 0.6 nm. That maximum resolution was being achieved was confirmed by scanning at slower

speeds (0.2 nm/s) with narrower slits (0.02 mm). The reductant was dithionite and the oxidant was ferricyanide for reduced-minus-oxidized difference spectra. Absolute spectra were obtained by measuring the spectra of dithionite- or ascorbate-reduced cytochrome against a reference solution containing the same buffer as the cytochrome solution. For measurement of (reduced-plus-CO)-minus-reduced difference spectra, cytochrome in both cuvettes was reduced with solid Na₂S₂O₄ followed by bubbling with CO through one of the cuvettes for 2 min, and the difference spectra were recorded immediately and at intervals up to 60 min. The cuvettes were sealed with SubaSeal caps to exclude air during incubations with CO. Base-lines were always checked before recording of spectra. Spectra at 77 K were obtained by placing Perspex [poly(methyl methacrylate)] cuvettes containing cytochrome samples in liquid N₂ and then quickly transferring them to an insulated liquid-N₂-containing box, placed in the sample position of the spectrophotometer. The cuvettes (2 mm light-path) were made by separating two sheets of Perspex, forming the optical surfaces, by an aluminium 'former', which also served as a heat conductor. The $(A_{550} - A_{575})/A_{280}$ ratios were calculated by using untreated (oxidized) cytochrome for the *A*₂₈₀ measurement and the dithionite-reduced cytochrome for the *A*₅₅₀–*A*₅₇₅ measurement (Ambler, 1963).

Purification of cytochromes *c*_H and *c*_L

These methods are adapted from those previously described (Anthony, 1975). Bacterial cells (200 g wet wt.) were suspended in 150 ml of 20 mM-Tris/HCl buffer, pH 8.0, and ruptured in a French pressure cell (American Instrument Co., Silver Spring, MD, U.S.A.) at 100 MPa followed by sonication at 2°C for a total of 9 min. Whole cells and cell debris were removed by centrifugation at 40 000 g for 2 h at 4°C. The resultant pellet was subjected to a second sonication and high-speed centrifugation, and the two high-speed supernatants were combined.

The pH of the high-speed supernatant was lowered to pH 4.0 with 1 M-HCl, and the resultant precipitate was removed by centrifugation. The supernatant was adjusted to pH 7.0 with 1 M-NaOH and then dialysed in a beaker dialyser (Biofibre 50; Bio-Rad Laboratories) against 4 litres of 20 mM-Tris/HCl buffer, pH 8.0, before application to a DEAE-cellulose column (26 cm × 3.5 cm) equilibrated with the same buffer.

Cytochrome *c*_H and methanol dehydrogenase did not bind to the DEAE-cellulose. The eluate containing the cytochrome and dehydrogenase was concentrated by ultrafiltration on an Amicon PM10 membrane (Amicon, High Wycombe, Bucks., U.K.) under N₂ before gel filtration on a Sephadex G-150

column (85 cm × 4 cm) equilibrated with a mixture of 100 mM-Tris/HCl buffer, pH 8.0, and 200 mM-NaCl. After gel filtration cytochrome c_H was dialysed against 10 mM-sodium phosphate buffer, pH 6.0, and then subjected to chromatography on a CM-cellulose column (24 cm × 4 cm) equilibrated with 10 mM-phosphate buffer, pH 6.0. The cytochrome was eluted at 40–45 mM-sodium phosphate when a linear gradient of 10–150 mM-sodium phosphate buffer, pH 6.0, was applied to the column in a total volume of 250 ml. The eluted cytochrome was again concentrated by ultrafiltration before gel filtration on a Sephadex G-50 column (93 cm × 1.8 cm) in 100 mM-Tris/HCl buffer, pH 8.0. The cytochrome was then dialysed against 10 mM-sodium phosphate buffer, pH 6.0, before chromatography and step-wise elution from a hydroxyapatite column (1.5 cm × 2.8 cm). The cytochrome was subsequently extensively dialysed against distilled water and stored at -17°C .

Cytochrome c_L was eluted from the DEAE-cellulose column in 220 mM-Tris/HCl when a linear gradient of 100–300 mM-Tris/HCl buffer, pH 8.0, was applied in a total gradient volume of 2 litres. The cytochrome was then concentrated by ultrafiltration and applied to a Sephadex G-50 column. The pooled cytochrome fractions were again concentrated by ultrafiltration and then applied to a Sephadex G-150 column. The resultant cytochrome was extensively dialysed against distilled water.

Although both cytochromes at this stage ran as single bands during SDS/polyacrylamide-gel electrophoresis, isoelectric focusing showed one very minor contaminant in cytochrome c_H and several protein bands in cytochrome c_L . The cytochromes were therefore subjected to preparative isoelectric focusing.

Electrofocusing of cytochromes c_H and c_L

Preparative isoelectric focusing of the cytochromes was performed in a Sephadex G-50 gel by using the LKB2117 Multiphor system. Cytochrome c_H was electrophoresed in the pH 8–9.5 range at an initial voltage of 0.39 kV at 20.5 mA. Cytochrome c_L was electrophoresed in the pH range 3.5–5.5 at an initial voltage of 0.5 kV at 16 mA. After electrophoresis for 15 h at 10°C at a limiting power of 8 W the gel was sectioned and the pH of each section was measured directly with a pH electrode. The cytochromes were easily visible as coloured bands and were eluted from the granular gel with distilled water. Ampholines used to create the pH gradient were removed by gel filtration on Sephadex G-50.

Analytical electrofocusing was done on LKB 804-101 Ampholine polyacrylamide-gel plates in the pH 3.5–9.5 range in the LKB 2117 Multiphor Universal apparatus. Before staining, gels were fixed in a mixture of trichloroacetic acid and sulpho-

salicylic acid (57.5 and 17.25 g respectively in 500 ml of water). Protein bands were stained with Coomassie Brilliant Blue (0.46 g in 400 ml of destaining solution). Destaining solution was an equal mixture of ethanol (25%, v/v) and acetic acid (8%, v/v).

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed by the method of Swank & Munkres (1971) with polyacrylamide gels (12.5% acrylamide) containing 6 M-urea at pH 6.8. For estimation of molecular weight the following protein standards were used (mol.wts. in parentheses): insulin (5700), horse heart cytochrome *c* (11 700), lysozyme (14 100), myoglobin (17 200), trypsin (23 000) and ovalbumin (42 000). Proteins were stained with Coomassie Brilliant Blue (see above).

Determination of haem

The haem content of the cytochromes was determined by the method of Rieske (1967) by using a mixture containing equal volumes of pyridine and 0.5 M-NaOH. The concentration of haem was calculated from the difference spectrum of the reduced and oxidized pyridine haemochrome by assuming an absorption coefficient of $19.1\text{ mm}^{-1}\cdot\text{cm}^{-1}$ for the 550 nm-minus-575 nm wavelength pair.

Measurement of midpoint redox potentials

Midpoint redox potentials were measured by the method of Dutton *et al.* (1970) by using platinum electrodes with a salt bridge completing the circuit to a calomel electrode. The reaction mixture contained 25 mM-buffer, 100 mM-KCl, 50 μM -2,3,5,6-tetramethylphenylenediamine and 4.3–8.6 μM -cytochrome *c*. Titrations were performed by injection of microlitre quantities of $\text{K}_3\text{Fe}(\text{CN})_6$ (which also acted as mediator) or sodium ascorbate through side arms in the redox cells. The reaction mixture was constantly stirred, and the extent of reduction of the cytochromes was determined by recording the spectrum between 535 and 575 nm at approx. 5 mV steps in potential. The difference in potential between the platinum and reference electrodes was measured with a digital voltmeter and a correction made to give the potential with respect to the hydrogen half-cell (an addition of 247 mV). All titrations were performed under O_2 -free N_2 . Residual traces of O_2 present in commercial 'white spot' N_2 were removed by passing the gas through an ' O_2 -scrubbing' solution as described by Sweetser (1967).

The following buffer solutions were used: acetic acid/sodium acetate (pH 4.0–5.8); $\text{NaH}_2\text{PO}_4/\text{NaOH}$ (pH 5.8–6.5); Mops (4-morpholinepropane-sulphonic acid) (pH 6.5–7.9); Hepes [4-(2-hydroxy-

ethyl-1-piperazine-ethanesulphonic acid] (pH 6.8–8.2); Tris (pH 7.5–8.8); 2-(*N*-cyclohexylamine)-ethanesulphonic acid (pH 9.0–10.1); cyclohexylaminepropanesulphonic acid (pH 9.7–11.1).

Results

Purification of cytochromes c_H and c_L

Table 1 summarizes the purification procedure used for the two soluble cytochromes c of *Pseudomonas* AM1. They were initially separated by chromatography on DEAE-cellulose at pH 8.0. Cytochrome c_H is the fraction having a high isoelectric point and thus unable to bind to DEAE-cellulose. Cytochrome c_L is the fraction binding to DEAE-cellulose (the subscript L indicating low isoelectric point). These subscripts are used because the α -band maxima are only 1.5 nm apart and, although reproducible, the cytochromes are not easily identifiable by wavelength maxima alone.

The results in Table 1 indicate that the molar ratio of cytochrome c_H to cytochrome c_L was about 2.6:1. When extract was passed through DEAE-cellulose without prior acid treatment, the proportion of cytochrome c_H to c_L was the same. Likewise the same proportion of the two cytochromes was demonstrated in acid-treated extracts of succinate-grown cells.

Isoelectric points of cytochromes c_H and c_L

Before the final stage of purification (preparative electrophoresis) both cytochromes c appeared homogeneous on SDS/polyacrylamide-gel electrophoresis. However, analytical electrofocusing on polyacrylamide-gel plates as described in the Materials and methods section showed a lack of homogeneity in the preparations, and so a final purification stage of electrofocusing was included. This separated a non-cytochrome impurity (less than 2% of the total protein) from both cytochromes c . After preparative isoelectric focusing, cytochrome c_H was eluted as a single cytochrome band of isoelectric point 8.8. Cytochrome c_L consisted of four cytochrome bands; the main component (90% of cytochrome c_L) had an isoelectric point of 4.2, and the three minor components had isoelectric points of 3.9 (1% of cytochrome c_L), 4.0 (4% of cytochrome c_L) and 4.3 (5% of cytochrome c_L). All properties described in the present paper relate to the major component of cytochrome c_L .

Spectral characteristics of cytochromes c_H and c_L

The two cytochromes c differed by 1.5 nm in their absorbance maxima and they had slightly different molar absorption coefficients (Fig. 1 and Table 2).

The $(A_\alpha - A_{375})/A_{280}$ ratios were 1.13 and 1.0 for cytochromes c_H and c_L respectively; these values are similar to those obtained for species of cytochrome c

Table 1. Purification of two cytochromes c from methanol-grown *Pseudomonas* AM1

Details of methods 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_L has a low isoelectric point and so binds to DEAE-cellulose. Before separation on DEAE-cellulose, the cytochrome concentrations were calculated by using an average molar absorption coefficient of $28.5 \text{ mm}^{-1} \cdot \text{cm}^{-1}$. After separation, the coefficients were 26 for cytochrome c_L and 31 for cytochrome c_H . Because accurate cytochrome determinations are impossible in the crude extract, the yield and purification factors for later purification stages were calculated from the amount and specific activity in the supernatant after acid treatment. This initial treatment yielded a purification of about 3-fold in about 100% yield. The final electrophoretic treatment of cytochrome c_L produced one major band plus four minor bands; these were measured separately and the total amounts were combined to give the value in the Table.

Purification step	Volume (ml)	Total protein (mg)	Total cytochrome (μmol)	Concn. of cytochrome (nmol/mg)	Yield (%)	Purification factor
Acid treatment	370	1655	6.0	3.6	100	1
Cytochrome c_H						
DEAE-cellulose	590	590	3.68	6.2	61	1.7
Sephadex G-150	140	448	3.52	7.9	58	2.2
CM-cellulose	200	226	3.2	14.2	53	3.9
Sephadex G-50	120	37.7	2.53	67.1	42	10.8
Hydroxyapatite	56	9.33	1.17	125	19	35
Electrofocusing	5.2	9.2	1.15	125	19	35
Cytochrome c_L						
DEAE-cellulose	162	790	1.42	1.79	19	0.5
Sephadex G-50	80	48.4	0.74	15.3	12	4.3
Sephadex G-150	102	17.3	0.67	39	11	11
Electrofocusing	10.4	13.4	0.64	48	11	13

containing two residues of tryptophan per molecule of protein (Ambler, 1963; Gürtler & Horstman, 1970). The ratio for cytochromes with only one

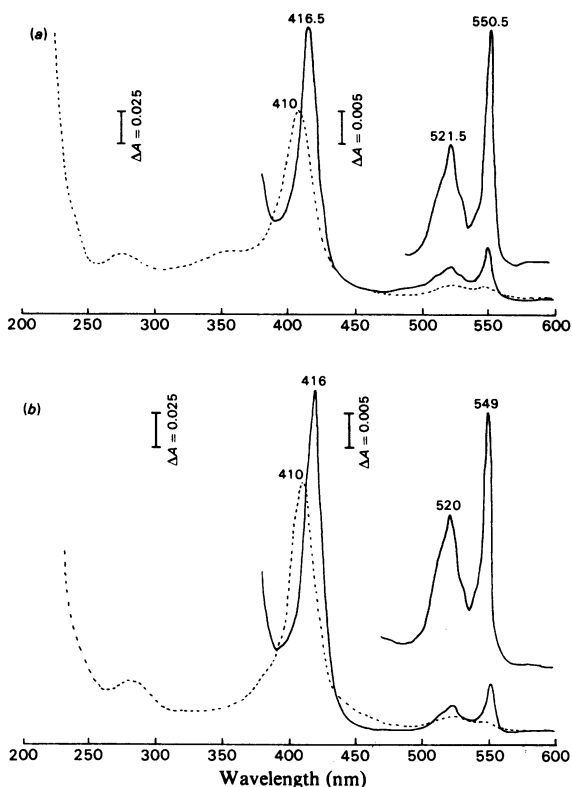


Fig. 1. Absorption spectra of cytochromes c_H and c_L (a) Cytochrome c_H (1.2 μM); (b) cytochrome c_L (1.46 μM). Continuous lines are spectra of cytochrome reduced with dithionite; broken lines are spectra of untreated (oxidized) cytochrome.

tryptophan residue per molecule is usually more than 2.

The α -band absorbance maxima of both cytochromes were lowered to about 548 nm at 77 K (Fig. 2); there was a 5–7.5-fold intensification of this band, but no splitting was observed. In this respect these cytochromes differ from most other soluble cytochromes *c*, in which splitting of the α -band at low temperatures is usual (Lemberg & Barrett, 1973), including two cytochromes *c* from the obligate methylotroph *Methylophilus methylotrophus* (Cross & Anthony, 1980) and the soluble cytochrome *c* found in supernatant fractions of the methanol-grown *Pseudomonas extorquens* (Tonge *et al.*, 1974).

Treatment with alkaline pyridine gave typical pyridine haemochrome spectra for the two cytochromes with absorbance maxima at 550 nm in dithionite-reduced-minus-ferricyanide-oxidized difference spectra. Assuming the molecular weights measured below and an absorption coefficient of $19.1 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ ($A_{550} - A_{575}$) (Rieske, 1967), then the haem/protein ratios were 0.79 and 0.97 for cytochromes c_H and c_L respectively, indicating that these cytochromes are similar to most other bacterial cytochromes *c* with high midpoint potentials in having one haem group per molecule of protein.

Molecular weights of cytochromes c_H and c_L

The molecular weights of cytochromes c_H and c_L were 11 000 and 20 900 respectively when measured by SDS/polyacrylamide-gel electrophoresis. Similar molecular weights were obtained by gel filtration on Sephadex G-50 and G-75, indicating that both of the cytochromes were monomers. That SDS/polyacrylamide-gel electrophoresis is able to denature dimeric cytochrome *c* has been shown for *Azotobacter vinelandii* cytochrome c_3 by Swank & Burris (1969).

Table 2. Properties of the two soluble cytochromes *c* of *Pseudomonas* AM1

	Cytochrome c_H	Cytochrome c_L
Relative proportions in crude extracts	72%	28%
Isoelectric point	8.8	4.2
Molecular weight	11 000	20 900
Midpoint redox potential at pH 7.0	+294 mV	+256 mV
Absorption maxima of ferrocytochrome (α , γ)	550.5 nm, 416.5 nm	549 nm, 416 nm
Absorption coefficients ($\text{mm}^{-1} \cdot \text{cm}^{-1}$) (α , γ)	31, 162	26, 163
Absorption maximum of ferricytochrome (γ)	410 nm	410 nm
Ratio of α -absorption/ γ -absorption (ferrocytochrome)	5.23	6.25
Absorption maxima of difference spectrum (α , γ)	550 nm, 416 nm	549 nm, 416 nm
Absorption coefficients ($\text{mm}^{-1} \cdot \text{cm}^{-1}$) (α , γ)	22.5, 77.35	21.8, 64.5
Absorption maxima at 77 K	548 nm	548 nm
Ratio of ($A_\alpha - A_{575}$)/ A_{280} at 20°C	1.13	1.0
Haem groups/molecule of protein	1	1
% CO-binding	36%	72%
Autoxidizability	Slow	Slow
Reduction by methanol dehydrogenase (see O'Keeffe & Anthony, 1980)	Slight	Extensive

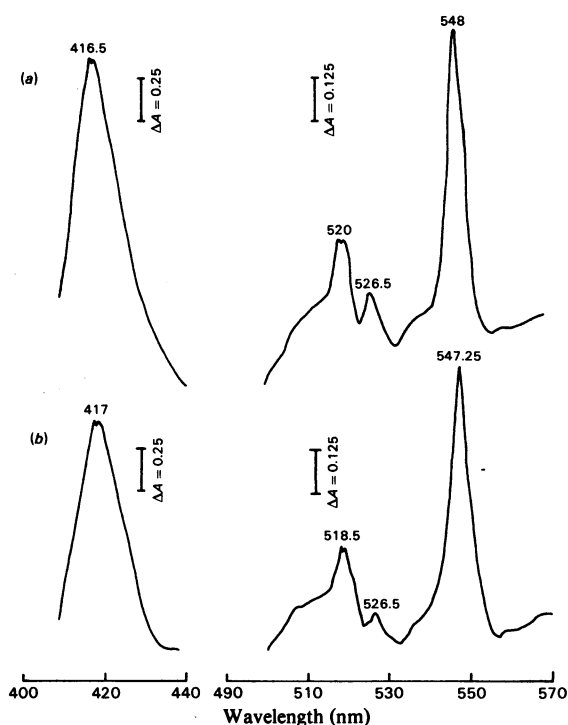


Fig. 2. *Reduced-minus-oxidized difference spectra of cytochromes c_H and c_L at 77 K*

(a) Cytochrome c_H ($4 \mu\text{M}$); (b) cytochrome c_L ($5.2 \mu\text{M}$). Cytochromes were dissolved in 30% (v/v) glycerol and 2 mm-light-path cuvettes were used (see the Materials and methods section).

Autoreduction of cytochromes c_H and c_L

Both pure cytochromes c were shown to be reduced by methanol dehydrogenase in the absence or presence of methanol (see Anthony, 1975). The significance of this in relation to the function of the cytochrome c in methylotrophic bacteria was made even more difficult to interpret by the observation that at pH 9.5 both of the cytochromes became rapidly reduced in the absence of added reductant or of methanol dehydrogenase. This autoreduction and the reduction by methanol dehydrogenase are discussed in detail elsewhere (O'Keeffe & Anthony, 1980).

Autoxidizability of the cytochromes and their reaction with CO

Neither cytochrome was rapidly autoxidizable. During the separation procedures, after removal of methanol dehydrogenase both cytochromes became slowly oxidized; whenever methanol dehydrogenase was present the cytochromes were fully reduced. After reduction with the minimum volume of dilute

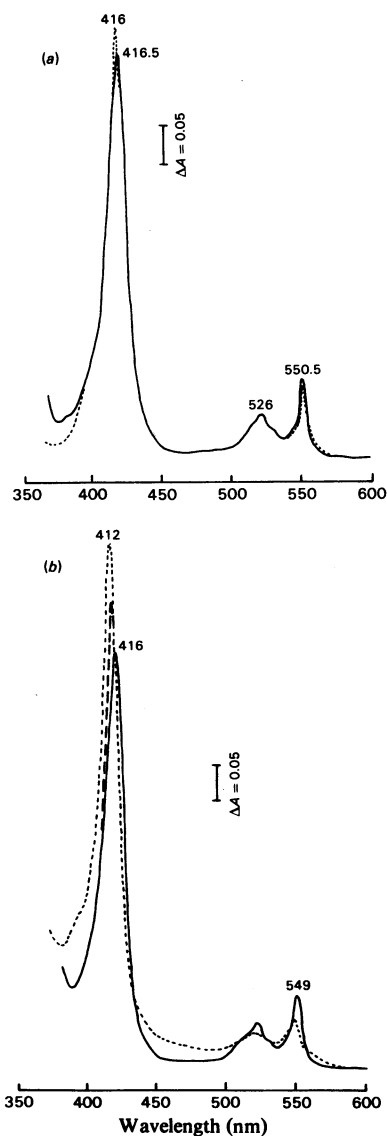


Fig. 3. *Reaction of cytochromes c_H and c_L with CO* (a) Cytochrome c_H ($3.5 \mu\text{M}$); (b) cytochrome c_L ($3.8 \mu\text{M}$). —, Reduced cytochrome c ; - - - - -, reduced cytochrome c after 30 min exposure to CO. The intermediate spectrum (— — —) was drawn after 7 min exposure to CO.

dithionite, the time taken for 50% oxidation of the pure cytochromes was 15 min and 8 min for cytochromes c_H and c_L respectively. These results argue strongly against the possibility of an oxidase function for these cytochromes.

The reaction of CO with cytochrome c of bacteria capable of oxidizing methane has led to the proposal that this cytochrome may have an oxygenase or

oxidase function, and this has been extended to propose an oxidase function for cytochrome *c* in the facultative methylotroph *Pseudomonas extorquens*, which does not grow on methane and is similar in most respects to *Pseudomonas* AM1 (Tonge *et al.*, 1975, 1977). In spite of this important conclusion with respect to reaction with CO, no full spectra of the CO-ferrocycytochrome complex have been published. Such spectra for the pure cytochromes *c* from *Pseudomonas* AM1 are presented in Fig. 3 and (reduced + CO)-minus-reduced difference spectra are shown in Fig. 4. The fact that the Soret absorption maximum of cytochrome c_L (412 nm) was the same wavelength as the Soret maximum for the (reduced + CO)-minus-reduced difference spectrum indicates that a high proportion of the cytochrome c_L was bound to CO. By contrast, the relatively slight shift in the Soret maximum of cytochrome c_H after reaction with CO indicates that relatively less of the cytochrome *c* was bound to CO. The simplest interpretation of the spectra presented here is the same as previously published (Widdowson & Anthony, 1975); both cytochromes *c* appear to form complexes with CO having absorption maxima at 412 nm, the dissociation constant of cytochrome c_H being considerably higher than that of cytochrome c_L . The rate of reaction of CO with cytochrome c_H was about half that with cytochrome c_L . About 50% of the cytochrome c_L that would finally bind had done so after 5 min reaction with CO.

For purposes of comparison it is convenient to estimate the percentage of cytochrome *c* binding to CO in CO-saturated solution, and for this purpose a molar absorption coefficient must be assumed. Bartsch & Kamen (1960) calculated a molar absorption coefficient of $165 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ for the 'peak-minus-trough' values in the (reduced + CO)-minus-reduced difference spectrum of *Chromatium* cytochrome *c*, which contains three haem groups per molecule. This was used by Weston & Knowles (1974) and by Tonge *et al.* (1974, 1975, 1977) for calculation of the percentage of cytochrome *c* binding CO in *Beneckea* and in the methylotrophs *Methylosinus trichosporium* and *Pseudomonas extorquens*. If the same coefficient of 55 (for monohaem cytochrome) is assumed in the present work for comparative purposes, then it can be concluded that 36% of the cytochrome c_H and 72% of the cytochrome c_L bind CO in CO-saturated solution at 25°C.

pH-dependence of the midpoint redox potentials of cytochromes c_H and c_L

The midpoint potentials presented in Fig. 5 and Table 3 show that cytochromes c_H and c_L are very similar in the response of their midpoint potentials to changes in pH. The E'_0 values at pH 7.0 were

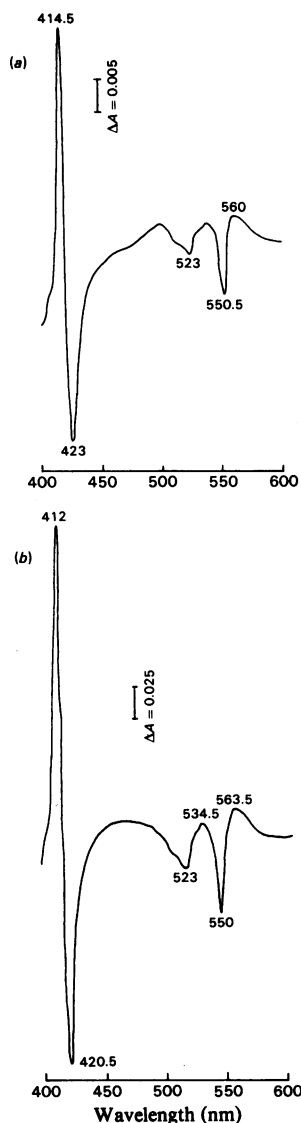


Fig. 4. (Reduced + CO)-minus-reduced difference spectra of cytochromes c_H and c_L (a) Cytochrome c_H ($3.5 \mu\text{M}$); (b) cytochrome c_L ($3.8 \mu\text{M}$). Cytochromes were reduced with dithionite and CO was bubbled through for 30 min.

+294 mV and +256 mV, and these values are typical of the high-potential monohaem bacterial cytochromes (Lemberg & Barrett, 1973). The apparent E'_0 values were +404 mV and +345 mV for cytochromes c_H and c_L respectively. These values are 'apparent' because potentials were not measured below pH 4.0, and further dissociations below this pH would lead to much higher real E'_0 values. Both cytochromes have two ionizing groups in the oxidized and reduced forms, the pK values being

Table 3. *pH-dependence of midpoint redox potentials of cytochromes c_H and c_L*
 These pK and E_0 values are calculated from the data in Fig. 5 by using the equation given in the legend.

	pK_{o1}	pK_{o2}	pK_{r1}	pK_{r2}	E_0 (mV)
Cytochrome c_H	3.5	5.5	4.5	6.5	404
Cytochrome c_L	3.6	5.6	4.4	6.4	345

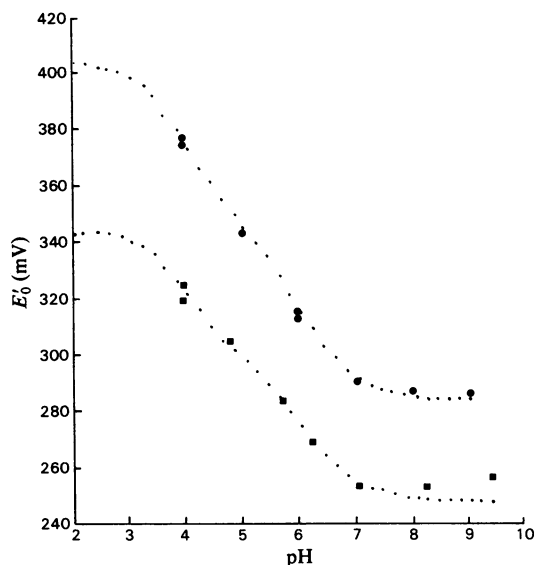


Fig. 5. *pH-dependence of the midpoint redox potentials of cytochromes c_H and c_L*

Details of the methods are given in the Materials and methods section. Oxidative and reductive titrations gave identical results. ●, Midpoint potentials of cytochrome c_H ; ■, midpoint potentials of cytochrome c_L . The dotted lines are theoretical curves calculated from the equation:

$$E'_0 = E_0 + 59 \log \left(\frac{[H^+]^2 + K_{r1}[H^+] + K_{r1}K_{r2}}{[H^+]^2 + K_{o1}[H^+] + K_{o1}K_{o2}} \right)$$

where K_{o1} and K_{o2} are the dissociation constants for the first and second ionizations in the oxidized species and K_{r1} and K_{r2} are the equivalent dissociation constants for the reduced species. The pK ($-\log K$) and E_0 values assumed for calculation of the theoretical curves are given in Table 3.

about 3.5 and 5.5 in the oxidized forms and 4.5 and 6.5 in the reduced forms. These similarities suggest that the ionizable groups and the haem environments are very similar in the two cytochromes.

If the dissociations arise from the haem, then the likely candidates are the propionate side chains. If the propionate groups are involved, then the higher of the pK values is likely to be due to the rear (inner) propionate group buried in the hydrophobic environment of the haem cleft; the lower pK would then be

due to the front (outer) propionate group in a more hydrophilic environment. Allocation of the pK values to propionate groups for these cytochromes would be consistent with proposals for the cytochromes c_2 of Rhodospirillaceae (Pettigrew *et al.*, 1975, 1978) and for cytochrome c_{551} of *Pseudomonas aeruginosa* (Moore *et al.*, 1980).

Discussion

The properties of the pure cytochromes c from *Pseudomonas* AM1 are summarized in Table 2. The previous description of a single cytochrome c from this organism (Anthony, 1975) was an accidental composite one, some properties being of cytochrome purified by one method and other properties being of cytochrome purified by an alternative method. Thus it was concluded that the 'single' cytochrome c had a high isoelectric point and a high molecular weight. As shown in the present work, the major cytochrome c has a high isoelectric point but a low molecular weight (cytochrome c_H), whereas the minor component that binds to DEAE-cellulose has a low isoelectric point and a high molecular weight (cytochrome c_L). The larger cytochrome c_L (mol.wt. 20900) is unlikely to be a dimer of cytochrome c_H (mol.wt. 11000) because these molecular weights were determined by SDS/polyacrylamide-gel electrophoresis, which dissociates dimers (Swank & Burris, 1969). The minor components of cytochrome c_L separating from it during preparative isoelectric focusing have very similar isoelectric points (all being between 3.9 and 4.3), and these may be isocytochromes produced by deamidation.

It is unlikely that cytochrome c_H is a breakdown product of cytochrome c_L because the proportion of the two cytochromes is the same whether they are separated on DEAE-cellulose within 1 h of cell disruption or after 24 h.

If, as appears to be the case, the two cytochromes c of *Pseudomonas* AM1 are fully distinct cytochromes, then the lack of both of them in the cytochrome c -deficient mutant PCT76 (Anthony, 1975) must be explained. That the mutation is not in a gene responsible for haem biosynthesis is shown by the normal concentrations of cytochromes b and $a + a_3$ in the mutant (Anthony, 1975; Widdowson & Anthony, 1975). It is possible that the mutation is in a regulatory gene affecting the concentrations of two

different cytochromes *c*. One may note a similar observation that both cytochromes *c* and *c*₁ were lost by a single mutation in *Paracoccus denitrificans* (Willison & John, 1979).

There are few full descriptions of pure cytochromes *c* from methylotrophs, and so extensive comparisons are impossible. In *Methylophilus methylotrophus* (an obligate methanol-utilizer) there are at least three soluble cytochromes *c*, and the characterization of these is extensively described in the following paper (Cross & Anthony, 1980). The best-characterized from an obligate methanotroph is that of *Methylosinus trichosporium* (Tonge *et al.*, 1975, 1977). This cytochrome *c* has been completely purified; it probably has a high isoelectric point, because it does not bind to DEAE-cellulose during the purification process. It has a low molecular weight (13 000), one haem-group molecule, a midpoint redox potential of +310 mV, an absorption coefficient of 22.5 M⁻¹ · cm⁻¹ and an absorption maximum at 551 nm.

The physiological role of the two cytochromes *c* of *Pseudomonas* AM1 is not completely clear, although some information on this is discussed elsewhere (O'Keefe & Anthony, 1980). It was thought previously that, because mutant PCT76 completely lacks cytochrome *c* but still grows on all substrates except methanol, ethanol and methylamine, cytochrome *c* may not function in the oxidation of NADH in *Pseudomonas* AM1 (Anthony, 1975; O'Keefe & Anthony, 1978). However, it has since been suggested that under conditions of carbon-limitation cytochrome *c* may be involved in the oxidation of all substrates (Keevil & Anthony, 1979). This conclusion leads to the possibility that one of the soluble cytochromes *c* may only be involved in the oxidation of methanol, whereas the other may be involved exclusively in the oxidation of other substrates in some growth conditions.

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