

Primary structure determination of two cytochromes c_2 : Close similarity to functionally unrelated mitochondrial cytochrome c

(photosynthetic bacteria/amino-acid sequence/molecular evolution)

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ABSTRACT The amino-acid sequences of the cytochromes c_2 from the photosynthetic non-sulfur purple bacteria *Rhodomicrobium vannielii* and *Rhodopseudomonas viridis* have been determined. Only a single residue deletion (at position 11 in horse cytochrome c) is necessary to align the sequences with those of mitochondrial cytochromes c . The overall sequence similarity between these cytochromes c_2 and mitochondrial cytochromes c is closer than that between mitochondrial cytochromes c and the other cytochromes c_2 of known sequence, and in the latter multiple insertions and deletions must be postulated before a match can be obtained. Nevertheless, these two cytochromes c_2 show no better reactivity with the mitochondrial cytochrome c oxidase than do the less well-matched cytochromes c_2 . The bearing of these findings on possible evolutionary relationship between mitochondria and prokaryotes is discussed.

The non-sulfur purple bacteria (*Rhodospirillaceae*) are a family of photosynthetic prokaryotes (1) which possess characteristic pigments and have the same type of metabolism. They share a unique ability to utilize a wide range of simple organic carbon sources and some members can grow non-photosynthetically under fully aerobic conditions. However, they do not exhibit a common morphology. *Rhodomicrobium vannielii* resembles the nonphotosynthetic *Hyphomicrobiaceae* in that daughter cells are formed as spherical buds at the ends of filaments growing out from the mother cell. *Rhodopseudomonas viridis* also uses a budding mode of cell division, but lacks the stalked and branched appearance of *Rhodomicrobium*. All but two of the remaining *Rhodospirillaceae* species, including rods, spirilla, and cocci, reproduce through binary fission.

All *Rhodospirillaceae* produce large amounts of soluble c -type cytochrome when growing photosynthetically. The best-studied example is the cytochrome c_2 from *Rhodospirillum rubrum* (2), for which both the amino-acid sequence (3) and three-dimensional structure (4) are known. *R. rubrum* cytochrome c_2 has a strong structural resemblance to mitochondrial cytochrome c (5) and is more closely related in sequence to the eukaryotic proteins than are any of the other bacterial cytochromes examined (6) before now.

In most species of *Rhodospirillaceae*, the predominant c -type cytochrome resembles *R. rubrum* cytochrome c_2 in optical spectra, in oxidation-reduction potential (7), and in limited reactivity with beef heart cytochrome oxidase (8). This is true also (B. Errede and M. D. Kamen, unpublished) for the only soluble cytochromes found in *Rm. vannielii* and *Rps. viridis* (10). In cells of *R. rubrum*, the cytochrome c_2 can be photooxidized (11), but in *Rps. viridis* light-induced absorbance changes have been ascribed (10, 12) to a pair of particulate cytochromes (analogous to those of the purple sulfur bacterium *Chromatium vinosum*).

We have been studying the amino-acid sequences of the *Rhodospirillaceae* cytochromes c_2 and find that they can be divided at present into at least two groups on the basis of the number of insertions and deletions which must be postulated to align them with mitochondrial cytochrome c . One of these, which includes the proteins from *Rps. palustris*, *Rps. capsulata*, *Rps. spheroides* (R. P. Ambler, T. E. Meyer, R. G. Bartsch, and M. D. Kamen, unpublished results, see ref. 13), as well as *R. rubrum* cytochrome c_2 , requires multiple deletions and insertions for alignment, whereas in this report we describe another group, including *Rm. vannielii* and *Rps. viridis* cytochromes c_2 , which require only a single residue deletion for optimal alignment with mitochondrial cytochrome c .

MATERIALS AND METHODS

Rm. vannielii (ATCC 17100) and *Rps. viridis* (NTHC 133) were grown anaerobically in the light on modified Hutner's medium (14), and the heme and non-heme proteins were isolated and purified by standard methods (15). In each case the cytochrome c_2 was the only soluble cytochrome. It was isolated in a yield of about 15 $\mu\text{mol/kg}$ from *Rm. vannielii* and 30 $\mu\text{mol/kg}$ from *Rps. viridis*.

The amino-acid sequences of the cytochromes c_2 were determined by the methods used for other bacterial cytochromes (16), and to similar standards. Protein (2-3 μmol) was treated with HgCl_2 in 8 M urea/0.1 M HCl at 37° for 16 hr to remove the heme, and after gel filtration and lyophilization the sample was digested with a protease. The peptides formed were fractionated by gel filtration followed by high-voltage paper electrophoresis and paper chromatography. Peptide sequences were investigated by the dansyl-Edman method, and by exo- and endo-peptidase digestion. The NH_2 -terminal sequence of the *Rm. vannielii* protein was also investigated with an automatic sequenator (Beckman model 890A) (17). Amide and acid groups were assigned from peptide electrophoretic mobilities, exopeptidase action, and *Staphylococcus aureus* protease (18) digestion.

RESULTS

The amino-acid sequences of the cytochromes c_2 from *Rm. vannielii* and *Rps. viridis* are shown in Fig. 1, aligned with representative mitochondrial cytochrome c sequences.

The sequence of the *Rm. vannielii* protein has been determined exhaustively. Peptides accounting for the whole of the postulated sequence have been purified and characterized from each of tryptic, chymotryptic, and thermolysin digests. The evidence from these peptides was not sufficient to establish the overlap at bond 97/98 (horse cytochrome numbering), and there was only a single residue overlap at bonds

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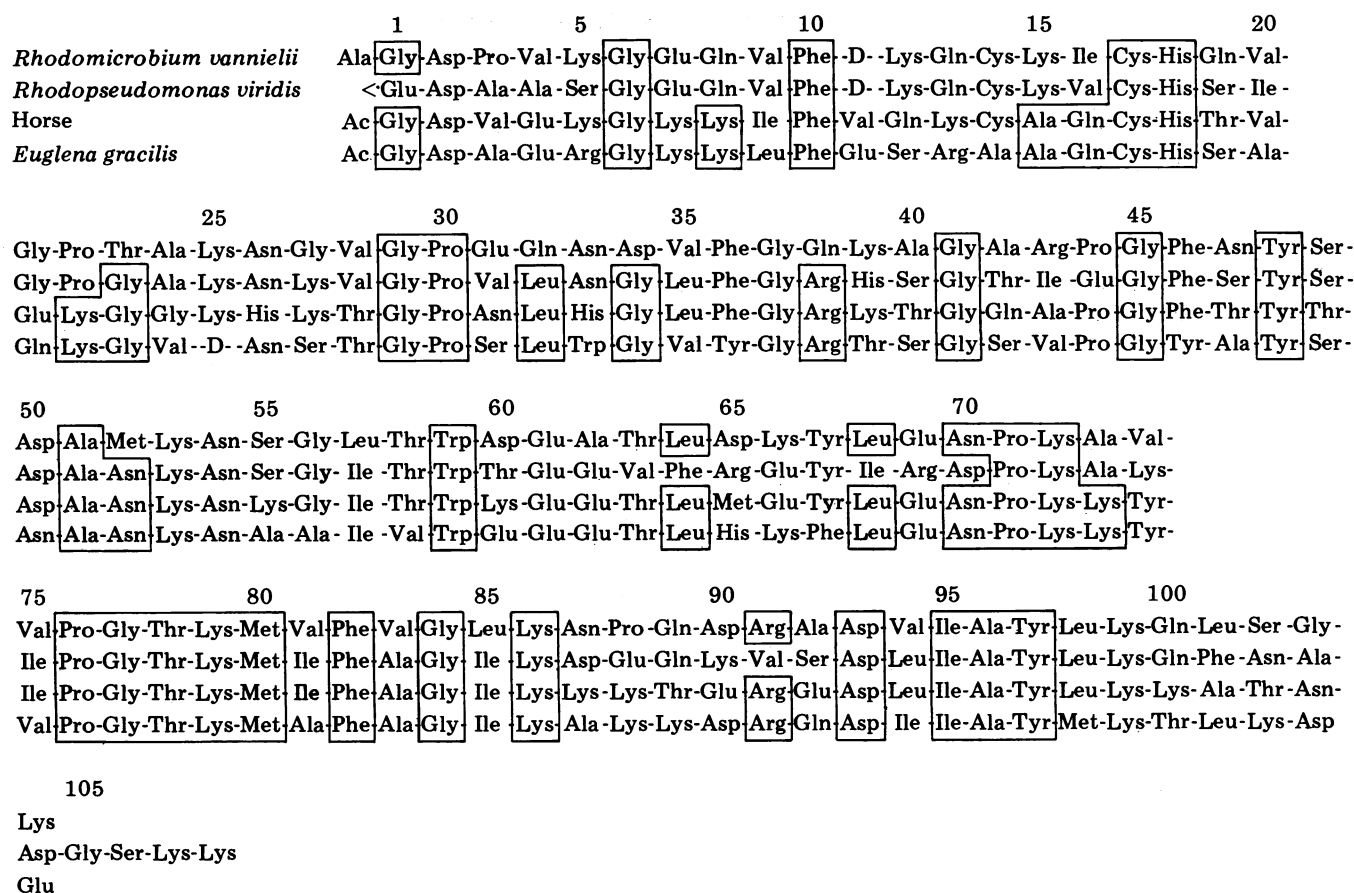


FIG. 1. Alignment of amino-acid sequences of the cytochromes *c*₂ from *Rhodomicrobium vannielii* and *Rhodopseudomonas viridis* (this work) and mitochondrial cytochrome *c* from horse (20) and the alga *Euglena gracilis* (21, 22). The residue numbers are for the horse sequence. The NH₂-terminal residue of the *Rps. viridis* protein is considered to be pyrrolidone carboxylic acid. In the protein as isolated, residue 86 in the *E. gracilis* is *ε-N*-trimethyllysine. Residues that are identical in all the four mitochondrial proteins considered in Table 1 are shown boxed, with the boxes extended if the residue is also identical in one or both of the bacterial sequences. The postulated deletions are shown as -D-.

66/67/68. However, purification and analysis of the products of CNBr cleavage of the protein provided firm evidence for these regions. The sequence of more than 90% of the residues in the protein was established by the dansyl-Edman method in two or more different peptides, and exopeptidase experiments provided duplicate evidence for most of the remaining positions.

The sequence presented for the *Rps. viridis* protein has been derived from the results of a tryptic and chymotryptic digest. All the postulated fragments were found. Overlaps of only single residues have been obtained at positions 10/12/13, 25/26/27/28, 64/65/66, and 79/80/81, and the sequence of most of the residues has been established by a single dansyl-Edman degradation. The evidence for acid or amide groups at Gln-1 and Asp-2, and at Asp-50 and Asn-52 is not yet as strong as for other positions. In the protein as isolated, the NH₂-terminus is blocked (probably by pyrrolidone of Gln-1), and no successful automatic sequenator degradations have been achieved.

The sequences shown for each protein agree well with the observed amino-acid compositions of the isolated proteins. No evidence has been found for the presence of methylated lysine residues in either protein, but this possibility has not been rigorously excluded.

A fuller account of the experimental sequence determination will be published elsewhere.

DISCUSSION

Mitochondrial cytochromes *c* from animals, plants, and fungi have been sequenced and a plausible phylogenetic tree has been suggested (19). Although many bacterial *c*-type cytochromes have been sequenced (6), the cytochrome *c*₂ from the photosynthetic bacterium *R. rubrum* (3) has been the only one to show extensive similarity to mitochondrial cytochrome *c*. Recently, another example—that of the cytochrome *c*-550 from the denitrifier *Paracoccus denitrificans*—has been added (25). During the course of sequencing other cytochromes *c*₂ from the *Rhodospirillaceae*, we now have found two which are more like mitochondrial cytochrome *c* than are either *R. rubrum* cytochrome *c*₂ or *P. denitrificans* cytochrome *c*-550.

The amino-acid sequences of *Rm. vannielii* and *Rps. viridis* cytochromes *c*₂ are shown in Fig. 1 aligned with mitochondrial cytochromes *c* from horse (20) and from the alga *Euglena gracilis* (21, 22). Optimal matching of the bacterial sequences is achieved with only a single residue deletion at position 11 (horse cytochrome *c* numbering) in each sequence. A single residue deletion apparently has occurred in *Euglena gracilis* cytochrome *c* (21, 22) at position 25 and is the only known internal deletion in a mitochondrial cytochrome *c*. The COOH-terminus of the *Rps. viridis* protein is three residues longer than any reported mitochondrial cyto-

Table 1. Comparison of the amino-acid sequences of selected mitochondrial cytochromes *c* (A–D) and bacterial cytochromes *c*₂ (E–G)

	(A)	(B)	(C)	(D)	(E)	(F)	(G)	Refs.
Eukaryotes: (A) Horse	104							(20)
(B) Mung bean	63	103						(29)
(C) <i>Crithidia oncopelti</i>	57	56	102					(30)
(D) <i>Euglena gracilis</i>	58	53	56	102				(12, 22)
Prokaryotes: (E) <i>Rhodomicrobium vannielii</i>	50	50	47	45	103			This work
(F) <i>Rhodopseudomonas viridis</i>	54	52	52	44	56	107		This work
(G) <i>Rhodospirillum rubrum</i>	40	38	44	36	42	41	112	(3)

The numbers shown are the identities scored when the sequences are aligned with the heme attachment sites in register. The deletions assumed for sequences (D), (E), and (F) are as shown in Fig. 1. For sequence (G), the following insertions and deletions are assumed: insertions, three residues after position 54, six residues after position 75, two residues after position 77; deletions of single residues at positions 11 and 84 (horse numbering).

chrome *c*, but differences in the lengths of proteins at the NH₂- and COOH-terminus are common, and some may be due to posttranslational modifications of the coded sequences.

The number of identical residues when these cytochrome sequences are compared is shown in Table 1. This matrix indicates that *Rps. viridis* and *Rm. vannielii* cytochromes *c*₂ are more similar to the selected mitochondrial cytochromes *c* than they are to *R. rubrum* cytochrome *c*₂. The *R. rubrum* protein is only slightly more like the other two cytochromes *c*₂ than it is like the mitochondrial cytochromes. A somewhat greater percentage of the "conserved residues" of mitochondrial cytochrome *c* are shared by the cytochromes *c*₂ than is indicated by the number of overall identities. The 40 residues common to the four very different mitochondrial proteins considered in Table 1 are shown boxed in Fig. 1. Twenty-five of these are identical in both of the bacterial proteins in Fig. 1, and of the 15 other residues "conserved" in the mitochondrial proteins, 10 (five in each) occur in one or other of the bacterial proteins. Four of the five remaining residues are very close in the sequence to the heme attachment site (residues 8, 15, 16, and 22), as is the postulated deletion (residue 11). There is a suggestion that the NH₂-terminal half of the *Rps. viridis* is more similar to the mitochondrial proteins than is the corresponding part of the *Rm. vannielii* protein, with the converse applying to the COOH-terminal halves. The *R. rubrum* protein is identical to the mitochondrial set of proteins (when aligned as described in Table 1) in 21 out of the 40 "conserved" sites.

The results obtained to date would suggest that a continuum of sequences between typically mitochondrial cytochrome *c* and cytochrome *c*₂ may be demonstrated eventually and that these two structural classes may merge into one. On the other hand, the functional properties of these two groups are quite distinct. The cytochromes *c*₂ average less than 10% of the reactivity of mitochondrial cytochromes *c* with beef heart cytochrome oxidase (8), and the oxidation-reduction potentials are reported to be higher and more variable than for the mitochondrial group of cytochromes *c* (7). It may eventually be possible by comparative study of these two groups of proteins to locate residues or regions which define the differences in properties.

The functional roles of mitochondrial cytochromes *c* and of cytochromes *c*₂ are quite different, although not fully understood in either instance. There is evidence to suggest that cytochrome *c*₂ has a direct role in photosynthesis as the immediate electron donor to photooxidized reaction center bacteriochlorophyll in a cyclic photophosphorylation pathway (23). However, in *Rps. viridis*, light-induced absorp-

ance changes are observed for a pair of particulate cytochromes, but not for the cytochrome *c*₂ (10, 12).

It was recently proposed (24) that the facultatively denitrifying aerobic bacterium *Paracoccus denitrificans*, more than any other bacterium, resembles a mitochondrion. A point of the argument in support of this proposal was that the major soluble cytochrome was likely to be related in sequence, in 3-dimensional structure, and in function to mitochondrial cytochrome *c*. In fact, *P. denitrificans* cytochrome *c*-550 is most similar to *Rps. capsulata* cytochrome *c*₂ in sequence (25), but both proteins, like *R. rubrum* cytochrome *c*₂, have longer sequences than those shown in Fig. 1. *P. denitrificans* cytochrome *c*-550 also resembles the cytochrome *c*₂ group in its limited reactivity with beef cytochrome oxidase (8). Although both *Rm. vannielii* and *Rps. viridis* cytochromes *c*₂ are considerably more like mitochondrial cytochromes *c* than is the *P. denitrificans* cytochrome *c*-550, there is no other reason to suspect that ancestral types of these bacteria rather than *P. denitrificans* are better candidates for the role of postulated endosymbiont ancestor to the mitochondrion (24, 26). The present data rather demonstrate that it is premature to speculate on the evolution of bacteria, much less on the possible bacterial origins of the mitochondrion.

There seems to be little doubt that cytochrome *c*₂ and mitochondrial cytochrome *c* share a common ancestor, but placement of cytochrome *c*₂ in the phylogenetic tree (19) for mitochondrial cytochrome *c* appears hazardous owing, in part, to the lack of functional continuity between the two classes and to ignorance of genetic factors controlling bacterial evolution. As an example of the possible complications, *P. denitrificans* cytochrome *c*-550 and *Rps. capsulata* cytochrome *c*₂ are structurally related (6, 25), yet the presence of a denitrification pathway including cytochrome *cd* (27) in *P. denitrificans* argues against its being a photosynthetically incompetent mutant of *Rps. capsulata* because neither denitrification nor cytochrome *cd* has been observed in the *Rhodospirillaceae* (28). Also, no cytochrome resembling cytochrome *c*₂ has yet been found in any denitrifying bacterium other than *P. denitrificans*. The anomalous presence of a cytochrome *c*₂ in *P. denitrificans* could well be the first example of cytochrome gene transfer in unrelated bacteria. Perhaps continued comparative sequence studies will provide unambiguous evidence for occurrence of genetic events, such as gene transfer, so that a distinction between their occurrence and processes of divergent evolution can be made.

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