Amino acid sequences of cytochrome *c*-554(548) and cytochrome *c'* from a halophilic denitrifying bacterium of the genus *Paracoccus*

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The amino acid sequences of the cytochromes *c*-554(548) and *c'* from the moderately halophilic bacterium *Paracoccus* sp., I.A.M. 203 (= A.T.C.C. 12084, N.C.I.B. 8669) have been determined. Cytochrome *c*-554(548) consists of a single polypeptide chain of 83 residues, and dimerizes strongly. The most similar protein of known sequence is the *N*-terminal half of the dihaem cytochrome *c*₄, and other related proteins include the cytochrome *c*-554(547) of *Thiobacillus neapolitanus* and the cytochrome *c*-553 of *Desulfovibrio vulgaris*. Cytochrome *c'*, which has a single polypeptide chain of 132 residues, is similar in sequence to cytochromes *c'* from phototrophic and denitrifying bacteria, but only shows about 36% sequence identity to the most similar protein of known sequence. Both of the *Paracoccus* proteins have a considerable excess of acidic amino acid side chains over basic ones, and a higher proportion of their basic amino acids is arginine than is usual in cytochromes *c*. Both these characteristics seem to be adaptations to increase the stability of the proteins in an environment of high ionic strength. Detailed evidence for the amino acid sequences of the proteins has been deposited as Supplementary Publication 50140 (24 pp.) at the British Library (Lending Division), Boston Spa, Yorkshire LS23 7BQ, U.K. from which copies are available on prepayment.

INTRODUCTION

Both denitrifying and phototrophic bacteria are notable for the complexities of their electron-transport systems, and for the large numbers of soluble electron-transport proteins that they can produce. Our studies (e.g. Ambler, 1985; Ambler *et al.*, 1981a) have shown that some sequence classes of electron-transport proteins are widely distributed in bacteria, with homologous proteins apparently functioning in quite different electron-transport chains. Their distribution, although wide, is erratic, and it seems that a limited range of types of homologous proteins can be assembled, in many different combinations, to give functionally different electron-transport chains.

The moderately halophilic denitrifying bacterium that we are now calling *Paracoccus* sp., I.A.M. 203 (= A.T.C.C. 12084, N.C.I.B. 8669) was isolated in 1950 from decaying whale meat (Egami *et al.*, 1953). It grows best in a medium containing 10% (v/v) NaCl. Before 1953, no soluble cytochrome had been isolated from a bacterium, and Egami and his colleagues believed that they had extracted a soluble *b*-type cytochrome from this organism containing non-covalently-bound haem which they called cytochrome *b*₄. Subsequent work by Hori (1961a) showed that 'cytochrome *b*₄' was a mixture of cytochromes *c*. Hori (1961b) found that the organism also produces large amounts of a soluble 'brown protein', which Tedro *et al.* (1977) have shown to belong to the class now known as HiPIP (high-potential iron-sulphur protein), which up until then had only been recognized in

phototrophic bacteria. Kono & Taniguchi (1960) characterized a haem protein from the organism that had hydroxylamine reductase activity, and this we now show to be a typical cytochrome *c'*.

The Egami organism is similar to the type-strain of *Paracoccus halodenitrificans* (A.T.C.C. 13511) (M. Kocur & L. B. Perry, personal communications) but has not yet been thoroughly characterized. It was mistakenly listed as *Staphylococcus epidermidis* in the 1975 N.C.I.B. catalogue.

In the present investigation we have determined the amino acid sequences of two of the most abundant cytochromes *c* produced by this strain of *Paracoccus* when it is grown anaerobically on a nitrate medium. These are a cytochrome *c*-554(548), which has an absorption spectrum in which the reduced α -band is markedly split (Fig. 1), and which we identify with the cytochrome *b*₄-I of Hori (1961a), and a cytochrome *c'*, the hydroxylamine reductase of Kono & Taniguchi (1960). The amino acid sequence of the HiPIP has already been published (Tedo *et al.*, 1977).

EXPERIMENTAL

The maintenance and growth of the organism, I.A.M. 203, has already been described by Tedro *et al.* (1977).

Purification of proteins

Wet cells (1500 g) were disrupted in a Ribi Cell Fractionator and membranes were removed by ultra-

Fig. 1. U.v.-visible-absorption spectra of *Paracoccus* cytochrome *c*-554(548).

Note the two α -peaks at 554 nm and 548 nm. The reduced form was obtained by adding solid $\text{Na}_2\text{S}_2\text{O}_4$.

centrifugation as described by Tedro *et al.* (1977). The 60–90 % saturation $(\text{NH}_4)_2\text{SO}_4$ precipitate was dissolved in a minimal volume of 20 mM-Tris/HCl buffer, pH 7.3, containing 0.1 M-NaCl, and subjected to gel filtration through Sephadex-G-100 (8 cm $\times$ 100 cm diameter). Five coloured bands were separated. The first band to be eluted contained residual membranes and bacterioferritin (cytochrome *b*-560; Yariv *et al.*, 1981). The second band was identified spectroscopically as containing mostly a cytochrome *cd*-type nitrate reductase. The third band contained a mixture of cytochrome *c'*, a cytochrome *c*-554, and a protein spectrally similar to the iron proteins protocatechuate oxygenase, transferrin and purple phosphatase (Whittaker *et al.*, 1984; Anderson *et al.*, 1987; Antanaitis & Aisen, 1982). The fourth band contained a mixture of cytochrome *c*-554(548) (Fig. 1), and a cytochrome *c*-549 (called cytochrome *c*-551 by Hori, 1961*b*). The fifth band contained only HiPIP. Some further cytochrome *cd*, cytochrome *c*-554(548) and cytochrome *c*-549 was obtained by gel filtration of the 40–60 % saturation $(\text{NH}_4)_2\text{SO}_4$ fraction.

The pooled fractions containing the mixture of cytochrome *c*-554(548) and cytochrome *c*-549 were chromatographed on DEAE-cellulose (Whatman DE-52; 15 cm $\times$ 7 cm diameter) in 20 mM-Tris/HCl buffer, pH 7.3, using a linear gradient of 0.1–0.6 M-NaCl.

Fig. 2. U.v.-visible-absorption spectra of *Paracoccus* cytochrome *c'*.

The reduced form was obtained by adding solid $\text{Na}_2\text{S}_2\text{O}_4$.

Cytochrome *c*-549 and traces of HiPIP could be seen eluting with 0.2 M-NaCl, just ahead of the main band of cytochrome *c*-554(548), which eluted with 0.25 M-NaCl, although the bands were not satisfactorily resolved. Some cytochrome *c*-554 and cytochrome *c'* were eluted at 0.5 M-NaCl. The traces of HiPIP were removed from the cytochrome *c*-554(548) fraction by gel filtration through Sephadex-G-100, and then the two cytochromes *c* were separated by chromatography on DEAE-cellulose (15 cm $\times$ 7 cm diameter), equilibrated with 0.1 M-ammonium acetate buffer, pH 6, and eluted with a linear gradient of 0.1–0.6 M-ammonium acetate buffer, pH 6. The cytochrome *c*-549 was eluted at 0.3 M, and the cytochrome *c*-554(548), at 0.4 M. The final preparation of cytochrome *c*-554(548) had a purity index (A_{276}/A_{410}) of 0.15, and the reduced and oxidized absorption spectra that are shown in Fig. 1. About 60 μmol of cytochrome *c*-554(548) was recovered from the 1500 g of wet cells, in about 50 % yield of the total cytochrome applied to the first Sephadex column. The third band from gel filtration, which contained the cytochrome *c'*, was chromatographed on DEAE-cellulose (15 cm $\times$ 7 cm diameter) equilibrated with 20 mM-Tris/HCl buffer, pH 7.3, using a linear gradient of 0.1–0.6 M-NaCl. Traces of cytochrome *c*-554(548) were eluted with 0.25 M-NaCl, cytochrome *c*-554 with 0.35 M-NaCl, ferredoxin with 0.4 M-NaCl, and the cytochrome *c'* together with nucleic acid with 0.5 M-NaCl. The cytochrome *c'* was again precipitated with $(\text{NH}_4)_2\text{SO}_4$ (70–90 % saturation), subjected to gel filtration as described above, and rechromatographed on DEAE-cellulose under similar conditions but was still

Table 1. Amino acid compositions of *Paracoccus halodenitrificans* cytochromes *c'* and *c-554(548)*

The hydrolysis values are derived from multiple analyses under different hydrolysis conditions. The sequence values are taken from the deduced sequence shown in Figs. 1 and 2.

Amino acid	Composition (mol/mol)			
	Cytochrome <i>c'</i>		Cytochrome <i>c-554(548)</i>	
	Hydrolysis	Sequence	Hydrolysis	Sequence
Glycine	14.2	14	10.9	11
Alanine	18.4	19	13.7	14
Valine	6.1	6	1.1	1
Leucine	7.0	7	5.0	5
Isoleucine	2.9	3	4.8	5
Serine	3.9	4	5.7	6
Threonine	5.8	6	4.6	5
Aspartic acid	16.1	13	8.9	7
Asparagine	—	3	—	2
Glutamic acid	23.5	15	9.3	4
Glutamine	—	8	—	5
Phenylalanine	5.9	6	< 0.07	0
Tyrosine	2.1	2	4.5	5
Tryptophan	3.0	3	—	0
Cysteine	2.0	2	2.0	2
Methionine	4.9	5	2.0	2
Proline	2.8	3	2.9	3
Lysine	3.0	3	2.9	3
Histidine	3.8	4	1.1	1
Arginine	5.8	6	1.8	2
Total		132		83

not satisfactorily resolved from the ferredoxin-like protein. Effective purification was finally obtained by using chromatography on hydroxyapatite (as described by Ambler *et al.*, 1986). The cytochrome was absorbed onto a column of hydroxyapatite (5 cm × 8 cm diameter) and equilibrated with 20 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-NaCl. The column was developed with a linear gradient of 20–200 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-NaCl, and the ferredoxin-like protein was eluted first. The cytochrome *c'* was eluted with 0.15 M-potassium phosphate, flanked by several colourless but u.v.-absorbing components. The final preparation had a purity index (A_{280}/A_{400}) of 0.37, and the oxidized and reduced absorption spectra that are shown in Fig. 2. The recovery was 8 μ mol of protein.

Neither the cytochrome *c-554* nor the cytochrome *c-549* have yet been purified to homogeneity.

Amino acid sequence determination

The methods used have been described before (Ambler & Wynn, 1973; Ambler *et al.*, 1979, 1984). The *N*-terminal sequences of the proteins were examined with an automatic sequencer (Beckman Model 890A) using a DMBA program (Beckman 102473). Amide groups were assigned from peptide electrophoretic mobilities and exopeptidase analysis.

RESULTS

Properties of the protein preparations

The cytochrome *c'* ran as a single band corresponding to M_r about 14000 when subjected to SDS/poly-

acrylamide-gel electrophoresis. The native protein behaved on gel filtration as if it has an M_r of about 34000. The amino acid composition is given in Table 1. The value of 0.37 attained for A_{280}/A_{400} is consistent with that expected for a pure cytochrome *c'* containing three tryptophan residues per haem.

The cytochrome *c-554(548)* behaved on gel filtration as if it had an M_r of 16000. When subjected to SDS/polyacrylamide-gel electrophoresis there was some material of M_r about 16000 and some of lower molecular mass, and the amount of lower- M_r material predominated if the protein was boiled in 2.0% (w/v) SDS for 2 min before application to the gel. The amino acid composition is shown in Table 1, and indicates a protein containing only one residue of valine and one residue of histidine per molecule, and having an M_r of 8500 plus haem.

Amino acid sequences

The evidence for the proposed amino acid sequences of the *Paracoccus* cytochromes *c'* and *c-554(548)* are summarized in Figs. 3 and 4. Details of the purification, analysis and sequence-determination experiments on the peptides are given in Supplementary Publication SUP 50140. The criteria for satisfactory results, and the nature and format of the Supplementary Publication are given in previous papers (Ambler & Wynn, 1973; Ambler *et al.*, 1979).

The amino acid sequence of the cytochrome *c-554(548)* was determined on five separate digests. The digests were: (a) 5.1 μ mol of apoprotein digested with chymotrypsin; (b) 0.6 μ mol of apoprotein digested with trypsin (c) 0.6 μ mol of apoprotein digested with staphylococcal

Fig. 3. Amino acid sequence of *Paracoccus* cytochrome *c*-554(548)

Peptides derived by digestion with trypsin (T), by trypsin after *S*-aminoethylation (AET), by trypsin followed by chymotrypsin after *S*-aminoethylation (AETC) and by digestion with staphylococcal protease (F) are shown above the sequence, and by digestion with chymotrypsin (C) below the sequence. Peptides from sub-digests are labelled with another letter to indicate the second method of cleavage: H, thermolysin; D, *Pseudomonas fragi* protease; S, subtilisin. All peptides were analysed quantitatively, but those marked * gave substandard, and those marked ** particularly bad, analyses. See Ambler & Wynn (1973) for a quantitative definition of these symbols. The peptides lines are doubled where the sequence has been determined by the dansyl phenylisothiocyanate method, with the lower line broken at residues where the identification was substandard. C-Terminal residues identified as free amino acids after the removal of the remainder by phenylisothiocyanate degradation are indicated by a vertical line joining the double lines at the end of the peptide. Peptides marked † were examined by carboxypeptidase A digestion. Under the sequence, → indicates residues identified as phenylthiohydantoin after degradation of the whole protein in an automatic sequenator. -→ Indicates only tentative identification.

protease; (d) 2.4 μ mol of apoprotein, treated with ethyleneimine under reducing conditions and then digested with trypsin; and (e) 2.1 μ mol of apoprotein treated with ethyleneimine under reducing conditions and then digested first with trypsin and then with chymotrypsin.

The amino acid sequence of the cytochrome *c'* was determined on five separate digests. These were (a) 1.3 μ mol of apoprotein digested with chymotrypsin; (b) 2.6 μ mol of apoprotein digested with staphylococcal protease; (c) 0.4 μ mol of apoprotein digested with trypsin; (d) 0.5 μ mol of native protein treated with CNBr; and (e) 1.3 μ mol of apoprotein digested with thermolysin. The amount of protein available was near the lower limit for satisfactory peptide purification with the methods used.

DISCUSSION

Accuracy of proposed sequences

Both of the proteins contain regions for which it was difficult to obtain sequence information from small overlapping peptides. In the cytochrome *c*-554(548), it was found difficult to characterize the region 20-35 (Fig. 3), but satisfactory peptides were eventually obtained from the double trypsin/chymotrypsin digest. This region, which immediately follows the haem attachment site, was also found difficult to sequence in *Azotobacter vinelandii* cytochrome *c*₄ (Ambler *et al.*, 1984) which shows some sequence similarity to the cytochrome *c*-554(548). We have considered the possibility that stable dimerization of the cytochrome might be due to an *OO'*-dityrosine cross-link (Verweij *et al.*, 1982) but none of the tyrosine-containing small peptides that were isolated eluted at unexpectedly early positions on gel filtration (behaviour that would be predicted were they to be involved in such cross-linking).

In the cytochrome *c'* sequence (Fig. 4), the evidence for the overlap at positions 24/25 comes only from the composition of peptide F-II, which gave a sub-standard analysis as the peptide was too hydrophobic to purify satisfactorily by paper electrophoresis. This analysis showed Asp = 2.7 instead of the expected 2. The evidence for ordering residues 80/81 as Asp-Trp is very weak and primarily based on the occurrence of peptide C514 (Glu-Gly-Phe), although this peptide is also believed to arise from another place in the sequence. If residues 80/81 were instead Trp-Asp, chymotryptic digestion should have produced detectable amounts of the readily recognizable peptide Asp-Glu-Gly-Phe, which was not observed. In most cytochromes *c'* of known sequence (Ambler *et al.*, 1981a) there is an aromatic residue at the position corresponding to Phe-84 in Fig. 4. The locations in the cytochrome sequences where aromatic residues can be accommodated seem to be limited, and the position corresponding to Trp-81 in Fig. 4 is the only site of aromatic residues in this region in other cytochrome *c'* sequences. Evidence for some of the acid or amide assignments, at positions 72, 79, 80 and 82 (all believed to be acids) has been taken from a quantitative consideration of electrophoretic mobilities on paper. Our experience has been that the graphs of Offord (1977) predict mobilities that are greater than those actually observed for small peptides that have multiple acidic charges. This discrepancy only becomes large enough to become potentially confusing for peptides that have a net charge of 4. The numerical values of mobilities, observed and predicted, are given in the Supplementary Publication.

Sequence affinities of *Paracoccus* cytochromes

The *Paracoccus* cytochrome *c'* has a sequence typical of this class of protein (Ambler *et al.*, 1981a), and while it is not close in sequence to any of the other characterized

Fig. 4. Amino acid sequence of *Paracoccus* cytochrome *c'*

Peptides derived by digestion with staphylococcal protease (F) or with thermolysin (H) are shown above the sequence, and by digestion with chymotrypsin (C) or by cleavage with CNBr, below the sequence. All peptides except X31 and T27/11 (shown with broken lines) were analysed quantitatively. Other symbols etc. are shown in the caption to Fig. 3.

Fig. 5. The relationship of excess charge to relative arginine content for some electron-transport proteins from halophiles and non-halophiles

Solid symbols are for proteins from halophiles, open symbols are for proteins from other bacteria. ●, ○, Class I cytochromes *c* [e.g. cytochrome *c*₂ and *c*-554(548)]; ■, □, Class II cytochrome (e.g. cytochrome *c*′); ▲, △, non-haem iron proteins (e.g. HiPIP, ferredoxin). Excess charge (%) = $\frac{(K+R)-(D+E)}{n} \times 100$; arginine proportion = $\frac{R}{(K+R)}$, where K, R, D and E are the numbers of residues of lysine, arginine, aspartic acid and glutamic acid in the protein respectively, and *n* is the total number of residues. All the proteins have been sequenced, so amide residues have been excluded, and no account is taken of histidine residues.

cytochromes *c'*, these proteins do not show strong conservation of sequence between organisms for which other evidence suggests a relatively close relationship. Thus the closest known sequence to that of the *Paracoccus* cytochrome *c'* is that of protein from the denitrifying *Alcaligenes* strain N.C.I.B. 11015, which shows 36% identity. The *Alcaligenes* protein shows comparable similarity (Ambler *et al.*, 1981a) to the cytochromes *c'* from the phototrophic bacteria *Rhodospirillum photometricum* and *Rhodopseudomonas sphaeroides*, so we attach no special phylogenetic significance to these connections.

The closest known sequence relation of the cytochrome *c*-554(548) is the *N*-terminal half of the dihaem cytochrome *c*₄ from *Azotobacter vinelandii* (Ambler *et al.*, 1984) with which it shares about 46% identity. The sequence can also be aligned with those of the cytochrome *c*-554(547) from *Thiobacillus neapolitanus*

(Ambler *et al.*, 1985) and the cytochrome *c*-553 from *Desulfovibrio vulgaris* (Nakano *et al.*, 1983), with about 35% identity. We include all these cytochromes in sequence class Ic (Ambler, 1982), a subset of class I cytochromes *c*. The normal characteristics of the class I cytochromes *c* are a single haem attached near the *N*-terminus of the polypeptide chain, and a methionine residue 40 to 70 positions further down the chain acting as the sixth ligand to the iron atom. The class I cytochromes would all seem to be homologous, although there is insufficient sequence similarity for an unequivocal alignment to be arrived at. Mitochondrial cytochromes *c* (in class Ib) cannot be aligned with the cytochrome *c*-554(548) to give an identity of greater than about 20%.

The electron transport system of *Paracoccus denitrificans* has been extensively studied, but the only sequenced protein from it is the cytochrome *c*₂ (or *c*-550; Timkovich *et al.*, 1976; Ambler *et al.*, 1981b), which is from a different sequence class (Ia) to the sequenced proteins from the present *Paracoccus* species. It is possible that sequence work on the still uncharacterized cytochromes *c*-554 and *c*-549 may yet show some genetic similarities between the two species of the genus, although spectral properties suggest that the cytochrome *c*-554 may well be a class II cytochrome.

Protein structure and haloadaptation

The proteins of halophilic bacteria have been characterized as being more acidic in nature than the proteins of typical bacteria (Baxter, 1959), but even extreme halophiles such as *Halobacterium cutirubrum* (Matheson, 1985) have some basic proteins. If a globular protein is to retain its structure in a solvent of high ionic strength, its surface must be able to compete effectively with the salt for water molecules, and this is a likely purpose for the extra acidic groups that are found (Rao & Argos, 1981). There is as yet little structural information available about proteins from halophiles, but Rao & Argos (1981) have considered the structures of the ferredoxins from *Halobacterium halobium* and *Halobacterium marismortui* in comparison with the homologous ferredoxin from the cyanobacterium *Spirulina platensis*.

Both the *Paracoccus* proteins whose sequences are reported in the present paper are acidic, as is the HiPIP (Tedro *et al.*, 1977). Although the organism is only a moderate halophile, the excess of acidic residues when compared with similar proteins from the non-halophilic bacteria are similar to the values reported by Werber *et al.* (1986) for proteins from the extreme halophile *H. marismortui*.

We have also noticed that there is a tendency, in proteins from halophilic bacteria, for the proportions of arginine among the basic amino acids to increase. While the relative proportions of lysine to arginine vary greatly between different types of proteins in normal organisms, among the electron-transport proteins that we have been particularly concerned with the arginine content has been almost always low. The exceptions have been entirely among proteins from bacteria that have adapted to growth in salt (Fig. 5). The generalization also seems to hold good for the *H. marismortui* proteins considered by Werber *et al.* (1986). A preference for arginine rather than lysine residues in locations where a basic residue is functionally necessary should also serve to increase the hydration of the protein surface, as the arginine side chain, by a variety of criteria (Creighton, 1984; Eisenberg

& McLachlan, 1986), is more hydrophilic than that of lysine.

The *Paracoccus* cytochrome *c'* has six arginine, four histidines, but only three lysine residues. Arg-10 is conserved in all the cytochromes of known sequence, and in the three-dimensional structure of the *Rhodospirillum rubrum* protein (Weber *et al.*, 1980; Finzel *et al.*, 1985) this residue can be seen to form a salt-bridge to a haem propionate. His-8 is a lysine in the *R. rubrum* protein, and has a role in the stabilization of the dimer through a salt bridge to the other propionate. Lys-123 is conserved in all the cytochromes *c'*, and occurs in a structurally-constrained location between the haem-linked cysteine residues. The side chain is disordered in the crystal, but may form a salt-bridge to the conserved acidic residue 127. Arg-130 of the *Paracoccus* sequence is lysine in several of the other cytochromes *c'*, and is also near the propionates.

It has been suggested (Rao & Argos, 1981; Werber *et al.*, 1986) that clusters of acidic residues are a particular feature of proteins from halophiles. While there are eight doublets of acidic residues in the 132-residue *Paracoccus* cytochrome *c'* sequence, this number of pairs is not significantly greater than that expected by chance in a protein of this size containing 20% acidic residues.

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