The amino acid sequence of the cytochrome c_2 from the phototrophic bacterium *Rhodopseudomonas globiformis*

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The amino acid sequence of the principal soluble cytochrome c from the phototrophic acidophilic bacterium *Rhodopseudomonas* (or *Rhodopila*) *globiformis* was determined. By the criteria of percentage sequence identity and fewness of internal insertions and deletions it is more similar in sequence to some mitochondrial cytochromes c than to any known bacterial cytochrome. The organism does not have any properties that commend it as being particularly similar to postulated prokaryotic precursors of the mitochondrion. We consider that the relatively high degree of sequence similarity is an instance of convergence, and is an example of the limitations that are imposed on attempts to deduce distant evolutionary relationships from sequence information. Detailed evidence for the amino acid sequence of the protein has been deposited as Supplementary Publication SUP 50136 (12 pages) at the British Library Lending Division, Boston Spa, West Yorkshire LS23 7BQ, U.K., from whom copies are available on prepayment [see Biochem. J. (1987) 241, 5].

INTRODUCTION

The purple phototrophic bacteria are a readily definable group of organisms, as they possess a characteristic mode of energy metabolism mediated by easily recognizable bacteriochlorophyll pigments and soluble electron-transport proteins. However, they vary greatly in their cellular morphology and in the appearance of their photosynthetic membranes, and in sequences of macromolecules. The group is being used as a test system for the exploration of the validity of using sequence information for constructing natural classifications of prokaryotes (Ambler *et al.*, 1979a,b; Dickerson, 1980; Woese *et al.*, 1984; Meyer *et al.*, 1986). Sequence results have also been interpreted as showing that the purple phototrophic bacteria are closely related to the prokaryote that is supposed to have been the mitochondrial ancestor (Dayhoff & Schwarz, 1981).

Rhodopseudomonas globiformis (Pfennig, 1974) was isolated from a warm acidic sulphur spring in Yellowstone Park, and only this single strain of the organism has been recognized so far. Imhoff *et al.* (1984) have proposed that this organism should be put into a new genus, *Rhodopila*, as it is sufficiently different from all other purple phototrophic bacteria.

We have isolated the predominant soluble cytochrome c from this organism, and determined its amino acid sequence. By the commonly used criteria of percentage sequence identity and number of insertions or deletions needed for optimal alignment we compare the sequence with those of mitochondrial cytochromes c and other bacterial cytochromes.

EXPERIMENTAL

Growth of organism

Rps. globiformis strain 7950 (D.S.M. 161) was kindly provided by Dr. H. Biebl and Dr. N. Pfennig. It was

grown phototrophically on the gluconate/mannitol medium recommended by Pfennig (1974) in 1-litre prescription bottles at 30 °C, and harvested after 3 days' growth, with a yield of about 2 g wet wt. of cells/l. Only about half the cultures grew at each transfer, and the cell yield was only about one-third of what we have come to expect from organotrophic purple bacteria.

Purification of protein

Wet cells (1250 g) were suspended in 4.5 litres of 0.1 M-Tris/HCl buffer, pH 7.3, and homogenized in a Ribi cell fractionator operated at 137 MPa (20000 lbf/in²). Almost all the membranes were removed by centrifugation at 145000 g for 4 h. The supernatant was passed through a column (15 cm $\times$ 7 cm diam.) of Whatman DE-52 DEAE-cellulose equilibrated with 0.2 M-Tris/HCl buffer, pH 7.3. Bacterial ferredoxin was adsorbed on the column, and eventually removed with 0.02 M-Tris/HCl buffer, pH 7.3, containing 0.5 M-NaCl. The cytochrome fraction, not retarded by this column, was desalted by gel filtration through Sephadex G-25 into 0.001 M-Tris/HCl buffer, pH 8.0, and applied to a column (15 cm $\times$ 7 cm diam.) of Whatman DE-52 DEAE-cellulose equilibrated with the same buffer. A pink cytochrome band was adsorbed less tightly than the green band of the high-potential non-haem iron protein (HiPIP). The coloured bands were not resolved when the column was developed with a 0–0.1 M-NaCl gradient in 0.02 M-Tris/HCl buffer, pH 8.0, and, when the coloured eluate was desalted and an attempt made to reabsorb the proteins on DEAE-cellulose, only a portion would bind, and in very broad bands. The coloured material that did not adsorb was adjusted to pH 5, diluted and adsorbed on a column (15 cm $\times$ 7 cm diam.) of Whatman CM-52 CM-cellulose equilibrated with 0.01 M-potassium phosphate buffer, pH 5.0. The cytochrome was adsorbed more strongly than the HiPIP. The DEAE-cellulose and

Abbreviation used: HiPIP, high-potential non-haem iron protein.

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Table 1. Amino acid composition of *Rhodopseudomonas globiformis* cytochrome *c*₂

Results are shown as mol of residues/mol, and were calculated on the basis that Σ (Ala + Leu + Asp + Glu + Phe + Lys + His + Arg) = (4 + 6 + 8 + 8 + 2 + 9 + 5 + 3) = 45. Values discarded because the hydrolysis conditions were not suitable for good recovery of the amino acid concerned are shown in parentheses. The 'best value' is the average of the acceptable estimates. Samples (2) and (5) were hydrolysed at 105 °C, the others (because of an oven fault; see the text) at about 100 °C. All were hydrolysed with 6 M-HCl except sample (6), which was hydrolysed with 3M-mercaptoethanesulphonic acid. Samples (2) and (6) were hydrolysed for 96 h, the others for 24 h. Samples (1), (2) and (6) and, in addition, sample (4) had been oxidized with performic acid and sample (5) aminoethylated.

	Composition (mol of residue/mol)						'Best value'	Sequence
	Hydrolysis and analysis							
	(1)	(2)	(3)	(4)	(5)	(6)		
Glycine	13.4	13.1	13.2	12.9	12.9	13.7	13.2	13
Alanine	4.2	4.1	3.9	4.1	3.9	4.1	4.1	4
Valine	(4.1)	7.3	(4.6)	(3.9)	(5.9)	(4.4)	7.3	7
Leucine	5.7	5.9	5.7	Lost	5.9	5.9	5.8	6
Isoleucine	(5.7)	9.9	(5.8)	Lost	(8.8)	(6.6)	9.9	10
Serine	6.0	(3.5)	4.9	5.4	4.8	5.0	5.2	5
Threonine	4.6	(3.9)	4.8	4.9	4.7	5.0	4.8	5
Aspartic acid	8.2	7.8	7.8	8.2	7.7	8.3	8.0	5
Asparagine								3
Glutamic acid	7.9	7.9	7.8	8.0	7.9	8.2	8.0	5
Glutamine								3
Phenylalanine	2.0	2.2	2.3	Lost	2.0	2.0	2.1	2
Tyrosine	5.8	5.7	5.7	Lost	5.8	6.0	5.8	6
Tryptophan					(0.4)	0.9	0.9	1
Cysteine				1.9*	1.9†		1.9	2
Methionine	0.8	1.0	1.1	1.1‡	1.0	1.0	1.0	1
Proline	9.9	10.4	10.7	10.6	10.6	(10.5)	10.4	11
Lysine	8.6	9.1	9.0	7.6	9.2	8.0	8.6	9
Histidine	4.8	5.1	5.4	5.0	5.2	5.0	5.1	5
Arginine	3.1	2.8	3.2	2.5	3.2	2.7	2.9	3
Total								106

* As cysteic acid.

† As S-aminoethylcysteine.

‡ As methionine sulphone.

CM-cellulose were extruded from their columns, and the HiPIP and cytochrome bands were cut out and eluted separately with 0.5 M-NaCl in pH 7.3 Tris buffer. The proteins were next precipitated with $(\text{NH}_4)_2\text{SO}_4$. HiPIP was precipitated in the 60–90% -saturated fraction, and the cytochrome in the 40–60% -saturated fraction. When redissolved, the $(\text{NH}_4)_2\text{SO}_4$ -precipitated fractions were extremely viscous. We believe that the explanation for the unsatisfactory chromatography of the proteins on DEAE-cellulose was the presence in the *Rps. globiformis* cell extracts of large amounts of acidic polysaccharide.

The cytochrome and HiPIP fractions were subjected to gel filtration through a column (100 cm × 8 cm diam.) of Sephadex G-75 (40–120 mesh) in 0.02 M-Tris/HCl buffer, pH 7.3, containing 0.1 M-NaCl. The cytochrome appeared to be slightly larger than the HiPIP, and they partially separated, but the 'polysaccharide' caused streaking and poor separation on the column if the viscosity was not greatly reduced by dilution before application of the sample to the column.

The cytochrome sample was desalted through Sephadex G-25, and adsorbed on CM-cellulose equilibrated with 0.01 M-potassium phosphate buffer, pH 5. An

attempt was made to oxidize the cytochrome on the column by passing through some 0.001 M- $\text{K}_3\text{Fe}(\text{CN})_6$, but when the cytochrome was eluted with a linear gradient of 0.01–0.05 M-potassium phosphate buffer, pH 6, it was still partly reduced. The CM-cellulose chromatography was repeated, and the resulting product was 20 μmol of cytochrome *c* with a purity index (A_{280}/A_{417}) of 0.18, with a yield of about 50% as judged by the spectrophotometric estimate of the cytochrome *c* content of the supernatant from the initial cell extract. About 15 μmol of HiPIP of purity index (A_{280}/A_{390}) of 1.8 was also recovered.

Despite the interference of the 'polysaccharide', the chromatographic behaviour of the cytochrome fraction suggested that the great majority of the material was of a single molecular species. The final preparation had an α -band maximum of 551 nm, an $A_{551(\text{reduced})}/A_{280}$ ratio of 1.3, and gave a single band of M_r about 11000 when examined by SDS/polyacrylamide-gel electrophoresis. It also gave only a doublet (oxidized and reduced forms) when subjected to fast protein liquid chromatography on a Mono-Q column in 0.02 M-sodium acetate buffer, pH 5.5, with a 0–0.5 M-NaCl gradient.

Amino acid sequence determination

The methods used have been described before (Ambler & Wynn, 1973; Ambler *et al.*, 1979c, 1984). The *N*-terminal sequence of the protein was 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. One peptide was subdigested with the proteinase from a mutant strain of *Pseudomonas fragi* (Drapeau, 1980) that specifically hydrolyses peptide bonds on the *N*-terminal side of aspartic acid and cysteine acid residues.

RESULTS

The amino acid composition of *Rps. globiformis* cytochrome c_2 is shown in Table 1. The evidence for the proposed amino acid sequence is summarized in Fig. 1. Details of the purification, analysis and sequence determination experiments on the peptides are given in Supplementary Publication SUP 50136. The criteria for satisfactory results and the nature and the format of the Supplementary Publication are given in previous papers (Ambler & Wynn, 1973; Ambler *et al.*, 1979c).

The amino acid sequence was deduced by characterizing peptides from four separate digests of the cytochrome, from which the haem had been removed before digestion, except in the case of the CNBr experiment. These digests were: (a) 2.3 μ mol, digested with trypsin; (b) 1.7 μ mol, treated with CNBr (native protein was used, to minimize operations that could result in partial oxidation of methionine residues); (c) 2.1 μ mol, digested with chymotrypsin; (d) 2.2 μ mol, digested with trypsin, after treatment with ethyleneimine under reducing conditions.

DISCUSSION

Accuracy of proposed sequence

In contrast with the purification of the protein, the determination of the amino acid sequence presented no particular difficulties. The peptides characterized are linked into a single sequence by good overlaps, and peptides accounting for the complete sequence were found in each digest. For no part of the proposed sequence does the evidence seem to be markedly weak. The general reliability of the procedures used has been demonstrated in a number of cases where amino acid sequences have been subsequently confirmed by DNA sequencing (e.g. Daldal *et al.*, 1986).

Structural affinities of the *Rps. globiformis* cytochrome c_2

The amino acid sequence (Fig. 1) is that of a 'small' cytochrome c_2 , like those of *Rhodospirillum rubrum* or *Rhodospirillum rubrum* (Ambler *et al.*, 1976), those of some other members of the Rhodospirillaceae (Ambler *et al.*, 1979a) and cytochromes from two non-phototrophic bacteria, *Agrobacterium tumefaciens* (Van Beeumen *et al.*, 1980) and *Nitrobacter agilis* (Tanaka *et al.*, 1982). The 'small' cytochromes c_2 are close in sequence to mitochondrial cytochromes c , and can be aligned to give good matching throughout the sequences with only the need for a very small number of insertions or deletions. A simple measure of the degree of similarity between related sequences is given by the

Fig. 1. Proposed amino acid sequence for *Rhodopseudomonas globiformis* cytochrome c_2

Peptides derived by digestion with trypsin (T) or by trypsin after *S*-aminoethylation (AET) are shown above the sequence, and by digestion with chymotrypsin (C) or by cleavage with CNBr (X) below the sequence. Peptides from subdigests are labelled with another letter indicating the second method of cleavage (H, thermolysin; F, staphylococcal proteinase; D, *Pseudomonas fragi* proteinase; π , dilute acid hydrolysis). All peptides except T-1H8, T29C2, X-IVH0, X-IVH4 and X-IVH1 were analysed for amino acid composition quantitatively, but those marked * gave substandard and those marked ** particularly bad analyses. See Ambler & Wynn (1973) for a quantitative definition of these symbols. The peptide lines are doubled where the sequence has been determined by phenyl isothiocyanate degradation with residues identified by the dansyl method. The lower line is broken under residues for which the identification was substandard. Peptides marked † were examined by carboxypeptidase A digestion, and those marked ‡ by aminopeptidase M digestion. The underlined residues at the *N*-terminus of the sequence were identified in an automatic sequencer degradation.

The sequences are aligned as shown in Fig. 3 of Meyer & Kamen (1982) except that no gap is placed between residues 77 and 78 of the mitochondrial sequences and the corresponding bacterial ones. The score is the percentage identity between pairs of sequences only counting positions that are occupied in both sequences, taking no account of insertions or deletions, or of extra residue on ends. The sequences are given in Meyer & Kamen (1982), apart from those of *Enteromorpha intestinalis* (Meatyard & Boulter, 1974), *Nitrobacter agilis* (Tanaka *et al.*, 1982) and *Aquaspirillum itersonii* (Woolley, 1987). Identity values of greater than 50% are shown in large numerals.

[illegible]

More sophisticated methods of comparison have also been used to try to relate the *Rps. globiformis* sequence to those of other cytochromes *c* (Dayhoff & Schwartz, 1981; Barnabas *et al.*, 1982; Dayhoff, 1983). Dayhoff interpreted the similarities as evidence that mitochondria had arisen from prokaryotes independently on more than one occasion, and constructed trees suggesting the lines in which these events might have occurred.

Meyer *et al.* (1986) have discussed the difficulties that exist in deducing phylogenetic trees from sequence similarities. They suggest that there are finite structural limitations on the variation of a macromolecule (be it 16 S rRNA or cytochrome *c*) that performs a particular function. The result is that evolutionary divergence can only go as far as this limit if function is to be retained, and as this limit is approached an increasing proportion of the acceptable changes will be convergent ones.

Woese and his associates have been attempting to deduce the affinities of the prokaryotic ancestor of the

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