Biochimica et Biophysica Acta, 1059 (1991) 233–238

ADONIS 000527289100199Q

BBABIO 40250

BBA Report

Cytochrome *c'* of *Paracoccus denitrificans*

Raymond Gilmour, Celia F. Goodhew and Graham W. Pettigrew

Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh (U.K.)

(Received 11 February 1991)

Key words: Cytochrome *c'*; (*P. denitrificans*)

Cytochrome *c'* was identified in periplasmic extracts of the *Paracoccus denitrificans* strains LMD 22.21 and LMD 52.44. The cytochrome *c'* was purified from the latter using the device of sequential molecular exclusion chromatography in the dimeric and monomeric states. Although showing the overall spectroscopic features of the cytochrome *c'* family, the *Paracoccus* cytochrome *c'* is unusual in having a red-shifted oxidised Soret band at 407 nm. Also unusual is the midpoint potential of 202 mV, well above the known cytochrome *c'* range. The amino-acid composition of *Pa. denitrificans* cytochrome *c'* showed the high alanine and low proline content characteristic of the group and reflecting the predominantly α-helical character of the protein. Comparison of the amino-acid compositions suggests some similarity to the cytochromes *c'* of *Chromatium vinosum* and halotolerant *Paracoccus*.

Cytochromes *c'* are polypeptides of 125–132 amino-acid residues containing a single covalently bound haem *c*. They form the Class IIa group of cytochrome *c* sequences, with the haem bound near the C-terminus of the protein [1]. The iron is coordinated by the porphyrin and by a single extraplanar histidine, giving rise to a predominantly high-spin spectroscopic character. Cytochrome *c'* is widespread among the Rhodospirillaceae and Chromatiaceae, but is also found in the non-photosynthetic facultative anaerobes, a halophilic *Paracoccus* species and a denitrifying *Alcaligenes* species [2,3]. It is also probably present in the strict aerobes *Azotobacter vinelandii* [4] and *Nitrosomonas europaea* [5] but has not been structurally characterised from these sources.

Because of its striking resemblance to the eukaryotic mitochondrion, *Paracoccus denitrificans* is one of the most intensively studied bacteria from the bioenergetic point of view [6,7]. However, it shows great bioenergetic versatility and synthesises a different complement of cytochromes *c* under different growth conditions [8–10]. Few of these cytochromes have been fully characterised and indeed many others may remain to be discovered under different nuances of growth. In our investigations of cytochrome *c* peroxidase of *Paracoccus denitrificans* [11], we have observed small amounts of a cytochrome *c* with the spectral features of cytochrome *c'*. This paper reports the purification and characterisation of this cytochrome as a member of the cytochrome *c'* family.

Correspondence: G.W. Pettigrew, Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, EH9 1QH, U.K.

Microorganisnis and growth conditions

Paracoccus denitrificans (ATCC 19367: NCIB 8944: LMD 52.44) and *Paracoccus denitrificans* (ATCC 13543: ATCC 17741: LMD 22.21) were grown aerobically in succinate medium as described in Ref. 11. The former strain was used for most of the investigation, the latter only for comparison of cytochrome content.

Purification of cytochrome c'

Periplasmic extracts and chromatography on DEAE-cellulose were performed as described in Ref. 11. Three coloured bands were eluted from the DEAE-cellulose column at 190 mM, 240 mM and 300 mM NaCl. The second of these (4–7 μmol per kg wet cells) was a haem-containing polypeptide of M_r 12000 on SDS-PAGE with the spectroscopic features of cytochrome *c'*.

This cytochrome *c'* fraction was concentrated and further purified by molecular exclusion chromatography as described in the legend to Fig. 2. Chromatography on Sephadex G75-40 was carried out firstly at pH 8 (conditions in which the cytochrome *c'* elutes at a position corresponding to M_r 30000–35000) and then repeated on the same column but in 5% (v/v) formic acid (conditions in which the cytochrome *c'* elutes as a monomer of M_r 12000). Fractions absorbing at 410 nm

were combined, freeze-dried and taken up in 5 mM sodium phosphate (pH 7). The cytochrome *c'* was pure at this stage but renaturation was confirmed by the reappearance of the dimer on passage down the Sephadex G75-40 column at pH 8. Redox potentiometry and spectroscopy were performed as described in the legends to Figs. 3 and 4.

Amino acid analysis was performed as described in Table II. N-terminal sequence analysis was attempted using the automated Applied Biosystems 477A Protein sequencer (Welmet Protein Characterisation Facility, University of Edinburgh).

Occurrence of cytochrome c' in Pa. denitrificans

It may seem strange at first that cytochrome *c'* has not been previously described in the well-studied bacterium, *Paracoccus denitrificans*. One possible explanation might be a difference between the LMD 52.44 strain that we have used here and the more commonly studied LMD 22.21 strain (ATCC 13543: ATCC 17741). However, we found that both strains contained a very similar *c*-type cytochrome complement which included cytochrome *c'* (Fig. 1). There are small differences between the two strains indicating that they are not isolates of the same organism. For example, the mobility of the membrane cytochrome *c* of M_r 22000 is slightly less in strain LMD 22.21. Also SDS-PAGE of purified cytochrome *c*-550 from the two strains shows small mobility differences (not shown).

The absence of cytochrome *c'* in previous studies of *Pa. denitrificans* may be due to different growth conditions. Our investigations of *Pa. denitrificans* have centred on the cytochrome *c* peroxidase which is induced by low aeration. The cytochrome *c'* was isolated from these poorly aerated cultures. Bosma et al. [9] observed a cytochrome *c* band of M_r 12000 in cells of the LMD 52.44 strain. This band was not studied further, but is probably the cytochrome *c'* that we have characterised here.

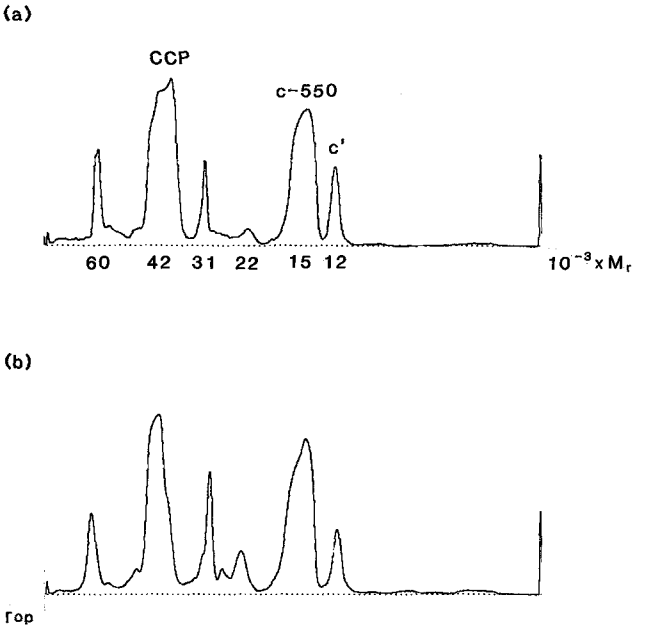

Fig. 1. The *c*-type cytochrome content of two strains of *Paracoccus denitrificans*. Cell suspensions of *Pa. denitrificans* strains LMD 22.21 and LMD 52.44, each containing 10 mg protein/ml, were disrupted by passage through a French pressure cell at $82.7 \cdot 10^6$ pascals (12000 psi). The broken cell suspensions were centrifuged at $100000 \times g$ for 2 h and samples (30 μl) of the supernatants were subjected to SDS-PAGE [21] in a gel containing 15% acrylamide and 0.4% bisacrylamide. Gels were stained for the presence of haem *c* [22] and densitometric scans shown are for (a) strain LMD 52.44 and (b) strain LMD 22.21. Because of the presence of small membrane vesicles, French press supernatants prepared in this manner contained both soluble and membrane-bound proteins. The soluble and membrane-bound *c*-type cytochromes were identified by reference to gels of periplasmic and membrane extracts of strain LMD 52.44 prepared by spheroplast formation (Fig. 1b in Ref. 11). The band of M_r 42000 is cytochrome *c* peroxidase (CCP), which is located both in the periplasmic and membrane fractions. The bands of M_r 60000, 31000 and 22000 are membrane-bound *c*-type cytochromes; the band of M_r 60000 is probably the cytochrome c_1 of the bc_1 complex [23] and the band of M_r 22000 is the *c*-type cytochrome of the ubiquinol oxidase complex [24]. The bands of M_r 15000 and 12000 are the periplasmic cytochromes *c*-550 and *c'*, respectively.

Purification of cytochrome c'

At 4–7 μmol/kg cells cytochrome *c'* is not an abundant protein in *Pa. denitrificans*. Two factors, however, made its purification relatively simple. The first is that it is a periplasmic protein and using periplasm as starting material gives a considerably enriched sample. The second factor is its aggregation state. We interpret the value of M_r of 30000–35000 on molecular exclusion chromatography at pH 8 to indicate the dimeric state. Almost all cytochromes *c'* are dimers and have been observed to behave as if larger than expected on molecular exclusion columns, presumably because of a non-spherical shape [3]. In 5% formic acid, however, the cytochrome *c'* elutes at a position corresponding to horse cytochrome *c* (M_r 12000) and contaminant proteins which co-chromatographed with the dimeric molecule (Fig. 2a) were well-separated from this monomeric form (Fig. 2b). This is a 'diagonal' purification in the sense used by Hartley [12].

The denatured form that is obtained from this chromatography in formic acid is satisfactory for structural analysis such as amino-acid composition but obviously not for investigation of properties such as midpoint redox potential. After removal of the formic acid by freeze-drying, the cytochrome *c'* dissolved completely in 5 mM sodium phosphate (pH 7) and eluted from a molecular exclusion column, run in 20 mM Tris-HCl/100 mM NaCl (pH 8) as the dimer. The midpoint potential (Fig. 3) and visible spectra (Fig. 4) of this material were indistinguishable from those of cytochrome *c'* prior to the formic acid chromatography

(not shown). We therefore conclude that denaturation in 5% formic acid is reversible and renaturation is complete.

The final purified product gives a single protein-staining band on SDS-PAGE (Fig. 2b) which contains haem *c* (not shown) and a single peak on reversed-phase HPLC (not shown). The purity index of 407/280 = 3.28 is within the range (3.1–4.8) observed for cytochromes *c′* with one tryptophan (Table I).

Properties of Pa. denitrificans cytochrome c′

In some ways, the *Pa. denitrificans* cytochrome *c′* has properties typical of the class, while in others it is quite distinctive. Particularly unusual features are the red-shifted oxidised Soret band at 407 nm, which is little different from that of a typical low-spin *c*-type cytochrome, and the midpoint potential of 202 mV which is well above the known cytochrome *c′* range. The low-spin relative of the cytochromes *c′* is *Rhodopseudomonas palustris* cytochrome c-556 which has a midpoint potential of 230 mV [13]. Further examination of the spectroscopic and magnetic features of *Pa. denitrificans* cytochrome *c′* should be made in the light of these unusual properties.

The amino acid composition of *Pa denitrificans* cytochrome *c′* shows the features of high alanine and low proline characteristic of the group and consistent with the known 4α-helical bundle motif of *Rhodospirillum molischianum* cytochrome *c′* [14]. $S\Delta N$ values calculated as described in Table III are proposed to give unbiased estimators of the number of sequence differences between pairs of amino-acid compositions [15].

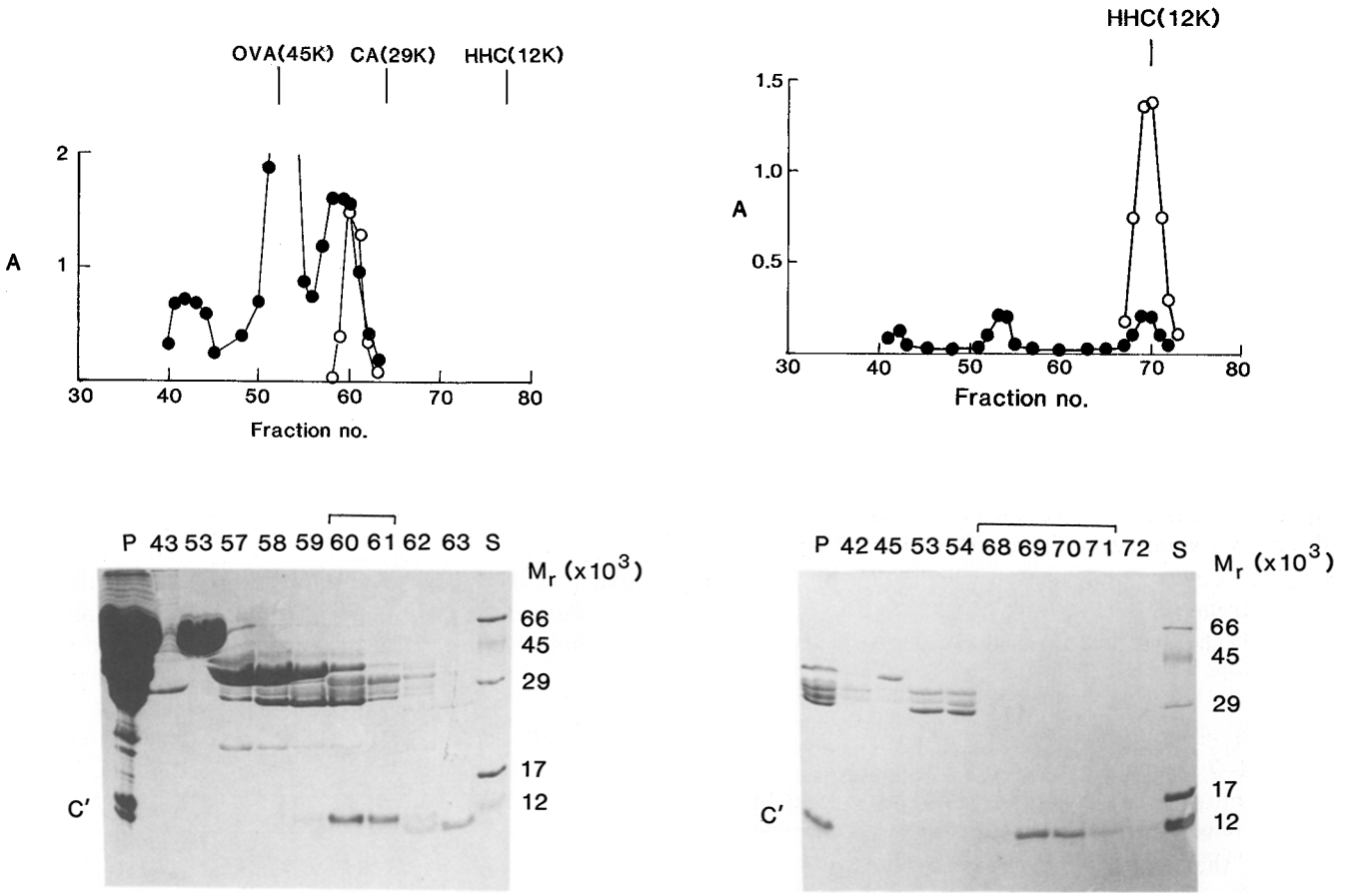

Fig. 2. Purification of *Pa. denitrificans* cytochrome *c′* by molecular exclusion chromatography in the oligomeric and monomeric states. Goodhew et al. [11] found that *Pa. denitrificans* periplasmic fraction contained three *c*-type cytochromes which could be bound to a DE-52 column and eluted with a linear salt gradient (Ref. 11, Fig. 2). The central peak absorbing at 410 nm contained material which spectroscopically resembled known cytochromes *c′*. This was diluted with 5 vol. of water and readsorbed on a small DE-52 column (2×1 cm) equilibrated in 10 mM Tris-HCl (pH 8) (4°C). The cytochrome *c′* was eluted from this column in the same buffer containing 0.5 M NaCl and the concentrated solution was applied to a Sephadex G75-40 column (100×1.5 cm) equilibrated in 20 mM Tris-HCl (pH 8) (4°C)/100 mM NaCl. (A) The A_{280} (●) and A_{410} (○) of fractions eluted from this column and the protein composition of selected fractions as determined by Coomassie blue staining [22] after SDS-PAGE as described in Fig. 1. The boxed fractions 60 and 61 were combined, desalted by passage through Sephadex G25-40 (10×1.5 cm), equilibrated in 5% (v/v) formic acid and freeze-dried. The residue was redissolved in 5% (v/v) formic acid (2 ml) and applied to a column of Sephadex (G75-40) (100×1.5 cm) equilibrated in 5% (v/v) formic acid. (B) The A_{280} (●) and A_{410} (○) of fractions eluted from this column and the protein composition of selected fractions as determined by Coomassie blue staining after SDS-PAGE. The boxed fractions (68–71) were combined and freeze-dried. The residue was redissolved in 5 mM sodium phosphate (pH 7).

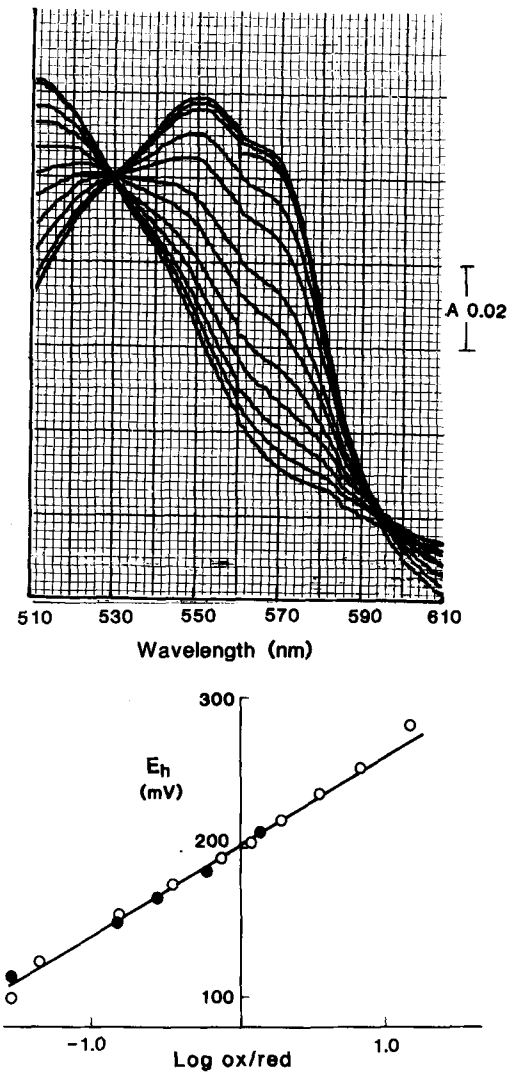

Fig. 3. Potentiometric redox titration of cytochrome c' from *Paracoccus denitrificans*. Potentiometric redox titrations were carried out in an anaerobic cuvette constantly bubbled with argon and magnetically stirred. The cuvette contained 13 μM cytochrome c' in 0.05 M sodium phosphate (pH 7), 17 μM phenazine methosulphate, phenazine ethosulphate and diaminodurol, and 20 μM ferric ammonium sulphate, 0.4 mM EDTA. The ambient redox potential (E_{obs}) was monitored by a Pt pin electrode in combination with an Ag|AgCl reference (Russell pH, Auchtermuchty, U.K.) and the potential with respect to the standard hydrogen electrode (E_h) was obtained by E_{obs} +196 mV. Oxidative and reductive titrations were performed by addition of 0.02 M potassium ferricyanide and 0.02 M sodium dithionite, respectively. (A) The oxidative titration of cytochrome c'. The ambient potential values are 56, 99, 124, 156, 175, 193, 204, 219, 236, 253, 282, 307 mV, going from the fully reduced peak at 550 nm to full oxidation. The titration was performed in both oxidative and reductive directions and both sets of results are plotted in (B). The line is theoretical for a slope of 59 mV, 25°C.

Values of $S\Delta N\%$ less than 42 are strongly indicative of sequence homology, while values less than 93 are weakly indicative of sequence homology. The conservative nature of the index [15] is indicated by the fact that cytochromes c' are indeed homologous from known sequences, yet several figures in the $S\Delta N$ matrix lie above 93. The very high figures in two of the halotolerant *Paracoccus* comparisons may be a special case. The halotolerant adaptation appears to involve a shift

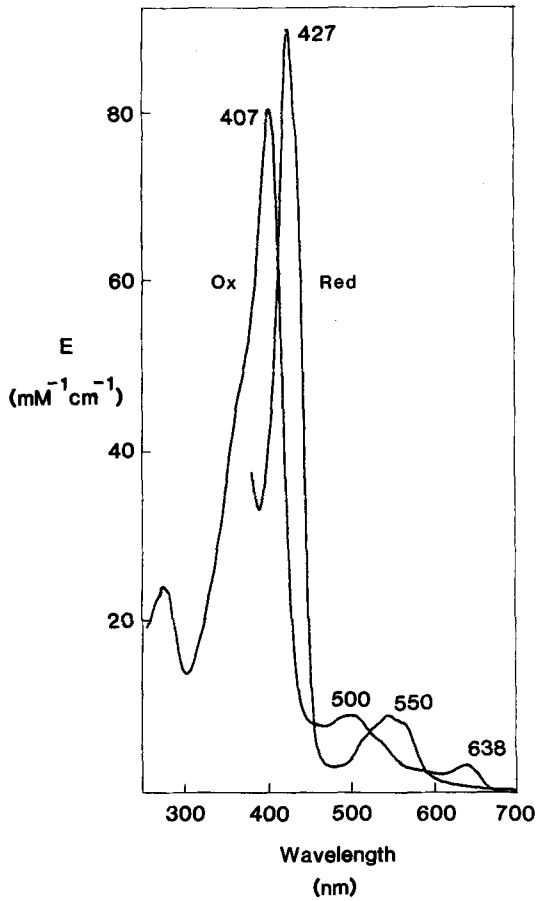

Fig. 4. The UV-visible absorption spectra of *Paracoccus denitrifcans* cytochrome c' Cytochrome c' (8 μM) in 0.1 M sodium phosphate (pH 7), was oxidised as isolated (ox) and then reduced with a pinch of sodium dithionite (red). The scale of extinction coefficients was calculated by determination of the pyridine ferrohaemochrome spectra of a second aliquot of cytochrome c' in 2 M pyridine/0.15 M NaOH ($E = 29.1$ mM^{-1} cm^{-1}), Ref. 25).

TABLE I

Properties of cytochrome c' from Pa. denitrificans and other bacteria

		Pa. denitrificans	Others [a]
Soret ox/280		3.3	2.5–2.8 (2–3 Trp) 3.1–4.8 (1 Trp)
Spectra	Soret ox.	407 nm (80 mM^{-1} cm^{-1})	390–402 nm
		500 nm	490–500 nm
		638 nm	632–643 nm
	Soret red.	427 nm	423–427 nm
		550 nm	546–556 nm
$E_{m,7}$		202 mV	−5 to 102 mV [b] 132 mV [c]

[a] Ref. 13
[b] Cytochromes c' from the purple and purple sulphur bacteria.
[c] *Alcaligenes* sp. cytochrome c' [26].

TABLE II

Amino-acid compositions of Pa. denitrificans cytochrome c′ and other cytochromes c′

Pa. denitrificans cytochrome *c′* (3 nmol) was hydrolysed in 6 M HCl/0.1% phenol in vacuo for 44 h and dried over sodium hydroxide pellets. The amino-acid content was determined using a Locarte amino-acid analyser. The haem content of the parent solution was determined by the pyridine ferrohaemochrome method using $E_{550nm} = 29.1\ mM^{-1}\ cm^{-1}$ [25] and used to calculate mol amino acid per mol haem. Tryptophan was not measured but the purity index is consistent with a tryptophan content of 1 (see Table I). Cysteine was not measured, but all known cytochromes *c′* have two cysteines involved in haem attachment. Compositions were obtained by summation from amino-acid sequences in Refs. 2, 16, 19 and 20.

	Pa. denitrificans		Halotolerant *Paracoccus*	*Chromatium vinosum*	*Rhodospirillum rubrum*	*Rhodobacter capsulatus*
	Mol per mol haem	integer				
Asp + Asn	13.5	14	16	12	9	13
Thr	4.8	5	6	6	8	9
Ser	9.4	9	4	3	7	5
Glu + Gln	15.6	16	23	18	12	12
Pro	5.3	5	3	3	4	3
Gly	13.5	14	14	15	7	11
Ala	23	23	19	24	30	32
Val	4.9	5	6	10	4	4
Met	2.7	3	5	4	2	4
Ile	3.0	3	3	5	7	3
Leu	6.9	7	7	4	8	8
Tyr	3.1	3	2	4	3	2
Phe	3.6	4	6	5	4	5
His	2.0	2	4	1	2	1
Lys	6.0	6	3	10	15	12
Arg	3.7	4	6	4	1	2
Trp		(1)	3	1	1	1
Cys		(2)	2	2	2	2
		126	132	131	126	129

Table III

Cornish-Bowden analysis of amino-acid compositions

The sequence divergence of a pair of amino-acid compositions was estimated by calculation of $S\Delta N$ according to the formula:

$$0.5\Sigma(n_iA - n_iB)^2 - 0.035(N_a - N_b)^2 + 0.535(N_a - N_b)$$

where n_iA and n_iB are the numbers of an amino acid in proteins A and B and N_a and N_b are the total amino-acids in each protein with N_a the larger of the two [15]. Figures in the table are $S\Delta N/N\%$. Superscripts are the actual % sequence differences from the known amino-acid sequences.

1. *Paracoccus denitrificans*

50	2. halotolerant *Paracoccus*			
43	^{72}64	3. *Chromatium vinosum*		
105	^{70}219	^{70}103	4. *Rhodospirillum rubrum*	
75	^{77}167	^{74}77	^{63}30	5. *Rhodobacter capsulatus*

in the amino acid composition to acidic residues from lysines [16] and this may unduly distort the calculation of $S\Delta N$.

One possible conclusion from the data is that *Pa. denitrificans* cytochrome *c′* more closely resemble those of the cytochromes *c′* from *Chromatium* and the halotolerant *Paracoccus* species than those of the Rhodospirillaceae. This would be surprising in view of the close relationship suggested between *Pa. denitrificans* and *Rhodobacter capsulatus* on the basis of their cytochrome c_2 and rRNA sequences [17,18], but the cytochrome *c′* dendrogram is anomalous in other respects that have been interpreted as due to possible gene transfer between species [19].

In any case, the matrix must be viewed with extreme caution. Indications of the inherent uncertainty are the $S\Delta N$ values for the halotolerant *Paracoccus* cytochrome *c′*. In sequence comparisons this cytochrome is slightly more similar to that from *R. rubrum* (30% identity) than to that from *Chromatium vinosum* (28% identity). Yet the $S\Delta N\%$ values suggest a weak indication of relatedness to the latter (64), but no indication

of relatedness to the former (219). Determination of the amino-acid sequence of *Pa. denitrificans* cytochrome *c'* will be necessary to clarify these problems. N-terminal sequence analysis of the holoprotein failed to give results, presumably due to cyclisation of an N-terminal glutamine as was observed for *Alcaligenes* sp. cytochrome *c'* [20]. On the other hand, *Rb. capsulatus* cytochrome *c'* gave the N-terminal sequence of ADTKE expected from the known sequence [2].

References

1 Ambler, R.P. (1980) in From Cyclotrons to Cytochromes (Robinson, A.B. and Kaplan, N.O., eds.), pp. 263–279, Academic Press, London.
2 Ambler, R.P., Bartsch, R.G., Daniel, M., Kamen, M.D., McClellan, L., Meyer, T.E., Van Beeumen, J. (1981) Proc. Natl. Acad. Sci. USA 78, 6854–6857.
3 Meyer, T.E. and Kamen, M.D. (1982) Adv. Protein. Chem. 35, 105–212.
4 Yamanaka, T. and Imai, S. (1972) Biochem. Biophys. Res. Commun. 46, 150–154.
5 Yamazaki, T., Fukumori, Y. and Yamanaka, T. (1986) Biochim. Biophys. Acta 871, 36–44.
6 John, P. and Whatley, F.R. (1975) Nature 254, 495–498.
7 Kurowski, B. and Ludwig, B. (1987) J. Biol. Chem. 262, 13805–13811.
8 Davidson, V.L. and Kumar, M.A. (1989) FEBS Lett. 245, 271–273.
9 Bosma, G., Braster, M., Stouthamer, A.H. and Van Verseveld, H.W. (1987) Eur. J. Biochem. 165, 665–670.
10 Long, A.R. and Anthony, C. (1991) J. Gen. Microbiol. 137, 415–425.
11 Goodhew, C.F., Wilson, I.B.H., Hunter, D.J.B. and Pettigrew, G.W. (1990) Biochem. J. 271, 707–712.
12 Hartley, B.S. (1970) Biochem. J. 119, 805–822.
13 Bartsch, R.G. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 249–279, Plenum, New York.
14 Weber, P.C. and Salemme, F.R. (1980) Nature 287, 82–84.
15 Cornish-Bowden, A. (1983) Methods Enzymol. 91, 60–75.
16 Ambler, R.P., Daniel, M., McLellan, L., Meyer, T.E., Cusanovich, M.A. and Kamen, M.D. (1987) Biochem. J. 248, 365–371.
17 Ambler, R.P., Daniel, M., Hermoso, J., Meyer, T.E., Bartsch, R.G. and Kamen, M.D. (1979) Nature 278, 659–660.
18 Fox, G.E., Stackebrandt, E., Hespell, R.B., Gibson, J., Maniloff, J., Dyer, T.A., Wolfe, R.S., Balch, W.E., Tanner, R.S., Magrum, L.J., Zablen, L.B., Blakemore, R., Gupta, R., Bonen, L., Lewis, B.J., Stahl, D.A., Luehrsen, K.R., Chen, K.N. and Woese, C.R. (1980) Science 209, 457–463.
19 Ambler, R.P., Meyer, T.E. and Kamen, M.D. (1979) Nature 278, 661–662.
20 Ambler, R.P. (1973) Biochem. J. 135, 751–758.
21 Laemlli, U.K. (1970) Nature 227, 680–685.
22 Goodhew, C.F., Brown, K.R. and Pettigrew, G.W. (1986) Biochim. Biophys. Acta 852, 288–294.
23 Ludwig, B., Suda, K. and Cerletti, N. (1983) Eur. J. Biochem. 137, 597–602.
24 Berry, E.A. and Trumpower, B.L. (1985), J. Biol. Chem. 260, 2458–2467.
25 Morton, R.K. (1958) Rev. Pure Appl. Chem. 8, 161–220.
26 Cusanovich, M.A., Tedro, S.M., Kamen, M.D. (1970) Arch. Biochem. Biophys. 141, 557–570.