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Biochemistry

Pseudomonas cytochrome c_{551} at 2.0 Å resolution: Enlargement of the cytochrome *c* family

(bacterial metabolism/protein evolution)

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ABSTRACT **The structure of respiratory cytochrome c_{551} of *Pseudomonas aeruginosa*, with 82 amino acids, has been solved by x-ray analysis and refined to a crystallographic *R* factor of 16.2%. It has the same basic folding pattern and hydrophobic heme environment as cytochromes *c*, c_2, and c_{550}, except for a large deletion at the bottom of the heme crevice. This same "cytochrome fold" appears to be present in photosynthetic cytochromes *c* of green and purple sulfur bacteria, and algal cytochromes *f*, suggesting a common evolutionary origin for electron transport chains in photosynthesis and respiration.**

Cytochrome c_{551} is found in various Pseudomonads and in *Azotobacter vinelandii*, where it plays a respiratory role analogous to mitochondrial cytochrome *c* in eukaryotes and cytochrome c_{550} in *Paracoccus denitrificans* (1–4). It is significantly smaller than these latter proteins, however, having only 82 amino acids instead of 103–134. Amino acid sequence comparisons (table 2 of ref. 4) have suggested a similarity of folding between c_{551} and the larger cytochromes, but in the absence of x-ray data it was difficult to decide where the "deletions" in the c_{551} chain should be placed to make the proper sequence alignment (5–8). The preliminary low-resolution x-ray analysis of *Pseudomonas aeruginosa* cytochrome c_{551} (discussed in ref. 4) showed that the folding patterns in *c*, c_2, c_{550}, and c_{551} were indeed the same. This has now been confirmed by x-ray analysis and constrained difference map refinement at 2.0 Å resolution.

METHODS

The original cytochrome c_{551} was the gift of Henry Harbury, who then trained one of us (R.J.A.) in the techniques of growth of culture of *P. aeruginosa* and purification of the cytochrome (ref. 2; H. Harbury, personal communication). Crystals were grown in 40–50% saturated ammonium sulfate solutions, with 1 M NaCl and 0.01 M ammonium phosphate buffer, pH 5.6–5.9. The crystals are space group $P2_12_12_1$ with unit cell dimensions: a = 29.43 Å, b = 49.00 Å, c = 49.66 Å. Three heavy-atom derivatives were prepared by soaking crystals in stock solutions: K_2PtCl_4, $UO_2(NO_3)_2$, and $NaAu(CN)_2$. All high-resolution data were collected on a modified General Electric XRD-490 x-ray diffractometer, to a resolution of 2.0 Å for native protein crystals and 2.4 Å for the isomorphous derivatives.

Full details of the structure analysis will be reported elsewhere, but the strategy may be outlined here. The mean figure of merit for multiple isomorphous replacement phase refinement (9) at 2.4 Å resolution was 0.924 for centric reflections and 0.763 for all data. Atomic coordinates were measured from a Kendrew wire model built in a Richards box. These were used as the starting point for refinement on a minicomputer, alternating cycles of (*a*) automated and occasionally manual shifts in atomic position based on Fourier difference maps, and (*b*) adjustment of bond distances, angles, and torsion angles toward standard values followed by calculation of a new difference map (10). At present, with stereochemically acceptable bond lengths and angles, a planar heme, and the addition of 42 water molecules per cytochrome molecule, the conventional crystallographic *R* factor* is 16.2% at 2.0 Å resolution.

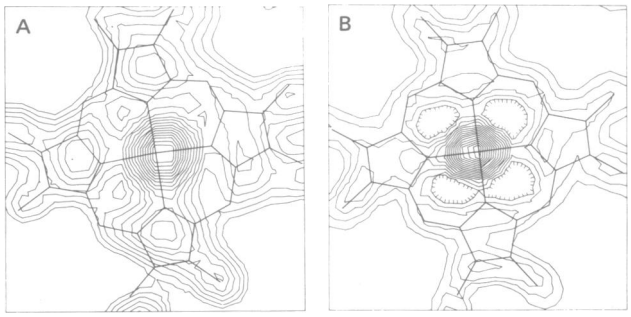

FIG. 1. (*A*) Section through the heme group, from the multiple isomorphous replacement electron-density map of *P. aeruginosa* cytochrome c_{551}. Heme coordinates as obtained from the multiple isomorphous replacement map are superimposed. (*B*) Section through the heme group in the final $(2F_o - F_c)$ map from the refined structure, with the refined coordinates superimposed. The five-membered rings appear as flat plates, with their substituent groups clearly defined; the six-membered rings including the heme iron have deep negative centers. The cysteine 12 attachment is at the left side of the lower pyrrole ring; the cysteine 15 attachment is at the top of the left pyrrole ring. The two propionic acid groups extend out of the sectioning plane at the upper right corner.

As a rough indication of the effect of refinement, two sections through the plane of the heme group are compared in Fig. 1, with heme skeletons superimposed. Fig. 1*A* shows the multiple isomorphous replacement map at 2.4 Å resolution, and Fig. 1*B* the refined $(2F_o - F_c) \exp i\phi_c$ map at 2.0 Å.

RESULTS

The α-carbon atoms, heme group, and a few key side chains of cytochrome c_{551} are shown in the stereo drawing of Fig. 2; the folding of c_{551} is compared with that of tuna *c* (11) in the ribbon drawings of Fig. 3. Among the common structural features are the amino-terminal α helix, cysteine and histidine attachments to the heme, the 20's loop at the right, the 60's helix (40's helix in c_{551}), methionine ligand to the heme, and the carboxy-ter-

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* $R \equiv \dfrac{\sum_{hkl} \| F_o | - | F_c \|}{\sum_{hkl} | F_o |} \times 100.$

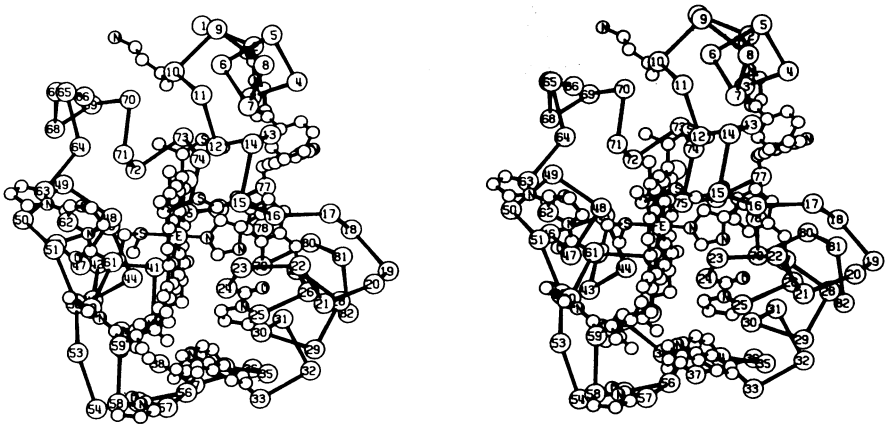

FIG. 2. Stereo drawing of the main chain backbone, heme, and key side chains in c_{551}. Similar drawings of tuna c, *Paracoccus* c_{550}, and *Rhodospirillum rubrum* c_2 can be found in ref. 4.

minal α helix. The principal differences are the deletion of the bottom of the c molecule in c_{551}, its replacement by a 30's helix at the lower rear, and the pulling downward of the 70's loop of c to close off the bottom of the c molecule. The deletion in cytochrome c required to produce the c_{551} molecule can be described approximately as a joining of residue 40 to 56, and removal of residues 41–55. Residues 58–63, with the amino acid sequence Pro-Ile-Pro-Met-Pro-Pro, are now seen to form an almost ideal polyproline threefold helix.

One interesting aspect, an apparent consequence of the deletion of chain at the bottom of the molecule, is the tilting of the heme within its polypeptide cage relative to that in cytochromes c, c_2, and c_{550}. If the polypeptide chains are made to coincide by rotating 44 α-carbon atoms of c_{551} onto corresponding atoms in homologous regions of tuna cytochrome c using a least-squares fitting program, then the heme in c_{551} is found to be rotated approximately 11° to the left about a vertical axis in Fig. 2, and tilted forward 16° about a horizontal axis, in comparison with its orientation in tuna c. (A similar tilt of the heme has subsequently been observed in cytochrome c_{555}; see *Discussion*.)

These structure comparisons now enable a precise alignment of amino acid sequences to be made, as shown in Fig. 4 for tuna c and *Pseudomonas* c_{551}.

The hydrophobic environment around the heme group is almost identical in the two proteins. Table 1 shows the comparison of structurally and sequentially equivalent hydrophobic side chains packed around the heme in 60 eukaryotic c sequences and 6 prokaryotic c_{551}. At only three places along the sequences is a hydrophobic heme contact in one protein not matched in the other. At two of these places the residue even remains hydrophobic, but is only turned so as to be in less obvious contact with the heme plane. This near-identity of heme environments is striking support for the evolutionary relatedness of the two proteins in spite of their size differences.

Aromatic side chains are less well conserved (Table 2). A cytochrome heme seems to demand the nearby presence of aromaticity, but with the placement in three dimensions being less critical. Only the two rings in what has been called the "right channel" (12), positions 10 and 97 in c or 7 and 77 in c_{551}, are invariant in all species. None of the aromatic rings that once were invoked in the electron transfer mechanism—59, 67, 74, 82—has an exact equivalent in c_{551}. However, the role of tryptophan 59, which is hydrogen bonded to the inner propionic

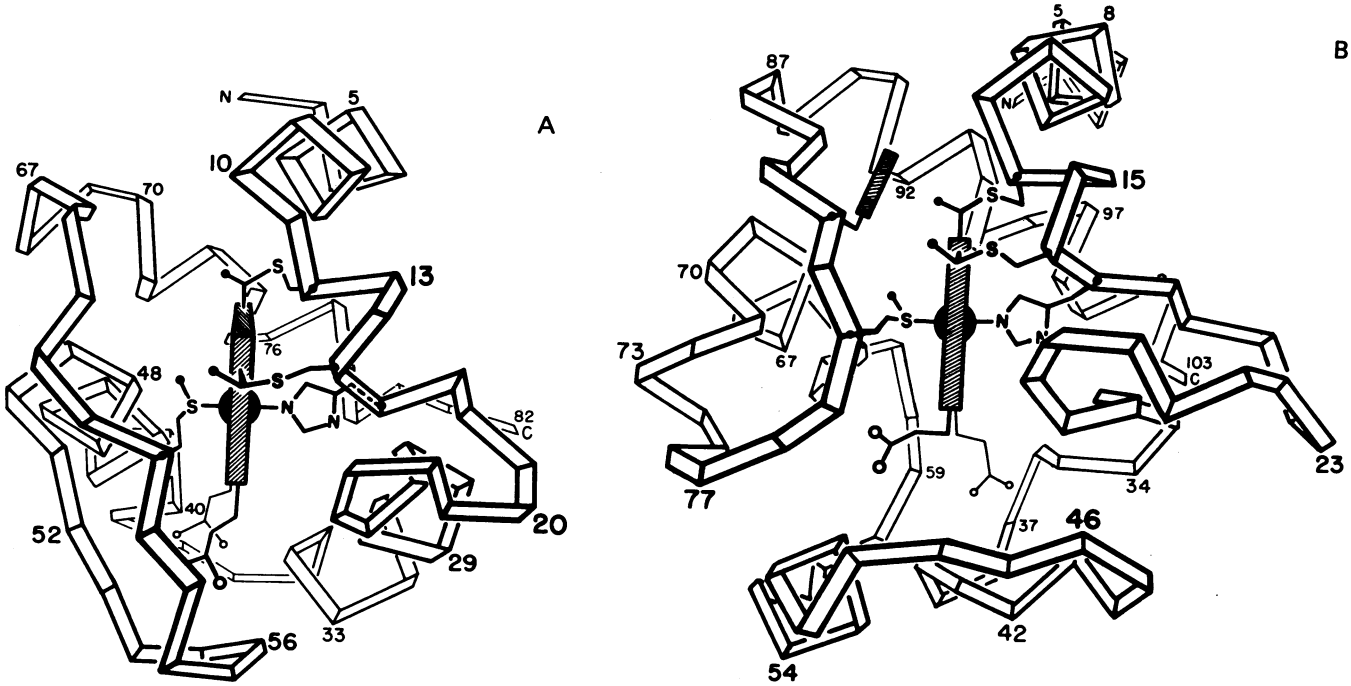

FIG. 3. Ribbon drawings of main chain pathways in (*A*) cytochrome c_{551} from *P. aeruginosa* and (*B*) cytochrome c from tuna. The basic molecular folding is the same. The heme group with its iron is represented by a cross-hatched slab with a central black ball. Covalent cysteine attachments, the methionine and histidine iron ligands, and the inner and outer heme propionic acid groups (at the bottom) are also shown.

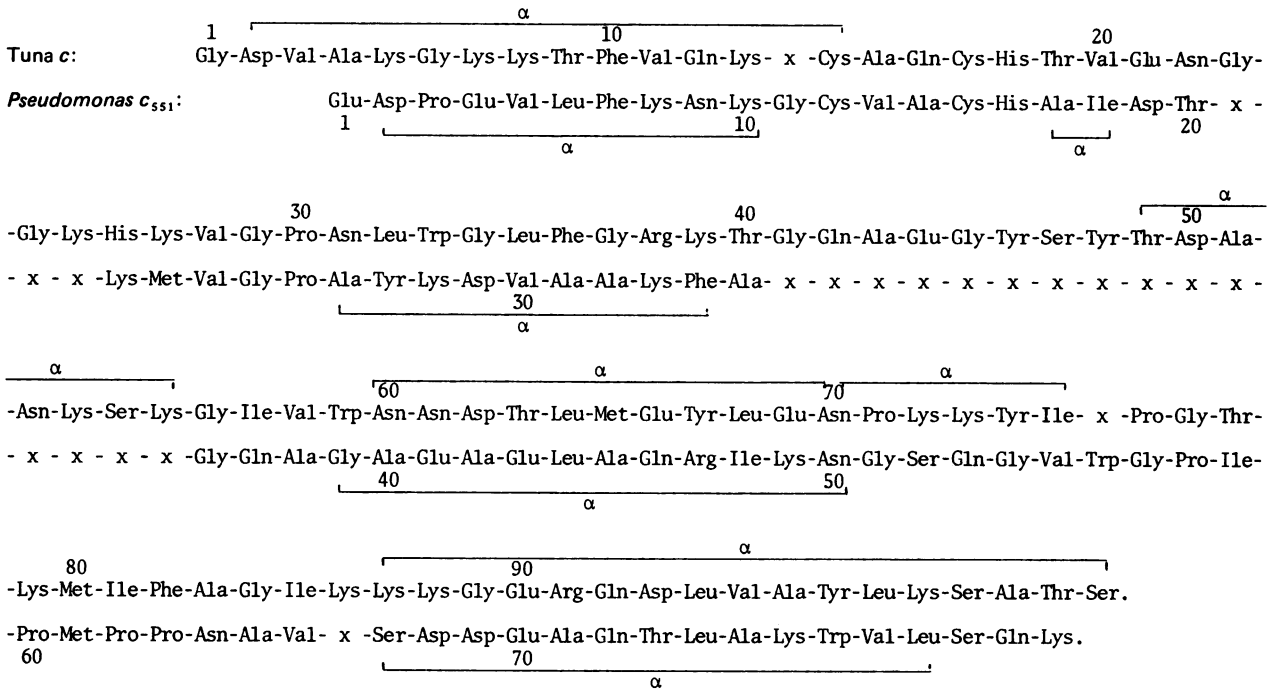

FIG. 4. Amino acid sequence alignments for tuna cytochrome *c* and *P. aeruginosa* c_{551}, made on the basis of the x-ray analyses. Brackets with α indicate regions of α helix. Ala 17-Ile 18 in c_{551} are considered α-helical because they make the proper hydrogen bonds to the beginning of helix 26–34.

acid group of the heme in all molecules of *c*, c_{550}, and c_2, is played by the sequentially unrelated tryptophan 56 of c_{551}. This appears to be an example of evolutionary convergence, as if the molecule, lacking an essential tryptophan at one position, developed a compensating residue somewhere else and made the requisite main chain adjustments to bring the tryptophan to its former position.

The environments of the buried and exposed propionic acid groups on the heme are similar in *c* and c_{551}, although the results are achieved in different ways from a sequence standpoint. The aromatic rings of invariant tyrosine 48 and tryptophan 59 in *c* are matched by invariant tryptophan 56 and semi-invariant phenylalanine/tyrosine/asparagine 34 around the buried propionic group in c_{551}. Serine/threonine 49 and invariant threonine 78 in *c*, hydrogen bonded to the outer propionic group, are paralleled by a semi-invariant serine/threonine at position 52 or 53 in c_{551}, also hydrogen bonded to the outer propionic group. In all known cytochrome *c* structures there seems to be a need for (*i*) a tryptophan residue hydrogen bonded to the buried heme propionic acid (*ii*) another nearby aromatic ring, (*iii*) a serine/threonine hydrogen bond to the outer propionate, and (*iv*) a pair of aromatic side chains to the right of the heme.

The highly asymmetric distribution of positively and negatively charged side chains that has been remarked for other cytochromes *c* (3, 12, 13) is present in cytochrome c_{551} also. The molecule has eight lysines, one arginine, five aspartic acids, and five glutamic acids, for a net charge count among side chains of −1. However, if a plane is drawn parallel to the page in Fig. 2 through the center of gravity of the molecule, then six positive charges (residues 8, 10, 28, 33, 47, and 49) and one negative

Table 1. Hydrophobic heme contacts in cytochromes *c* and c_{551}

Cytochrome *c*		Cytochrome c_{551}	
Tuna	60 Eukaryotes	*P. aeruginosa*	6 Prokaryotes
Phe^{10}	60 Phe	Phe^{7}	5 Phe, 1 Tyr
Pro^{30}	60 Pro	Pro^{25}	6 Pro
Leu^{32}	60 Leu	Tyr^{27}	3 Phe, 2 Leu, 1 Tyr
Leu^{35}	51 Leu, 5 Ile, 2 Val, 2 Phe	Val^{30}	6 Val
Leu^{64}	57 Leu, 2 Met, 1 Phe	Leu^{44}	5 Leu, 1 Ile
Tyr^{67}	59 Tyr, 1 Phe	No heme contact in c_{551}	
Leu^{68}	60 Leu	Ile^{48}	6 Ile
Pro^{71}	60 Pro	Gly^{51}	6 Gly
No heme contact in *c*		Pro^{62}	6 Pro
Phe^{82}	60 Phe	No heme contact in c_{551}	
Ile^{85}	36 Leu, 24 Ile	Val^{66}	6 Val
Leu^{94}	58 Leu, 2 Ile	Leu^{74}	6 Leu
Val^{95}	55 Ile, 4 Val, 1 Leu	Ala^{75}	6 Ala
Leu^{98}	57 Leu, 3 Met	Val^{78}	4 Val, 2 Ile

Eukaryotic cytochrome *c* sequences from tables IX and X of ref. 18; prokaryotic c_{551} sequences from table 2 of ref. 4.

Table 2. Aromatic side chains in cytochromes *c* and c_{551}

Cytochrome *c*		Cytochrome c_{551}	
Tuna	60 Eukaryotes	*P. aeruginosa*	6 Prokaryotes
Phe^{10}	60 Phe	Phe^{7}	5 Phe, 1 Tyr
Not aromatic		Tyr^{27}	3 Phe, 2 Leu, 1 Tyr
Phe^{36}	53 Phe, 3 Ile, 2 Val, 2 Tyr	Not aromatic	
Not aromatic		Phe^{34}	3 Tyr, 2 Asn, 1 Phe
Tyr^{46}	33 Tyr, 27 Phe	Deletion region	
Tyr^{48}	60 Tyr	Deletion region	
Trp^{59}*	60 Trp	Trp^{56}*	6 Trp
Tyr^{67}	59 Tyr, 1 Phe	Not aromatic	
Tyr^{74}	58 Tyr, 2 Phe	Not aromatic	
Phe^{82}	60 Phe	Not aromatic	
Tyr^{97}	59 Tyr, 1 Phe	Trp^{77}	6 Trp

* Molecular convergence: same position in three dimensions, although different positions along polypeptide chains.

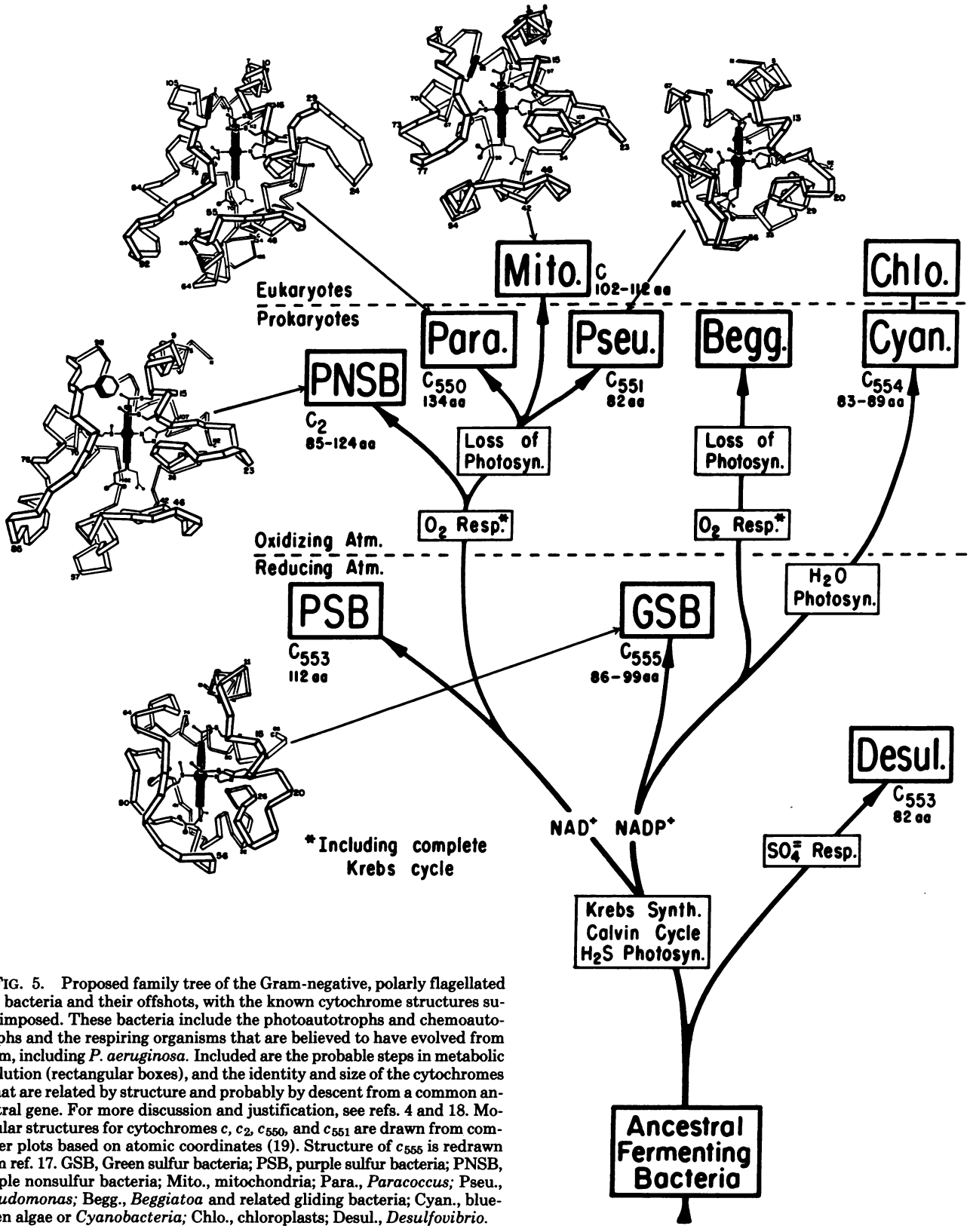

FIG. 5. Proposed family tree of the Gram-negative, polarly flagellated rod bacteria and their offshots, with the known cytochrome structures superimposed. These bacteria include the photoautotrophs and chemoautotrophs and the respiring organisms that are believed to have evolved from them, including *P. aeruginosa*. Included are the probable steps in metabolic evolution (rectangular boxes), and the identity and size of the cytochromes *c* that are related by structure and probably by descent from a common ancestral gene. For more discussion and justification, see refs. 4 and 18. Molecular structures for cytochromes *c*, c_2, c_{550}, and c_{551} are drawn from computer plots based on atomic coordinates (19). Structure of c_{555} is redrawn from ref. 17. GSB, Green sulfur bacteria; PSB, purple sulfur bacteria; PNSB, purple nonsulfur bacteria; Mito., mitochondria; Para., *Paracoccus;* Pseu., *Pseudomonas;* Begg., *Beggiatoa* and related gliding bacteria; Cyan., blue-green algae or *Cyanobacteria;* Chlo., chloroplasts; Desul., *Desulfovibrio.*

charge (residue 29) lie on this bisecting perimeter, eight negative charges (residues 1, 2, 4, 41, 43, 68, 69, and 70) and two positive (residues 76 and 82) lie on the back half of the molecular surface, and only one side chain of each charge (aspartic 19 and lysine 21) is found on the front hemisphere. The cytochrome c_{551} molecule is essentially a sphere with the heme crevice opening to a hydrophobic front hemisphere, diametrically opposed to a negatively charged back hemisphere, with a belt of positive charges separating the two. The positive charges around the perimeter of the heme face of the molecule, which have been commented upon in other cytochromes *c*, are present here also. Lysines 10 at the top of the crevice, 21 at the right, and 47 at the left correspond to lysines 13, 25/27, and 72/73 in eukaryotes. These residues are evolutionarily invariant

among the six c_{551} sequences known (as are most of the other lysines). The acidic side chains are more variable, as was also the case for the eukaryotic cytochromes *c*.

DISCUSSION

The x-ray crystal structure shows clearly that cytochrome *c* belongs in the same evolutionary family with eukaryotic, mitochondrial *c*, *Paracoccus* c_{550}, and c_2 from purple nonsulfur photosynthetic/respiratory bacteria such as *Rhodospirillum*. Possible evolutionary relationships between these proteins, and between their host organisms, have been discussed in ref. 4. At the time of that paper it appeared as if cytochromes *c*, c_2, and c_{550} were the most closely related group and that the c_{551}s with their chain deletion at the bottom of the molecule were more distantly related. Among the cytochromes c_2 from various purple nonsulfur photosynthetic bacteria whose sequences had been determined by Ambler and others (14, 15) were molecules that were as small as eukaryotic *c* or as large as c_{550}, but with nothing in the size range of c_{551}. Recent work has changed this picture (R. Ambler, private communication). Sequences have been determined for at least one c_2 from every formal species listed in *Bergey's Manual* (16). In at least two of the three genera, *Rhodospirillum* and *Rhodopseudomonas*, Ambler found species containing cytochromes c_2 that in both size and amino acid sequence appear to be homologous with *Pseudomonas* c_{551}. It appears that all of these small cytochromes, from c_{551} with 82 amino acids to c_{550} with 134, should be considered as one evolutionary family, with a standard pattern of acceptable insertions and deletions that is followed in diverse organisms: respirers and photosynthesizers, prokaryotes and eukaryotes.

Other members of this family probably include cytochrome c_{553} of purple sulfur bacteria (Chromatiaceae) with 112 amino acids, c_{555} of green sulfur bacteria (Chlorobiaceae) with 86 amino acids, cytochromes *f* or c_{554} of prokaryotic and eukaryotic algae with 83–89 amino acids, and perhaps even the single-heme cytochrome c_{553} of the sulfate-respiring *Desulfovibrio* with 82 amino acids. Korszun and Salemme have recently reported the folding of the polypeptide chain backbone in cytochrome c_{555} of *Chlorobium thiosulfatophilum* (17) as obtained from an unrefined multiple isomorphous replacement analysis at 2.7 Å resolution, and it is apparent that this backbone is virtually identical with that reported for *Pseudomonas* c_{551} in ref. 4 and this paper. The same large deletion is observed at the bottom of the molecule, and from the ribbon drawing of the c_{555} chain path, the heme appears to be tilted forward as in c_{551}. One interesting difference observed in c_{555} is that the tryptophan that appears to be hydrogen bonded to the buried propionic acid (17) is present in the *same* position along the chain in eukaryotic cytochromes *c* and all published c_{555} sequences, even though these two proteins probably are the most distantly related in an evolutionary sense. The shifted tryptophan observed in c_{551} may represent a special adaptation in the Pseudomonads.

The probable evolutionary relationships between the cytochrome *c*-containing metabolic pathways in bacteria and eukaryotes are outlined in Fig. 5, which is an extension of an earlier metabolic tree in ref. 4. At least the main chain pathway is now known for five of the cytochromes indicated in this figure; c_{555}, c_2, c_{551}, c_{550}, and *c*, in the probable order of evolution. The "small" form of cytochrome *c* with the bottom chain deletion is found in green sulfur and purple nonsulfur photosynethetic bacteria, respiratory bacteria (c_{551}), and cyanobacteria or blue-green algae. The "large" form of cytochrome *c*, in contrast, has been observed so far only in the branch of Fig. 5 leading to the purple sulfur and nonsulfur bacteria. Hence, it seems more likely that the small cytochrome is the ancestral form and that one branch has seen the *addition* of more chain at the bottom of the heme crevice.

All of the bacteria discussed in this paper and shown in Fig. 5 are similar enough in general morphology to be grouped by Brock (20) into one category: the Gram-negative, polarly flagellated rods. It is interesting to find this morphological similarity matched by a biochemical similarity: all members perform either photosynthesis or respiration or both, all possess electron transport chains with a cytochrome *c* near the high-potential end, and all of these cytochromes *c* appear to be structurally or sequentially homologous. This class of cytochromes arose soon after bacteria developed the ability to trap light and use it for chemical purposes, and has been retained in the divergent evolution of present-day photosynthesis and respiration.

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1. Horio, T., Higashi, T., Sasagawa, M., Kusai, K., Nakai, M. & Okunuki, K. (1960) *Biochem. J.* **77**, 194–201.
2. Ambler, R. (1963) *Biochem. J.* **89**, 341–349 and 349–378.
3. Timkovich, R. & Dickerson, R. E. (1976) *J. Biol. Chem.* **251**, 4033–4046.
4. Dickerson, R. E., Timkovich, R. & Almassy, R. J. (1976) *J. Mol. Biol.* **100**, 473–491.
5. Needleman, S. B. & Blair, T. T. (1969) *Proc. Natl. Acad. Sci. USA* **63**, 1227–1233.
6. Dickerson, R. E. (1971) *J. Mol. Biol.* **57**, 1–15.
7. McLachlan, A. D. (1971) *J. Mol. Biol.* **61**, 409–424.
8. Dayhoff, M. O. (1976) *Atlas of Protein Sequence* (Georgetown Univ., Washington, DC), Vol. 5, Suppl. 2, pp. 26–27.
9. Dickerson, R. E., Weinzierl, J. E. & Palmer, R. A. (1968) *Acta Crystallogr. Sect. B* **24**, 997–1003.
10. Chambers, J. L. & Stroud, R. M. (1977) *Acta Crystallogr. Sect. B* **33**, 1824–1837.
11. Mandel, N., Mandel, G., Trus, B. L., Rosenberg, J., Carlson, G. & Dickerson, R. E. (1977) *J. Biol. Chem.* **252**, 4619–4636.
12. Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A. & Margoliash, E. (1971) *J. Biol. Chem.* **246**, 1511–1535.
13. Salemme, F. R., Freer, S. T., Xuong, Ng. H., Alden, R. A. & Kraut, J. (1973) *J. Biol. Chem.* **248**, 3910–3921.
14. Ambler, R. P. (1976) in *CRC Handbook of Biochemistry and Molecular Biology*, ed. Fasman, G. D. (CRC Press, Cleveland, OH), 3rd Ed., Proteins Section, Vol. III, pp. 294–298.
15. Ambler, R. P., Meyer, T. E. & Kamen, M. D. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 472–475.
16. Buchanan, R. E. & Gibbons, N. E., eds. (1974) *Bergey's Manual of Determinative Bacteriology* (Williams and Wilkins, Baltimore, MD), 8th Ed.
17. Korszun, Z. R. & Salemme, F. R. (1978) *Proc. Natl. Acad. Sci. USA* **74**, 5244–5247.
18. Dickerson, R. E. & Timkovich, R. (1975) in *The Enzymes*, ed. Boyer, P. (Academic Press, New York), Vol. 11, pp. 397–547.
19. Dickerson, R. E. (1978) in *International Symposium on Biomolecular Structure, Conformation, Function and Evolution Proceedings*, Madras, 4–7 January 1978, ed. Srinivasan, R. (Pergamon Press, New York).
20. Brock, T. D. (1970) *Biology of Microorganisms* (Prentice Hall, New York), pp. 579–606.