**SUMMARY**

The structure of ferricytochrome $c_2$ from the non-sulfur purple photosynthetic bacterium *Rhodospirillum rubrum* has been determined at 2 A resolution by x-ray crystallographic methods. The 112-residue polypeptide chain encloses a single covalently bound heme in a predominantly hydrophobic environment, leaving only one edge exposed to the solvent at the front of the molecule. Distributed around the exterior perimeter of the heme crevice are 11 lysine residues which form an essentially uninterrupted positively charged patch. The molecule contains four segments of α-helix, with the conformation of the remainder of the chain being primarily governed by interactions with the heme. A hypothetical scheme for folding of the molecule is presented, based primarily upon the assumption that the helical regions are formed first and are subsequently distorted by hydrophobic interactions with the heme. The heme iron is coordinated to nitrogen Nε2 of His 18 and to the sulfur atom of Met 91 in the fifth and sixth positions. A notable feature of the structure is an apparently "off axis" or bent Met 91 sulfur-iron bond. The non-axial nature of this bond is attributed to a charge-pair interaction between the Tyr 70 hydroxyl oxygen, bearing a partial negative charge, and the Met 91 sulfur atom, bearing a partial positive charge detached from the ferriheme iron. This interaction serves to stabilize the oxidized heme. The additional participation of Tyr 70 in a hydrogen bond network via Tyr 52 to Ser 89 on the front surface of the molecule suggests that the physiological mechanism for reduction of cytochrome $c_2$ involves concerted protonation of Ser 89 concomitant with electron donation to the heme. The process is reversed for oxidation. This proposed mechanism has several chemical and structural implications, the most important being that the site of interaction with physiological oxido-reductants is at the front of the cytochrome $c_2$ molecule. These conclusions appear to be substantiated by a variety of published findings, including pH dependence of the midpoint potential of cytochrome $c_2$ and polylysine inhibition of cytochrome $c_2$ reactivity in physiological oxidation-reduction systems.

Cytochrome $c_2$ of *Rhodospirillum rubrum* was the first $c$-type cytochrome to be isolated and characterized from a procaryotic organism (1). *R. rubrum* is a common purple non-sulfur photosynthetic bacterium which can live anaerobically as a photoheterotroph dependent upon a cyclic photophosphorylating electron transport chain or alternatively in the dark in the presence of oxygen and reduced organic substrates, utilizing an oxidative electron transport chain for the production of ATP. The organism in either case produces large amounts of cytochrome $c_2$, approximately 75% of which is found in the cytoplasmic fraction. The remainder is firmly bound in the membrane fraction where it may play a role in either oxidative or photosynthetic electron transport. Although all of the specific functions of this protein have yet to be established unequivocally, it is believed to function as the primary donor to bacteriochlorophyll in the photosynthetic electron transport chain (2).

Cytochrome $c_2$ consists of a single polypeptide chain of 112 residues and contains a single covalently bonded protoheme IX. The amino acid sequence as determined by Das et al. (3) is shown in Fig. 1. The calculated molecular weight is 12,450. The $E_{1/2}$ of the protein is +320 millivolts. As there is substantial structural and sequence homology (3) with eucaryotic cytochrome $c$, the three-dimensional structure of cytochrome $c_2$ is of considerable interest relative to the evolution of $c$-type cytochromes (4). A detailed structural comparison of *R. rubrum* cytochrome $c_2$ with eucaryotic mitochondrial cytochrome $c$ (5-7) is in preparation.

**EXPERIMENTAL PROCEDURE**

**Protein Crystallization and Isoemorphous Derivative Preparation—**Cytochrome $c_2$ was isolated from anaerobically grown photosynthetic cultures of *R. rubrum* by a modification of the method of Horio and Kamen (8). Although cytochrome $c_2$ is isolated from the bacterium in the reduced form (Fe$^{2+}$), upon prolonged contact with air in the absence of reducing agents it is approximately 95% converted to the oxidized form (Fe$^{3+}$). Suspensions of oxidized cytochrome $c_2$ microcrystals precipitated from 90% saturated ammonium sulfate (pH 5.8) were centrifuged, and the pellet was dissolved in cold distilled water to obtain a concentrated (30 mg per ml) protein solution for crystallization, containing some residual ammonium sulfate at a concentration of about 0.3 M. Crystals were grown in small test tubes (7 × 20 mm) by a free interface diffusion technique (9). Initial crystallization experiments were carried out by layering 50 μl of the concentrated protein solution over 100 μl...
of 80% saturated ammonium sulfate at room temperature. Crystals suitable for x-ray intensity measurement (average dimension ≈ 0.3 mm) grew as rhombohedral plates within 1 to 2 weeks.

Many attempts to prepare heavy atom isomorphous derivatives of these protein crystals were carried out by soaking a few crystals in a 10- to 100-fold molar excess of heavy atom reagent in 80% saturated ammonium sulfate, buffered at various pH levels. After 1 to 2 weeks of soaking, a representative crystal was mounted in a Lindemann glass capillary in the conventional manner and 21° precession photographs of the 0kl or hkl reciprocal lattice nets were compared with the corresponding projections obtained from native parent protein crystals in order to ascertain whether or not a derivative had been formed. In approximately 200 attempts, only one useful heavy atom isomorphous derivative (AuCl-) of the room temperature form was found.

Further attempts were made to obtain heavy atom derivatives by transferring these crystals to a medium consisting of saturated potassium phosphate plus 1.0 M ammonium sulfate containing heavy atom reagents. This procedure yielded two additional derivatives, Pt(SCN) 42- and [Os(NH3) &. Derivatives isomorphous derivative (AuCl-) of the room temperature form, but they grow as slightly truncated rhombohedral plates. They were prepared by layering 50 ~l of oxidized protein crystals in a lo- to 100-fold molar excess of heavy atom reagents. This procedure yielded two additional derivatives, Pt(SCN) 42- and [Os(NH3) &.

Crystal damage was considered justification for replacing the crystal. The maximum x-ray exposure received by any single crystal was about 200h. A small set of reflections was continuously monitored at intervals throughout data collection. An average 15% decrease in intensity of these reflections due to crystal damage was considered justifiable for replacing the crystal.

Four sets of parent intensities obtained from precession films sealed together with an over-all R factor (11) of 8.8%. Two sets of 2-A diffractometer data sealed together with an R factor of 5.1%. The over-all scaling R factor for combined film and diffractometer parent data was 8.6%. In summary, each unique intensity was replicated at least four times. Data from 2.0 to 2.5 A resolution were measured at least twice.

The four heavy atom derivatives used in the structure deter-
duplicated. Overall scaling R factors for the derivatives ranged from 6 to 9%.

Phase Refinement—Intensities of hkI and Bijvoet related reflections were averaged during the initial stages of phase refinement, which were carried out using only data to 2.5 Å resolution by the usual method of minimizing \( R = \sum_{hkl} |F_{hkl}(\text{obs}) - F_{hkl}(\text{calc})|^2 \) in alternating cycles of phase calculation and refinement of heavy atom parameters. The iridium derivative was eliminated, since its sites appeared nearly identical with those occupied by the osmium derivative. Later cycles of phase refinement included all data to 2 Å resolution, since 2.0- to 2.5 Å shell difference Patterson maps of the gold chloride and uranyl nitrate derivatives showed peaks 2 to 16 times above background at all heavy atom sites. To account for anomalous scattering effects due to the heme iron atom, final centroid phases were calculated by a program which combined the closure errors for each \( F_{hkl} \) and the complex conjugate of its Friedel mate. This program also calculates a least-squares value of an anomalous scale constant (anom K), which is the ratio of imaginary to real scattering power for each heavy atom type. Statistics from the last cycle of refinement are shown in Table I.

### Table I

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<th>Derivative</th>
<th>Site No.</th>
<th>( \alpha )</th>
<th>( \beta )</th>
<th>( \gamma )</th>
<th>( \delta_{\alpha} )</th>
<th>( \delta_{\beta} )</th>
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<th>( k )</th>
<th>( c )</th>
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<th>( \text{rms}_{E} )</th>
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<th>( R_{2} )</th>
<th>( \text{anom. K} )</th>
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<td>101.8</td>
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<td>24.8</td>
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</tbody>
</table>

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**Mean figure of merit to 2.0 Å resolution = 0.74.**

**R** as given here is defined by: \( R = \sum_{hkl} |F_{hkl}(\text{obs}) - F_{hkl}(\text{calc})|^2 \).

**R** is the scale factor to put the structure factor for a derivative on the same scale as the parent.

**k** is the scale factor to put the structure factor for a derivative on the same scale as the parent.

**c** is the scale factor that compensates for any difference in the temperature factor between the derivative and the parent: \( k' = k \exp \left( \frac{c \sin \theta}{\lambda} \right) \).

**E** is the closure error, \( |k'F_{hkl}(\text{obs}) - F_{hkl}(\text{calc})| \).

**R_{1} = \sum_{\text{all hkl}} |k'F_{hkl}(\text{obs}) - F_{hkl}(\text{calc})|**

**R_{2} = \sum_{\text{all hkl}} |k'F_{hkl}(\text{obs}) - F_{hkl}(\text{calc})|**

\( \text{rms}_{R} \) sums over all reflections.

\( \text{rms}_{E} \) sums over centric zone reflections only.

\( \text{anom. K} = \text{ratio of imaginary to real scattering power.} \)

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**Interpretation of Electron Density Map**—The correct structural enantiomorph was determined by calculating a parent Bijvoet difference Fourier map (14) which showed a single positive peak six times higher than background at the position of the heme iron determined from previous 5 Å resolution studies (15). A small preliminary map on a scale of 0.163 inch per Å was contoured on Plexiglas sheets (3.5 × 11 inches) to allow the boundaries of the molecule to be determined prior to production of a large scale map.

A 2 cm per Å map was calculated from centroid phases but without centroid weighting factors. Forty-eight sections perpendicular to the a axis at intervals of 0.87 Å were calculated on a 32 × 72 grid and plotted directly on transparent acetate plastic sheets with a CalComp flatbed plotter. The map was contoured at a minimum level of 0.27 e per Å³ and at every 0.18 e per Å³ thereafter. An absolute scale in e per Å³ was estimated from a subsequent structure factor calculation. The plastic sheets were taped to aluminum screen frames and inserted into a horizontal Richards optical comparator (16).

Model construction was begun at the heme group, since the positions of the iron atom and cysteinyl sulfur atoms of residues 14 and 17 appeared as prominent features in the map. The chain was subsequently built according to the published sequence (5). There appeared to be no breaks in the backbone chain density. The majority of backbone carbonyl groups and most of the internal amino acid side chains were well defined, but external residues were generally less clear. Density corresponding to lysine residues in most cases faded appreciably beyond the β or γ side chain carbon atom, although in some cases these residues appeared to have branched density suggesting the existence of...
two or more alternative conformations in the crystal lattice. The over-all quality of the map appeared to be poorer in the section of chain from residue 71 through 95. This effect may be due to greater flexibility of this part of the chain.

The following structural analysis is subject to the usual caveats with respect to interpretation of electron density maps based on multiple isomorphous replacement phases. It is not likely, however, that crystallographic refinement procedures which are currently in progress to improve the quality of this map will significantly alter the description of the molecule presented in this paper.

RESULTS

Heavy Atom Binding Sites—All of the principal heavy atom binding sites to the cytochrome c2 molecule in the crystal lattice occur on the back side (relative to the heme crevice) of the molecule. Inspection of the electron density distribution in the unit cell shows that this part of the molecule forms the border of the largest solvent channel in the crystal lattice.

The principal osmium-binding site (No. 1, see Table I) occurs in the vicinity of the carboxylate group of Glu 37 and the ammonium group of Lys 112. This position is also a secondary site (No. 4) for the uranyl nitrate derivative. Additional osmium-binding sites occur in the vicinity of the ammonium side chain groups of Lys 56 (No. 2) and Lys 109 (No. 3). Lysine 56 is adjacent to Met 55 which also could be involved in the osmium-binding site.

The two principal uranium-binding sites occur near the carboxylate group of Glu 64 (No. 1) and near the hydroxyl group of threonine 63 (No. 2). A secondary site (No. 3) in addition to the one occupied in common with the osmium derivative is located near the partially exposed Ile 101 side chain in the vicinity of the ammonium group of Lys 97.

The most heavily occupied gold chloride-binding site (No. 1) is adjacent to the imidazole ring of His 42. The small secondary gold-binding site (No. 2) lies in the vicinity of the carboxylate group of Asp 3.

Description of Molecular Conformation—The over-all shape of the molecule is a roughly ellipsoidal figure of dimensions 25 × 33 × 40 Å. A stereooscopic front view (facing the home crevice) of the molecule is shown in Fig. 2A and a schematic drawing of the structure showing covalent and hydrogen-bonded interactions is shown in Fig. 3. The polypeptide chain begins at the upper right of the molecule, forming a regular β-helix from residues 2 through 10. Residues 10 through 14 form a pair of type IIa hairpin loops (17) as the chain makes the transition from α-helical to a more extended conformation. Cysteines 14 and 17 are covalently bonded to the heme through thioether linkages resulting from condensation of the side chain thiol groups with the heme vinyl groups. The short loop formed by these residues forms a structure intermediate between a type IIa bend and a turn of an α-helix as the carboxyl oxygen of Cys 14 can form equally good hydrogen bonds with the amide nitrogens of residues 17 and 18. The imidazole N(2) of His 18 forms the fifth coordinate bond with the heme iron atom. The succeeding residues 18 through 40, which make up the lower right side of the molecule, form a meandering series of loops devoid of any regular extended hydrogen-bonded structures. Two type IIa hydrogen bonds from Gly 24 to Asp 21 and from Val 35 to Leu 52 occur in this region, as well as a type Iα hydrogen bond between residues Asn 38 and Val 35 and interchain hydrogen bonds from the amide of Gly 29 to the carbonyl of Cys 17, the side chain hydroxyl of Thr 19 to the carbonyl oxygen of Ala 25, and between the side chain amides of asparagines 21, 26, and 45 (Fig. 3). This section also contains several residues which form hydrogen bonds with the heme. The hydroxyl oxygen of Tyr 46 is positioned to form a hydrogen bond with either the carbonyl oxygen of Val 28 or an oxygen of the front heme propionic acid group. The hydroxyl group of Tyr 48 is hydrogen bonded to the rear heme propionic acid group. The side chain hydroxyl of Ser 49 donates its proton to form a hydrogen bond to the remaining front propionic acid oxygen. The backbone chain forms a pair of type IIa loops at residues 50 through 57 before ascending at the rear of the molecule to Trp 62, the indole nitrogen of which forms a hydrogen bond with an oxygen atom of the rear propionic acid group. Thus, the polypeptide chain from residues 14 through 62 is held together by an extensive lattice of bonded interactions and serves as a template supporting the heme. The rigidity of this region is manifest by its clarity in the electron density map.

Residues 64 through 71 form a regular α-helix which bounds the heme crevice on the upper left side. The polypeptide chain makes a sharp reversal at Pro 74 before forming another short length of α-helix from residues 75 through 80. The succeeding polypeptide chain then follows a meandering course to Pro 85, where it reverses direction and ascends in extended conformation up the left side of the heme crevice to residue 95, at the top front of the molecule. The sulfur atom of Met 91 forms the sixth coordinate bond with the heme iron atom. Residues 96 through 110 form a regular α-helix which descends toward the rear of the molecule, forming an "X" with the NH2-terminal helix. The COOH-terminal residues 111 and 112 are not well defined in the map.

The over-all backbone chain configuration of the molecule is organized into three sections.

The first section, which roughly comprises the right side of the molecule, consists of the NH-terminus α-helix and the succeeding chain from residues 11 through 62. This latter segment of the chain furnishes all of the bonded interactions to the heme with the exception of the iron-methionine 91 sulfur bond. As indicated previously, the conformation of this segment is dictated by the stereochemical requirements for rigidity supporting the heme.

The second section, comprising the left side of the molecule, consists of residues 63 through 95 and contains two short α-helices as well as several sections of extended or less structured polypeptide chain. The over-all conformation of this region is principally maintained by interactions between buried hydrophobic amino acid side chains and the heme. The internal polar residues Tyr 52 and Tyr 70, which are nearest neighbors to the heme on the left side, are shielded from the solvent by the polypeptide chain in this region. The proximity of these residues to the heme and their apparent interaction with Met 91 implicate the importance of these residues for the mechanism of electron transport in this cytochrome, as is further discussed below.

The final section of the chain consists of the COOH-terminus α-helix, which crosses over the top of the molecule from left to right, and whose interactions with the 64-71 α-helix, the heme, and the NH2-terminal helix appear to be essentially hydrophobic in nature. The polar residue Tyr 107 is buried at the upper rear of the heme crevice by the "X" configuration formed by the descending NH2-terminal helix and the ascending COOH-terminal helix.

In summary, it is clear that although several locally hydrogen-bonded structures exist in the molecule, the principal factors governing its over-all conformation are bonded and hydrophobic interactions with the heme.
Fig. 2. Computer-generated stereo drawings of the cytochrome c$_2$ molecule showing $\alpha$-carbon positions, heme ligands, and aromatic side chains. A, front view of the molecule; B, top view of the molecule; C, right side view; D, left side view.
These observations tempt speculation upon the possible mechanisms of chain folding in this molecule. Lewis and Scheraga (18) have predicted the $\alpha$ helical content of several homologous eucaryotic cytochromes c, based on the assumption that short range interactions stabilize the $\alpha$ helical conformation of a polypeptide chain in solution and predominate in establishing the polypeptide chain configuration in a globular protein. We have applied a very simplified version of Lewis and Scheraga's helicity predicting method, and we find good correlation with the observed helical regions in cytochrome c2. Therefore, it is perhaps not too presumptuous to speculate that an early event in the folding of the cytochrome c2 molecule is spontaneous condensation of the helical regions of the chain, which would at this stage include residues 11 through 18. As has been noted previously (19), the sequence Cys-X-X-Cys-His, if built in an $\alpha$ helical conformation, establishes the proper stereochemical relationships to allow simultaneously both condensation of the protoheme IX vinyl groups with the cysteine side chains and ligation at the fifth heme coordination position by the imidazole $N\epsilon2$ nitrogen atom of the histidine side chain. Thus, an ensuing step in the postulated folding of the molecule would involve attachment of the heme and histidine coordination to the heme iron atom. A subsequent step would result in formation of hydrogen bonds to the heme propionic acid side chains by Tyr 46, Tyr 48, Ser 49, and perhaps the ring nitrogen of Trp 62 and a main chain amide at His 42. Once these constraints have been established, structuring of the extended chain on the right side of the molecule could ensue, burying Phe 20 and Leu 32 next to the heme, stacking Pro 30 on top of the ring of Tyr 46, and forming the hydrogen bonds stabilizing the lower right side of the molecule.

The next step would involve association of the four $\alpha$ helical sections of the chain which form the top and upper left side of the molecule, primarily by hydrophobic interaction between themselves and the heme. This step would necessitate bending the NH$_2$-terminal $\alpha$ helix toward the rear of the molecule, breaking the $\alpha$ helical hydrogen bonds in the region from residues 11
through 18, as is actually observed in the cytochrome c₅₅ structure.

The remainder of the molecule folding is probably driven by a tendency to form hydrophobic interactions among aromatic and aliphatic side chains of the 64–71 and 75–80 α helices, the extended chain from 81 through 96, and the heme. Notable is the stacking interaction of Phe 77 with Trp 62 and the tucking of Phe 93 into the crevice. Concomitant with attainment of this configuration is the formation of the iron-methionine 91 sulfur bond.

**Heme Crevice**—The heme is rigidly constrained in a deep crevice formed by the polypeptide chain in such a way that only one edge is accessible to the solvent. The interior of the crevice is populated primarily by side chains of aliphatic or aromatic amino acids, including Val 10, Pro 30, Leu 32, Val 35, Ala 40 and 41, Leu 67, Val 71, Leu 95, and Val 104, and the backbone of Gly 29. The positions of α helices and interactions formed by these residues are summarized in Table II.

The molecule contains 5 phenylalanine residues in sequence positions 20, 33, 36, 77, and 93. Only Phe 93 is contiguous to the porphyrin ring. The ring of Phe 93 is situated at about a 45° angle to the heme plane at the upper left front of the crevice (Fig. 4) and appears to be in van der Waals contact with the heme ring, preventing access to the heme iron from this direction. The ring of Phe 20 is located at the right rear of the heme crevice, 6.5 Å from the heme and roughly coplanar (< ~20°) with the ring of Tyr 107. (Distances are measured from the nearest atomic position in the case of nonaromatic residues or from the center of the ring of aromatic residues to the plane defined by the porphyrin ring.) Phenylalanine 77 lies 10 Å from the heme plane and is nearly co-planar (< ~10°) with the ring of Trp 62. The rings of these residues are in van der Waals contact, and overlap edge to edge at an interplanar distance of 3.5 Å. The remaining phenylalanine residues 33 and 36 lie partially buried at the rear of the molecule, forming no obvious interactions with the heme or residues associated with it. The remainder of the residues bounding the heme crevice are polar in nature. These include His 42, Ser 49, Trp 62, Ser 89, Asn 103, and all five of the tyrosine residues of the molecule (see Table II). As described above, residues Ser 49, Trp 62, Tyr 46, and Tyr 48 are involved in hydrogen bonds to the heme propionic acid side chains (Fig. 4). Tyrosine 107 is located at the upper rear of the heme crevice, with its ring roughly normal to the heme plane at a distance of 6 Å. Curiously, the hydroxyl oxygen of this residue does not appear to interact with any proximal chemical groups.

Tyrosine residues 52 and 70 are both nearest neighbors to the heme on the left side of the crevice. The ring of Tyr 70 lies 6 Å from the heme in a plane approximately 45° to the heme plane. The ring of Tyr 52 is nearly co-planar with the heme at a distance of 5.5 Å. The hydroxyl oxygen of Tyr 70 is hydrogen bonded to the hydroxyl of Tyr 52, which is in turn hydrogen bonded to the hydroxyl oxygen of Ser 89 (Fig. 4).

**Heme Ligands**—The heme iron atom is coordinated on the right side to the imidazole Nε2 nitrogen atom of His 18. The imidazole ring is oriented in a plane perpendicular to the heme and intersecting it on a line defined by the iron atom and the methylene carbon atom bridging the pyrrole rings connected to the polypeptide backbone at residues Cys 14 and Cys 17. The ring of His 18 is fixed in this orientation by the Cα—Cε bond and a hydrogen bond from Nδ1 to the carbonyl oxygen of proline 30 (Fig. 4).

The sixth iron coordination position is filled by the sulfur atom of Met 91. The best fit to the electron density map is obtained by assuming the sulfur atom has a tetrahedral bonding configuration. It is especially noteworthy that the iron-sulfur coordinate bond does not appear to lie exactly perpendicular to the heme plane but is rather displaced by about 0.5 Å toward the rear of the heme crevice. This feature, although it involves a very small displacement of the Met 91 sulfur atom, may be seen in an appropriately chosen section through the electron density map containing the heme iron and its axial ligands (Fig. 5). The immediate question posed, therefore, is what is the source of the

### Table II

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<th>Residue</th>
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<td>Cys 17</td>
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<td>Fifth coordinate bond to heme iron, H-bond from Nδ1 to Pro 30 carbonyl oxygen</td>
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</tr>
<tr>
<td>Pro 30</td>
<td>Right side</td>
<td>5.7 A</td>
</tr>
<tr>
<td>Leu 32</td>
<td>Right side of crevice, near Phe 20, Tyr 107</td>
<td>6.5 A</td>
</tr>
<tr>
<td>Val 35</td>
<td>Right rear of crevice, near Leu 32, His 42, Trp 62</td>
<td>6.0 A</td>
</tr>
<tr>
<td>Ala 40</td>
<td>Left rear crevice</td>
<td>Ca, 8.0 A</td>
</tr>
<tr>
<td>Ala 41</td>
<td>Left rear crevice</td>
<td>Ca, 8.1 A</td>
</tr>
<tr>
<td>His 42</td>
<td>Right rear exterior, near Val 35, Asn 38</td>
<td>Ring 8.5 A</td>
</tr>
<tr>
<td>Tyr 46</td>
<td>H-bond to front heme propionic acid group</td>
<td>Ring 7.0 A</td>
</tr>
<tr>
<td>Tyr 48</td>
<td>H-bond to rear heme propionic acid group</td>
<td>Ring 6.5 A</td>
</tr>
<tr>
<td>Ser 49</td>
<td>H-bond to front propionic acid group</td>
<td>Ca, 9.5 A</td>
</tr>
<tr>
<td>Tyr 52</td>
<td>Approximately (&lt; ~15°) co-planar with heme H-bonds to Tyr 70, Ser 89</td>
<td>Ring 5.5 A</td>
</tr>
<tr>
<td>Trp 62</td>
<td>H-bond from Nε1 to rear heme propionic acid group, near Leu 67, Leu 80, Phe 77, Ala 40</td>
<td>OH 5.4 Å</td>
</tr>
<tr>
<td>Ring 6.0 A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu 67</td>
<td>Left rear of crevice, near Tyr 107, Val 104</td>
<td>8.0 A</td>
</tr>
<tr>
<td>Tyr 70</td>
<td>Ring at ~45° &lt; to heme plane, H-bond to Tyr 52</td>
<td>Ring 7.0 A</td>
</tr>
<tr>
<td>Val 71</td>
<td>Upper left side of crevice</td>
<td>OH 2.5 A (VW)</td>
</tr>
<tr>
<td>Phe 77</td>
<td>Ring interaction with Trp 62</td>
<td>6.7 Å</td>
</tr>
<tr>
<td>Ser 89</td>
<td>H bond to Tyr 52</td>
<td>Ring 10 A</td>
</tr>
<tr>
<td>H ε1</td>
<td>H bond to Tyr 52</td>
<td>OH 6.5 Å</td>
</tr>
<tr>
<td>Phe 93</td>
<td>Upper left front of heme crevice—ring at ~45° &lt; to heme</td>
<td>Ring 4.5 A (VW)</td>
</tr>
<tr>
<td>Leu 95</td>
<td>Upper left front of heme crevice, near Phe 93</td>
<td>5.7 A</td>
</tr>
<tr>
<td>Val 104</td>
<td>Top of heme crevice, H-bond Ser 11 (?)</td>
<td>6.5 A</td>
</tr>
<tr>
<td>Cys 104</td>
<td>Upper left rear of crevice</td>
<td>Ring 8.0 A</td>
</tr>
<tr>
<td>Tyr 107</td>
<td>Upper rear of crevice, ring nearly perpendicular to heme plane</td>
<td>OH 4.7 A</td>
</tr>
</tbody>
</table>

* Tabulated values are shortest distances to heme plane from individual indicated atoms, nearest side chain atoms for aliphatic side chains, and the ring center of aromatic residues. VW indicates that a group is in Van der Waals contact with the heme.
FIG. 4. Closeup of the heme region in cytochrome c₅₅ showing interactions stabilizing the heme and its ligands. Of particular note is the hydrogen bond network involving residues 89, 52, and 70, which is postulated to stabilize the ferriheme and distort the Met 91 sulfur-iron bond as described in text. A, front view; B, top view. Oxygen atoms are black, nitrogen hatched, and sulfur dotted. Hydrogen bonds are indicated by dashed lines.

strain which results in this distortion of the sulfur-iron bond from the expected octahedral geometry associated with six-coordinated iron. One source could be strain induced by interactions of residues immediately preceding or following Met 91 in the sequence. Notable would be the hydrogen bonded interaction between Ser 89 and Tyr 52, and the hydrophobic interaction of the ring of Phe 93 with the heme. Fitting the model in this region to the electron density map did not, however, require forming any strained bonds or unfavorable contacts. This region is, in fact, somewhat smeared in the map which suggests that it is flexible in the crystalline lattice.

An alternative to the possibility that the strain is transmitted through the backbone would require that a local force be exerted at or near the methionine sulfur atom. This alternative appears consistent with the observation that the Met 91 sulfur atom is displaced from the axial position toward the hydroxyl oxygen of Tyr 70, from which it is approximately 3 Å distant. Whereas this distance is much longer than the 1.47 Å sulfur-oxygen bond distance found in dimethylsulfoxide (20), it is in the range of the sum of the van der Waals radii of these atoms (21) and is not inconsistent with the existence of either a sulfur-oxygen hydrogen bond or a sulfur (δ⁺)-oxygen (δ⁻) ionic interaction. The observed values for sulfur-oxygen hydrogen bonds range from 3.21 to 3.37 Å (21), which is within experimental error of the observed 3.0 Å at the current state of refinement. However, given the proximity of the sulfur atom to the ferric iron atom, it would be expected that the sulfur atom might bear a partial positive charge, a situation which is not conducive to the formation of a hydrogen bond with the tyrosine hydroxyl group.

Thus, an attractive alternative hypothesis is that an interaction exists between the tyrosine hydroxyl oxygen bearing a partial negative charge and the methionine sulfur atom bearing a partial positive charge due to the ferric iron atom. A perhaps similar interaction is observed in the crystal structure of S-methylisothiourea sulfate (22), in which a divalent sulfur atom bearing a partial positive charge delocalized from adjacent imino groups forms approximately tetrahedral ionic interactions at distances of 3.03 and 3.44 Å to sulfate anion oxygen atoms.

This situation is analogous to that found in oxidized cytochrome c₅₅, since although it is customary to think of oxidized iron as bearing a +3 charge, two of these charges are neutralized by association with the porphyrin dianion, thus leaving the heme iron with a net +1 charge. This single positive charge which, by the above hypothesis may be partially delocalized to the Met 91 sulfur atom, could then be stabilized by interaction with a partial negative charge on the Tyr 70 hydroxyl oxygen.

DISCUSSION

Possible Interactions of Cytochrome c₅₅ with Cellular Oxidation-Reduction Components—The principal or at least most unam-
FIG. 5. Fourier section of electron density in plane of axial iron ligands perpendicular to heme porphyrin ring. Electron density corresponding to the sulfur atom of Met 91 appears to be displaced off axis of the iron-His 18 Nc2 bond by approximately 0.5 A.

FIG. 6. A schematic drawing of the front surface of the cytochrome c2 molecule, showing the uninterrupted positive charge distribution surrounding the perimeter of the heme crevice. This positive cluster involves 11 of the total 17 lysine residues of the molecule.

The ambiguous function served by cytochrome c2 in the living organism is donation of an electron from the reduced form of the molecule to an excited membrane-bound positively charged bacteriochlorophyll molecule (23). This electron is subsequently boosted in potential by the primary photoevent in photoprotein photosynthetic cyclic electron transport. The question immediately posed is the location on the molecular surface of the site of interaction between cytochrome c2 and the excited bacteriochlorophyll+ molecule.

One of the most striking structural features of the cytochrome c2 molecule is the preponderance of bonded interactions between the polypeptide backbone and the heme which rigidly fix the heme in what initially appears an unnatural configuration, that is to say, with the hydrophobic edge of the ring exposed to the solvent and the hydrophilic propionic acid chains hydrogen bonded to several internal polar groups of the molecule. The situation of the heme in this molecule differs from that found in the oxygen-binding heme proteins (24, 25) and in calf liver microsomal cytochrome b5 (26), both of which contain a non-covalently bonded protoporphyrin IX with its propionic acid groups extending into the external solvent.

Exposure of the hydrophobic edge of the heme at the molecular surface suggests that this is the site of interaction with the membrane-bound bacteriochlorophyll molecule. Presumably this interaction would take place by partial intercalation of the chlorophyll pheophytin and cytochrome porphyrin rings. Support for this hypothesis is provided by examining the external amino acid side chains bounding the perimeter of the heme crevice. Fig. 6 shows the distribution of 11 of the 17 lysine residues of the molecule. It can be clearly seen that the external side chains of these residues are distributed around the perimeter of the heme crevice and that this charge distribution is uninterrupted by the presence of any other external ionic side chains with the possible exception of aspartic acid residues 98 and 99 which lie close together at the very top exterior of the heme crevice.

This clustering of the lysine side chains can be rationalized if it is supposed that the bacteriochlorophyll molecule is situated in a phospholipid membrane, with its phytol side chain buried in the hydrophobic interior of the membrane and with its magnesium-pheophytin ring partially exposed, and that the membrane surface is populated by negatively charged phosphate groups of the membrane phospholipid components. That is, ionic interactions between the positively charged lysine ε-ammonium groups and the negatively charged membrane-bound phosphate groups would stabilize the juxtaposition of the heme and chlorophyll rings in an orientation facilitating efficient electron transport between these two molecules.

The remainder of the molecular surface does not exhibit any additional marked and extensive clustering of charged groups, with the possibly significant exception of a line of 4 acidic residues 3, 98, 99, and 102 at the intersection of the terminal helices at the top of the molecule. Both the right and left sides of the molecule have hydrophobic surface patches, most notably where the rings of Phe 20 and 36 on the right and Phe 77 on the left are partially exposed to the solvent. Co-planar interactions between the rings of Phe 20 and Tyr 107 and between Phe 77 and Trp 62 could conceivably serve as electron conduction routes from some external oxidant (or reductant) molecule to the heme. However, electron conduction by these routes would necessitate the transient formation of phenyl free radicals (27-29), which would not appear readily feasible owing to the large energy required for their formation.

Any speculation on the mechanism of the reduction of the cytochrome c2 molecule in the organism centers upon the question of whether or not the molecule interacts with the reductant at the same site as that at which it interacts with the oxidase (bacteriochlorophyll2). Although relatively little is known about the mechanism of reduction of cytochrome c2 in vivo, current data imply that either a membrane-bound cytochrome b (30) or ubiquinone 50 (31) may serve as the physiological reductant for...
cytochrome \( c \). Some recent studies (29), however, have confirmed the observation (1) that cytochrome \( c \) is active in the mitochondrial cytochrome oxidase and DPNH dehydrogenase systems at 5% and 56% of the respective mammalian cytochrome \( c \) levels of activity. It was further observed that the activity of cytochrome \( c \) in both of these systems was competitively inhibited by polylysine. This suggests that the site of interaction between cytochrome \( c \) and its physiological reductase is at the front of the molecule in both its oxidase and reductase reactions. Verification of these hypotheses awaits further biochemical and crystallographic investigation of binding to cytochrome \( c \) by physiological oxidation-reduction agents.

**Speculations on Mechanism of Cytochrome \( c \) Oxidation and Reduction**—Cytochrome \( c \) exhibits magnetic and spectral (33, 34) properties characteristic of low spin heme complexes in both its oxidized and reduced states. In the terminology of crystal field theory, this implies that the five normally degenerate iron d orbitals are energetically split by the octahedral ligand field into two nondegenerate sets: the high energy \( d_{x^2-y^2}, d_{z^2} \) orbitals whose lobes are directed at ligands at the apices of the octahedral complex, and the lower energy \( d_{xy}, d_{xz}, d_{yz} \) orbitals whose lobes are directed between the ligands. If the ligands are sufficiently strong, as they are in cytochromes in general, Hund’s rule will not be obeyed and all five electrons will occupy the energetically lower set of orbitals. Thus the net observed spin for ferricytochromes is \( \frac{1}{2} \).

Redfield and Gupta (35) have concluded from an elegant NMR double resonance experiment that the unpaired spin density in ferricytochrome \( c \) is localized in a region between the nitrogen atoms of pyrrole rings 2 and 4. (Pyrrole ring 2 is toward the front and pyrrole ring 4 is toward the rear of the heme crevice in Fig. 4.)

Inspection of the NMR contact shift data of Redfield and Gupta (Table I, Reference 35) reveals that large contact shifts are also observed for the axial ligand protons and suggests that in fact the unpaired spin density is distributed more or less uniformly in the heme \( xy \) plane (where the coordinate system is defined with the Fe-N2 bond lying along \( x \), the Fe-N3 bond lying along \( y \), and the Fe-N2 His 18 bond lying along \( z \)).

In the simple approximation of crystal field theory, the only lower energy iron d orbital which lies in this plane is the iron \( d_{yz} \) orbital. Thus, in the simplest picture, the unpaired spin distribution in ferricytochrome \( c \) can be identified with the iron \( d_{yz} \) orbital.

The ligands and general structure in the vicinity of the heme in cytochrome \( c \) are remarkably similar to those observed in mitochondrial cytochrome \( c \) (5). Hence it is assumed that the same distribution of the unpaired spin which exists in ferricytochrome \( c \) as found by Redfield and Gupta (35) also exists in cytochrome \( c \); that is, the unpaired spin occupies the iron \( d_{yz} \) orbital.

Inspection of Fig. 4 immediately yields the interesting observation that one lobe of this orbital points in the direction of the shortest route to the molecular exterior at the front edge of the heme. Further, since reduced cytochrome \( c \) exhibits a net spin of zero, it is implied that the reducing electron enters this orbital upon reduction of the ferriheme to ferroheme, as shown schematically in Fig. 7.

An additional interesting correlation is based on the observation that the Met 91 sulfur atom is displaced 0.5 Å off axis in the negative \( x \) direction toward pyrrole N4 at the rear of the heme crevice, such that the Fe-S bond lies in the \( xy \) plane. It was postulated above that this displacement was due to an interaction between the partially negatively charged Tyr 70 oxygen and the Met 91 sulfur atom bearing a partial positive charge delocalized from the ferric iron. The proposed delocalization of the ferric iron charge to the Met 91 sulfur atom may be supported by the single crystal absorption studies carried out by Eaton and Hochstrasser (36). In their study of crystalline ferricytochrome \( c \), it was noted that the 695 nm absorption band characteristic of all ferriytochromes \( c \) was \( z \)-polarized, consistent with the existence of a d-d charge transfer transition between the ferric iron and an axial ligand, which has here been identified with the Met 91 sulfur atom in cytochrome \( c \). It is
also interesting to note that the direction of displacement of the Met 91 sulfur atom is such as to increase overlap of the iron d_{xz} orbital with an unfilled d orbital of the sulfur atom, perhaps further stabilizing the unpaired spin density in the iron d_{xz} orbital.

Another curious feature of the heme ligation is the fixed orientation of the His 18 imidazole ring (described above) such that N2 of p_{xy} orbital lies parallel to a pair of iron d_{xy} orbital lobes but between the lobes of the closer d_{xz} and d_{yz} orbitals. This characteristic of heme imidazole ligation is also seen in the structure of eucaryotic cytochrome c (5), microsomal cytochrome b (26), and myoglobin (24). Its significance is as yet unclear.

The proposed over-all scheme for the reduction and oxidation of the cytochrome c_{2} molecule, based on these observations, is shown in Fig. 7, a and b. Fig. 7a shows a schematic representation of the molecule in the oxidized state, with the ferriheme iron having its unpaired spin localized in the d_{xz} orbital as described above. A partial positive charge on the Met 91 sulfur atom interacts with a partial negative charge on the Tyr 70 oxygen, causing the sulfur to be pulled off axis. The Tyr 70 oxygen accepts a hydrogen bond from the hydroxyl oxygen of Tyr 52, which in turn accepts a hydrogen bond from Ser 89 located at the lower left exterior of the heme crevice. Reduction of the heme would then take place by transfer of an electron from an approaching moiety, such as a metalloporphyrin, interacting with the cytochrome heme at its front exposed edge. The reduction process may be facilitated by the simultaneous approach of a chemical group capable of protonating Ser 89. This would tend to shift the hydrogen-bonding pattern of Ser 89, Tyr 52, and Tyr 70 to that postulated for the reduced state, thus destabilizing the oxidized heme and facilitating reduction.

Fig. 7b shows the heme in the reduced state. The net charge on the iron is now zero, abolishing the charge pair interaction between Met 91 and Tyr 70, and resulting in much stronger bonding between the iron and Met 91 sulfur. Serine 89 has been protonated and accepts a hydrogen bond from Tyr 70, which in turn accepts a hydrogen bond from Tyr 70. Reoxidation of the reduced heme takes place essentially by reversing the reduction process. In this case oxidation may be facilitated by the approach of a proton withdrawing group to Ser 89, causing the oxygen of Tyr 70 to assume the partial negative charge stabilizing the oxidized state.

The proposed mechanism has several structural and functional implications. 1. If the proposed hydrogen bond network involving Ser 89, Tyr 52, and Tyr 70 is involved in the stabilization and destabilization of the heme oxidation state, a pH effect should be manifest in the pH versus midpoint potential behavior of cytochrome c_{2}, according to the equation:\[c_{2}(ox) + H^{+} + e^{-} \rightleftharpoons c_{2}(red)\]. The midpoint potential curve of cytochrome c_{2} (37) shows a steady decline in potential from +370 millivolts at pH 5 to 300 millivolts at pH 8, after which the potential remains constant to pH 9.5. This behavior is consistent with successively decreasing protonation of Tyr 70 via Ser 89 as the pH is raised, resulting in increasing stabilization of the oxidized state relative to the reduced and a shift to lower observed potential.

2. The proposed mechanism implies that a charge-charge interaction between the Tyr 70 oxygen and Met 91 sulfur, which stabilizes the oxidized state, contributes to the somewhat off-axial orientation of the sulfur-ion bond. In the reduced molecule the net iron charge is zero, eliminating the Tyr 70-Met 91 charge pair. In fact, it has been shown (38) that the coordinate bond formed between methionine and the ferroheme octapeptide of horse cytochrome c is approximately 100 times stronger than that formed with the ferriheme peptide. These observations suggest that the Met 91 sulfur-iron bond should be essentially axial in reduced cytochrome c_{2}, owing to loss of the charge pair and the stronger interaction formed by the sulfur with the reduced heme iron. Crystalline reduced cytochrome c_{2} is isoformous with the oxidized form, and determination of the reduced structure by difference Fourier methods is in progress.

3. The proposed mechanism implies that the physiological oxidase and reductase both interact at the front of the cytochrome c_{2} molecule, since this region offers the only direct access to the heme, is the location of Ser 89, and is extensively populated with lysine residues. Although the polylysine inhibition studies cited previously suggest that this is true at least for the reactions of cytochrome c_{2} with mitochondrial reductase and oxidase preparations, further work will be required to establish that the same is true for the reactions of cytochrome c_{2} with its own (bacterial) reductase and oxidase.

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REFERENCES
4. DICKERSON, K. E. (1971) J. Mol. Biol. 67, 1
7. DICKERSON, R. E. (1972) Sci. Amer. 226, 58
16. RICHARDS, F. M. (1968) J. Mol. Biol. 37, 225
17. OZANAKA, S. (1968) Biopolymers 6, 1425
The Structure of Oxidized Cytochrome $c_2$ of *Rhodospirillum rubrum*

F. R. Salemme, S. T. Freer, Ng. H. Xuong, R. A. Alden and J. Kraut


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