Molecular Structure of Cytochrome $c_2$ Isolated from *Rhodobacter capsulatus* Determined at 2.5 Å Resolution

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The molecular structure of the cytochrome $c_2$, isolated from the purple photosynthetic bacterium *Rhodobacter capsulatus*, has been solved to a nominal resolution of 2.5 Å and refined to a crystallographic R-factor of 16.8% for all observed X-ray data. Crystals used for this investigation belong to the space group $R32$ with two molecules in the asymmetric unit and unit cell dimensions of $a = b = 100.03$ Å, $c = 162.10$ Å as expressed in the hexagonal setting. An interpretable electron density map calculated at 2.5 Å resolution was obtained by the combination of multiple isomorphous replacement with four heavy atom derivatives, molecular averaging and solvent flattening. At this stage of the structural analysis the electron densities corresponding to the side-chains are well ordered except for several surface lysine, glutamate and aspartate residues. Like other $c$-type cytochromes, the secondary structure of the protein consists of five $\alpha$-helices forming a basket around the heme prosthetic group with one heme edge exposed to the solvent. The overall $\alpha$-carbon trace of the molecule is very similar to that observed for the bacterial cytochrome $c_2$, isolated from *Rhodopseudomonas rubrum*, with the exception of a loop, delineated by amino acid residues 21 to 32, that forms a two stranded $\beta$-sheet-like motif in the *Rb. capsulatus* protein. As observed in the eukaryotic cytochrome $c$ proteins, but not in the cytochrome $c_2$ from *Rsp. rubrum*, there are two evolutionarily conserved solvent molecules buried within the heme binding pocket.

*Keywords:* redox proteins; cytochromes; biological electron transfer; X-ray diffraction; protein conformation

1. Introduction

The cytochromes $c_2$ are a family of heme-containing proteins found in most photosynthetic non-sulfur purple bacteria and some non-photosynthetic bacteria. They are the nearest bacterial equivalents to the mitochondrial cytochrome $c$ proteins and are characteristically low-spin with histidine and methionine residues as the extraplanar heme ligands. The heme group is covalently attached to the protein via thioether linkages to two cysteine residues located near the N terminus (for a review, see Meyer & Kamen, 1982). While structurally similar to the mitochondrial cytochrome $c$ proteins, they are functionally distinct in their dual role as electron carriers in both photosynthesis and respiration (Bartsch, 1978). On the basis of amino acid sequences, these bacterial cytochromes can be divided into two major groups: one subgroup...
contains a polypeptide chain that is essentially the same size as the mitochondrial cytochrome c proteins while the other group is larger and contains at least a three- and an eight-residue insertion in addition to a single-residue deletion (Ambler et al., 1979). The molecular structure of one bacterial cytochrome \( c_2 \) isolated from *Rhodospirillum rubrum* has been determined and refined at 1.68 Å resolution and it shows considerable three-dimensional similarity to horse heart cytochrome c (Salemme et al., 1972a; Bhatia, 1981). The structure of a second bacterial cytochrome \( c_2 \) isolated from *Paracoccus denitrificans* (referred to as \( c_{550} \)) has also been solved (Timkovich & Dickerson, 1976) although there are some minor ambiguities in its three-dimensional fold (Ambler et al., 1981).

One of the interesting aspects of the bacterial cytochrome \( c_2 \) proteins is that, in general, they display higher and more variable oxidation-reduction potentials than their mitochondrial counterparts (Meyer & Kamen, 1982). For example, while mitochondrial cytochromes from a wide variety of sources have typical redox potentials of approximately +260 mV (Petitgrew et al., 1975), the cytochrome \( c_2 \) proteins isolated from *Rhodopila globiformis, Rsp. rubrum* and *P. denitrificans* have oxidation-reduction potentials of +470 mV, +340 mV and +250 mV, respectively (Meyer et al., 1983). Consequently, the bacterial cytochrome \( c_2 \) proteins present a unique opportunity to investigate factors, such as hydrogen bonding and solvent structure, that are thought to modulate the redox potentials and electron transfer rates in cytochrome systems in general.

We have been studying the molecular structure of the cytochrome \( c_2 \) isolated from *Rhodobacter capsulatus*, strain St Louis, by X-ray crystallographic analysis (Holden et al., 1987a). This particular cytochrome was selected for study for a variety of reasons. Like the protein from *P. denitrificans*, it is one of the larger bacterial cytochrome \( c_2 \) proteins containing 116 amino acid residues but with a redox potential similar to the *Rsp. rubrum* molecule of 350 mV (Tollin et al., 1984). The structural gene for the protein has been cloned (Daldal et al., 1986) and mutant proteins have been expressed in the natural host rather than in a different organism such as *Escherichia coli* (Caffrey, 1991). Consequently, it has been possible to generate site-directed mutants of this cytochrome to test the importance of various conserved amino acid residues to redox potentials, electron transfer rates and protein stabilities (Caffrey, 1991; Caffrey & Cusanovich, 1991; Caffrey et al., 1991). It has also been possible to solve the three-dimensional structures of these mutant proteins by X-ray diffraction analysis (M. M. Benning, B. L. Jacobson, M. S. Caffrey, T. E. Meyer, M. A. Cusanovich & H. M. Holden, unpublished results).

One further advantage of this particular cytochrome \( c_2 \) system is that, although the protein is most likely important for efficient photosynthetic electron transport, it is not absolutely required for the survival of the *Rb. capsulatus* bacterium (Prince et al., 1986). Thus, it is possible to prepare mutations of the gene that would be lethal in an organism for which it was an obligatory cytochrome. Here, we describe the initial structure determination to 2.5 Å resolution of the *Rb. capsulatus* cytochrome \( c_2 \). The X-ray co-ordinates for this cytochrome have been deposited in the Brookhaven Protein Data Bank (accession no. 1C2R) or may be obtained immediately via

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or

**HOLDEN@WISCMACC (BITNET).**

### 2. Materials and Methods

(a) Crystalization and preparation of heavy atom derivatives

The protein used for crystallization trials was isolated and purified according to the method of Bartsch (1971) and was reduced by the addition of sodium dithionite. Large single crystals were grown at room temperature in the presence of atmospheric oxygen as described (Holden et al., 1987a) by the sitting drop method of vapor diffusion using 3-2 to 3.5 mM ammonium sulfate solutions containing 250 mM-NaCl and buffered to pH 7.5 with 50 mM-Na+/K+ phosphate. The protein concentration was typically 10 to 30 mg/ml in 10 mM-Na+/K+ phosphate. The crystals belong to the space group R32 with unit cell dimensions of \( a = b = 100.03 \) Å, \( c = 162.10 \) Å, \( (1 \times 2 \times 1) \) nm as expressed in the hexagonal setting. There are 2 cytochrome molecules per asymmetric unit. Protein crystals that had been left at room temperature for several months were redissolved and the resulting protein shown to be 80% reduced by measuring appropriate difference spectra at 550 nm. All X-ray data were collected from crystals less than 3 months old.

For heavy-atom derivative searches, crystals were transferred to a synthetic mother liquor containing 3.5 mM ammonium sulfate, 300 mM-NaCl, 50 mM-Na+/K+ phosphate (pH 7.4) and various heavy metal compounds. Isomorphous heavy-atom derivatives were prepared using 10 mM-K3PtCl4, 5 mM-mercuric acid, 5 mM-K3Pt(CN)6, and 20 mM-trimethyllead acetate. Crystals were soaked in these heavy-atom-containing solutions anywhere from 3 to 5 days except for the lead derivative which was soaked for 2 weeks.

(b) X-ray data collection and processing

X-ray data to 2.5 Å resolution were collected from the native crystals and the 4 heavy-atom derivatives by the method of oscillation photography with an oscillation angle of 2° and an X-ray exposure time of 4-2 h per film pack. Each crystal was rotated about the c-axis for a net rotation of 30°. Only 1 crystal was required for each X-ray data set. The X-ray source was nickel-filtered copper Kα radiation from an Elliot GX20 rotating anode X-ray generator operated at 35 kV and 40 mA with a 200 µm focal cup. All X-ray data collection was conducted at room temperature.

The X-ray films were digitized with an Optronics P1000 film scanner with a 100 µm raster and subsequently processed with a set of programs developed by Rossmann (1979) and modified by Schmid et al. (1981). Partial reflections were added between adjacent film packs. The derivatives were scaled to the native data in shells of equal volume in reciprocal space based on resolution. Relevant data processing and scaling statistics may be
found in Table 1. The native X-ray data set contains 86% of the total theoretical number of observations to 2.5 Å resolution. The missing X-ray data were primarily located close to the c-axis, a direct result of rotating only about this axis in the data collection scheme.

(c) Computational methods

The positions of the heavy-atom binding sites within the crystalline lattice were determined by inspection of difference Patterson maps and placed on a common origin by difference Fourier maps. The crystal soaked in 20 mM-trimethyllead acetate was by far the best derivative, and its binding site within the unit cell was quite easily solved by inspection of the difference Patterson map. Relevant Harker sections for this derivative are given in Fig. 1.

Positions, occupancies and thermal parameters for the heavy-atoms were refined with the origin-removed Patterson-function correlation method (Rossman, 1960; Tserwilliger & Eisenberg, 1983). These refined parameters are listed in Table 2. Centroid protein phases were calculated by the method of multiple isomorphous replacement and relevant phase calculation statistics may be found in Table 3. The phasing did not include anomalous scattering information since the oscillation film X-ray data did not yield a reliable estimate of the anomalous signal.

An initial electron density map calculated with X-ray data from 300 Å to 50 Å resolution clearly showed the molecular boundaries of the 2 molecules within the asymmetric unit. One of the molecules packed around a crystallographic 3-fold rotation axis while the other packed around the 2-fold symmetry axis lying along the cell diagonal at z = 0. With an electron density map calculated at 2.5 Å resolution, the heme groups for both molecules in the asymmetric unit were visible, as were the histidine and histinone heme ligands. However, the overall folds of the 2 cytochrome molecules in the asymmetric unit could not be unambiguously traced in the higher resolution map.

Since there were 2 molecules in the asymmetric unit, it was possible to improve the quality of the electron density by the technique of molecular averaging and solvent flattening. The heme groups and the heme ligands were fitted into the original electron density map calculated to 2.5 Å resolution using the Evans and Sutherland Computer Graphics System and the molecular modeling

Figure 1. Relevant Harker sections for the trimethyllead acetate derivative. (a) The section of the difference Patterson map shown here was calculated with coefficients of the form |F_{HA} − F_{DA}|^2 where F_{HA} was the heavy-atom derivative structure factor amplitude and F_{DA} was the native structure factor amplitude. The map was contoured starting at a height of approximately 3σ and increasing in increments of 1σ. An entire unit cell has been plotted. The Harker peak arising from the lead binding site at a = x+y, v = 2y−x and w = 0 is indicated by the arrow. (b) The Harker peaks at (1) a = x−y, v = y−x, w = 2z; (2) a = y, v = 2y, w = 2z; and (3) a = x, v = x, w = 2z arising from the lead binding site are indicated by the arrows. Other than the Harker peaks shown here, the difference Patterson map was essentially flat.
Table 2
Refined heavy-atom parameters

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Site no.</th>
<th>Relative occupancy</th>
<th>(x)</th>
<th>(y)</th>
<th>(z)</th>
<th>(B (\text{Å}^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{K}_2\text{PtCl}_4)</td>
<td>1</td>
<td>0.2207</td>
<td>0.6377</td>
<td>0.2880</td>
<td>0.0025</td>
<td>13.60</td>
</tr>
<tr>
<td>Mersalyl acid</td>
<td>1</td>
<td>0.0042</td>
<td>0.4341</td>
<td>0.0372</td>
<td>0.0290</td>
<td>12.85</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0778</td>
<td>0.4184</td>
<td>0.0574</td>
<td>0.0300</td>
<td>20.86</td>
</tr>
<tr>
<td>Trimethyllead acetate</td>
<td>1</td>
<td>0.5302</td>
<td>0.4620</td>
<td>0.0703</td>
<td>0.0372</td>
<td>22.06</td>
</tr>
<tr>
<td>(\text{K}_2\text{Pt(CN)}_6)</td>
<td>1</td>
<td>0.3202</td>
<td>0.4208</td>
<td>0.0551</td>
<td>0.0277</td>
<td>27.58</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.1580</td>
<td>0.3867</td>
<td>0.0387</td>
<td>0.0000</td>
<td>30.25</td>
</tr>
</tbody>
</table>

\(x, y, z\) are the fractional atomic co-ordinates, \(B\) is the thermal factor.

program FRODO (Jones, 1985). These molecular co-ordinates were then used to generate a set of rotational and translational matrices to superimpose the electron density of I molecule onto the other within the asymmetric unit. A molecular envelope was generated from this "averaged" electron density map and protein phases were refined by iterative molecular averaging and solvent flattening (Bricogne, 1976; Holden et al., 1987b). Averaging was initially performed at 30 Å resolution for 15 cycles. The initial phases based on the isomorphous heavy-atom derivatives were discarded after the 1st cycle. In the subsequent cycles, structure factors were weighted according to the fit between the observed and calculated structure factors where the algorithm used was of the form: \(w = e^{-[P_o - P_c]^2/\sigma_i^2}\), where \([P_o]\) was the observed structure factor amplitude and \([P_c]\) was the calculated structure factor amplitude (Raymont, 1983). The resolution was extended to 2.5 Å in 0.1 Å increments by including the protein phases based on the heavy-atom derivatives for each new wedge of X-ray data for the 1st cycle, followed by 10 cycles of refinement at the corresponding resolution. The final \(R\)-factor between the calculated structure factors from the averaged electron density map and the observed X-ray data to 2.5 Å resolution was 19.0%. The resulting electron density map confirmed the choice of hand of the heavy-atom constellations in the \(z\)-helices were right-handed.

An initial protein model was fitted into this averaged electron density map calculated from 30.0 Å to 2.5 Å resolution with the aid of the amino acid sequence determined by Ambler et al. (1979) for the cytochrome \(c_2\) isolated from \(\text{Rh. capsulatus}\), strain St Louis. The positions and isotropic thermal parameters for all atoms in the model were then refined by a least-squares refinement package developed in Dr Brian Matthews laboratory (Tronrud et al., 1987). "Ideal" stereochemistry for the heme group was based on the small molecule structure determination of Anderson et al. (1982). Refinement statistics may be found in Table 4. Small peaks of electron density observed in maps calculated with \(|\Delta F_o - \Delta F_c|\) coefficients and contoured at a height of 1σ were modeled as solvent molecules if they were within 4.0 Å of potential hydrogen bonding groups. The positions and the temperature factors for these putative solvent molecules were refined in the same manner as the protein atoms. A total of 93 solvent molecules were built into the electron density and the final \(R\)-factor for all observed X-ray data was 16.8%. A plot of the mean main-chain temperature factors versus amino acid residue number for each molecule in the asymmetric unit is shown in Fig. 2.

3. Results and Discussion

The molecular structure of the reduced cytochrome \(c_2\) isolated from \(\text{Rh. capsulatus}\), strain St Louis, has now been determined and refined to a nominal resolution of 2.5 Å. A representative portion of the electron density map is shown in Figure 3 and a Ramachandran plot of the main-chain dihedral angles is shown in Figure 4. The electron density for all atoms is well defined except for the side-chain densities of amino acid residues Lys5, Lys14, Lys29, Glu70, Lys90, Lys91 and Lys93 in

Table 3
Phase calculation statistics

<table>
<thead>
<tr>
<th>Resolution range</th>
<th>(-8) to 8</th>
<th>5 to 6</th>
<th>4 to 5</th>
<th>3 to 4</th>
<th>2 to 3</th>
<th>1 to 2</th>
<th>0 to 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of reflections</td>
<td>520</td>
<td>985</td>
<td>1147</td>
<td>1282</td>
<td>1412</td>
<td>1539</td>
<td>1592</td>
</tr>
<tr>
<td>Figure of merit</td>
<td>0.79</td>
<td>0.72</td>
<td>0.66</td>
<td>0.65</td>
<td>0.59</td>
<td>0.58</td>
<td>0.55</td>
</tr>
<tr>
<td>Phasing power ((\text{K}_2\text{PtCl}_4))</td>
<td>acentric reflections</td>
<td>1.72</td>
<td>2.41</td>
<td>1.48</td>
<td>1.29</td>
<td>1.60</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td>centric reflections</td>
<td>1.34</td>
<td>1.65</td>
<td>1.22</td>
<td>1.04</td>
<td>0.93</td>
<td>0.96</td>
</tr>
<tr>
<td>Phasing power (merysyl acid)</td>
<td>acentric reflections</td>
<td>0.62</td>
<td>0.99</td>
<td>0.89</td>
<td>1.31</td>
<td>1.10</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>centric reflections</td>
<td>0.71</td>
<td>0.55</td>
<td>0.55</td>
<td>0.83</td>
<td>0.71</td>
<td>0.66</td>
</tr>
<tr>
<td>Phasing power (trimethyllead acetate)</td>
<td>acentric reflections</td>
<td>2.18</td>
<td>2.08</td>
<td>1.95</td>
<td>1.84</td>
<td>1.73</td>
<td>1.82</td>
</tr>
<tr>
<td></td>
<td>centric reflections</td>
<td>1.38</td>
<td>1.47</td>
<td>1.26</td>
<td>1.03</td>
<td>1.69</td>
<td>1.40</td>
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<tr>
<td>Phasing power ((\text{K}_2\text{Pt(CN)}_6))</td>
<td>acentric reflections</td>
<td>1.69</td>
<td>1.73</td>
<td>1.63</td>
<td>1.61</td>
<td>1.53</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>centric reflections</td>
<td>1.14</td>
<td>1.14</td>
<td>1.08</td>
<td>0.95</td>
<td>1.06</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Phasing power is the ratio of the root-mean-square heavy-atom scattering factor amplitude to the root-mean-square lack of closure error.
Table 4
Refinement statistics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Resolution limits (Å)</td>
<td>1000-2.5</td>
</tr>
<tr>
<td>Final R-factor (%)†</td>
<td>16.8</td>
</tr>
<tr>
<td>No. of reflections used</td>
<td>94936</td>
</tr>
<tr>
<td>No. of atoms</td>
<td>1920</td>
</tr>
<tr>
<td>Weighted root-mean-square deviations from ideality</td>
<td>0.016</td>
</tr>
<tr>
<td>bond length (Å)</td>
<td>2.625</td>
</tr>
<tr>
<td>bond angle (deg.)</td>
<td>0.010</td>
</tr>
<tr>
<td>planarity (trigonal) (Å)</td>
<td>0.007</td>
</tr>
<tr>
<td>planarity (other planes) (Å)</td>
<td>17.688</td>
</tr>
<tr>
<td>torsion angle (deg.)‡</td>
<td></td>
</tr>
</tbody>
</table>

† R-factor = Σ|Fo-|Fc|/Σ|Fc|.
‡ The torsion angles were not restrained during refinement.

The molecule packed around the crystallographic threefold rotation axis and for amino acid residues Lys55, Lys82, Glu85, Lys91, Lys93, Lys102 and Glu106 in the molecule packed around the crystallographic dyad lying along the cell diagonal at z = 0. These amino acid residues are all located at the surfaces of the two molecules. The C-terminal lysine residues for both molecules are also weak in electron density. Since the α-carbon positions of the two molecules in the asymmetric unit superimpose with a root-mean-square deviation of 0.33 Å, the following discussion will refer only to the molecule packed around the crystallographic threefold rotation axis in the asymmetric unit. Also, the following structural comparisons will focus primarily on the bacterial cytochrome c_{2} proteins. For comparisons with eukaryotic cytochrome c proteins, the protein isolated from tuna was selected since both the oxidized and reduced forms have been determined by X-ray diffraction analysis and the three-dimensional co-ordinates are available from the Brookhaven Protein Data Bank (Bernstein et al., 1977).

There are five α-helical regions surrounding the heme group in the Rb. capsulatus cytochrome c_{2} as shown in Figure 5. According to hydrogen bonding patterns and main-chain dihedral angles, those amino acid residues found in α-helical conformations include residues 3 to 16, 55 to 63, 69 to 77, 80 to 87 and 104 to 114. Other regular secondary structural elements include two approximate type I turns delineated by amino acid residues 21 to 24 (φ_{2} = -53, ψ_{2} = -27; φ_{2} = -92, ψ_{2} = 8) and 89 to 91 (φ_{2} = -65, ψ_{2} = -19; φ_{3} = -91, ψ_{3} = 1) and two approximate type II turns formed by amino acid residues 37 to 40 (φ_{2} = -58, ψ_{2} = 127; φ_{3} = 72, ψ_{3} = 16) and 40 to 43 (φ_{2} = -71, ψ_{2} = 109; φ_{3} = 112, ψ_{3} = -11).

As mentioned, the molecular structures of two other bacterial cytochrome c_{2} proteins, namely those isolated from Rsp. rubrum (Salemme et al., 1973a; Bhatia, 1981) and P. denitrificans (Timkovich & Dickerson, 1976) were determined several years ago. Superpositions of the backbone α-carbon atoms of these cytochromes with the Rb. capsulatus cytochrome c_{2} may be found in

Figure 2. Plot of the mean B-value versus amino acid residue for all main-chain atoms. (a) The mean B-value for all main-chain atoms versus amino acid residue is shown for the molecule packed around the crystallographic 3-fold rotation axis. (b) The mean B-value for all main-chain atoms versus amino acid residue is shown for the molecule packed around the crystallographic dyad along the cell diagonal at z = 0.

Figure 6(a) and (b). With respect to amino acid sequence, the Rb. capsulatus cytochrome is approximately 40% homologous to the Rsp. rubrum protein and 50% homologous to the P. denitrificans molecule. X-ray co-ordinates for the Rsp. rubrum protein were obtained from the Brookhaven Protein Data Bank (Bernstein et al., 1977; co-ordinate listing no. 3C2C) whereas those for the P. denitrificans molecule were determined in this laboratory by collecting X-ray data from native crystals and refining the structure to 1.7 Å resolution using as a starting model the X-ray co-ordinates originally determined by Timkovich & Dickerson (1976); co-
Figure 3. Representative portion of the electron density map. The electron density map shown in stereo here was calculated with coefficients of the form (2\(F_o\) - \(F_c\)) where \(F_o\) is the observed structure factor amplitude and \(F_c\) is the calculated structure factor amplitude. The molecular modeling program FRODO was used to build the model into the electron density map on the Evans and Sutherland PS300 computer graphics system. Those amino acid residues fitted into the electron density shown here correspond to Gly64, Phe65, Ala66, Trp67, Thr68 and Glu69. Trp67 is 1 of the conserved amino acid residues found in cytochrome c proteins where it is involved in hydrogen bonding to 1 of the heme propionates. The quality of the electron density displayed here is consistent throughout the entire protein map with the exception of several surface amino acid residues.

ordinate listing no. 155C). The crystallographic R-factor for the \textit{P. denitrificans} cytochrome model is 18.1% using all observed X-ray data and maintaining good stereochemical geometry (M. M. Benning, T. E. Meyer & H. M. Holden, unpublished results). The superpositions shown in Figure 6(a) and (b) were generated from the algorithm described by Rossmann & Argos (1975).

As can be seen in Figure 6(a), the overall molecular folds of the cytochrome \(c_2\) proteins from \textit{Rsp. rubrum} and \textit{Rb. capsulatus} are very similar except for a six amino acid residue insertion in the \textit{Rb. capsulatus} protein (located between residues 20 to 32; \textit{Rb. capsulatus} numbering) and a two amino acid residue insertion in the \textit{Rsp. rubrum} molecule (located between residues 97 to 100, \textit{Rsp. rubrum} numbering). The x-carbon positions for these two cytochromes superimpose with a root-mean-square value of 1.02 \(\text{Å}\) for 90 structurally equivalent amino acid residues. This corresponds to a three-dimensional similarity of approximately 78\% for the main-chain x-carbons. The structural similarity between the cytochrome \(c_2\) proteins from \textit{Rb. capsulatus} and \textit{P. denitrificans} is even more striking, as shown in Figure 6(b). These two molecules superimpose with a root-mean-square value of 1.04 \(\text{Å}\) for 106 structurally equivalent x-carbon atoms or

Figure 4. Ramachandran plot of all non-glycinyl main-chain dihedral angles for the cytochrome \(c_2\) model. Fully allowed \(\phi, \psi\), values are enclosed by dashed lines; those only partially allowed are enclosed by continuous lines. The dihedral angles for both molecules in the asymmetric unit are plotted.
Figure 5. Stereo view of the α-carbon model of 1 of the cytochrome c₂ molecules in the asymmetric unit. The positions of the α-carbon atoms are shown together with the heme prosthetic group. Also indicated in the Figure are the positions of 2 heme ligands (Met96 and His17) and the 2 cysteine residues (Cys13 and Cys16) that form thioether linkages to the heme vinyl groups. Amino acid residues are labeled at various positions to aid the reader in following the course of the polypeptide chain. This Figure was generated with the plotting software package PLUTO, originally written by Dr Sam Motherwell and modified for proteins by Drs Eleanor Dodson and Phil Evans.

Figure 6. Superpositions of the α-carbon traces of the bacterial cytochrome c₂ proteins. (a) The superposition of the α-carbon atoms of the cytochrome c₂ proteins from Rh. capsulatus and Rep. rubrum is shown. The Rh. capsulatus molecule is shown in filled bonds while the Rep. rubrum protein is shown in open bonds. The only major structural difference between these 2 cytochromes resides in the extra loop in the Rh. capsulatus structure delineated by amino acid residues 21 to 32. X-ray co-ordinates for the Rep. rubrum were obtained from the Brookhaven Protein Data Bank (3C2C). (b) The superposition of the α-carbon atoms of the cytochrome c₂ proteins from Rh. capsulatus (filled bonds) and P. denitrificans (open bonds) is shown. X-ray co-ordinates for the P. denitrificans molecule were determined in this laboratory.
approximately 91% of the *Rb. capsulatus* tertiary structure.

A close-up view of the *Rb. capsulatus* heme binding pocket, displaying only those amino acid residues within 4.0 Å of atoms in the heme, is shown in Figure 7(a). The extraplanar heme ligands are Met96 and His17. Cysteine residues 13 and 16 form thioether linkages to the heme group. There are four buried water molecules located in the heme binding pocket, all within hydrogen bonding distance of the oxygen atoms of the heme propionate side-chains, as indicated in Figure 7(a) by the dashed lines. The water molecule located near Tyr75 and Thr94 on the "left" side of the heme binding pocket is well-ordered with a B-value of 27.4 Å² and is in the same general position as the conserved water molecule found in the eukaryotic cytochrome *c* proteins, such as those isolated from tuna (Takano & Dickerson, 1981a,b), yeast (Louie & Brayer, 1990) and horse heart (Bushnell et al., 1990). It is this particular solvent molecule that has been shown to move closer to the heme iron of eukaryotic ferrocyanochromes as they are oxidized to ferricytochromes (Takano & Dickerson, 1981a,b; Louie & Brayer, 1990; Bushnell et al., 1990). Another solvent molecule, located on the "right" side of the *Rb. capsulatus* heme binding pocket, is also evolutionarily conserved in the above-mentioned eukaryotic cytochromes. This water molecule has a B-value similar to that of the main-chain atoms of 13.4 Å² and interacts with both the heme propionate and the guanidinium group of Arg43. These two
conserved solvent molecules have not been observed in the *Rps. rubrum* cytochrome c₃ as will be discussed later (Salemme et al., 1973a,b; Bhatia, 1981). The other two solvent molecules found in the heme binding pocket of the *Rh. capsulatus* protein are not observed in the eukaryotic cytochrome c proteins.

Tyr75, located on the left side of the binding pocket, is a highly conserved amino acid residue in cytochrome c architecture. It has been suggested that this amino acid residue may be involved in hydrogen bonding to the sulfur of the methionine heme ligand and consequently may play a role in the modulation of the redox potential of the pros-
thetic group (Takano \& Dickerson, 1981a,b). Furthermore, in the cytochrome c\textsubscript{2} from *Rsp. rubrum*, it has been postulated that this tyrosine residue, by interacting with the sulfur atom of the methionine heme ligand, serves to stabilize the oxidized state (Salemm\ et al., 1973a). In the *Rb. capsulatus* protein the oxygen of Tyr75 is 3.8 Å from the sulfur atom of Met96, which is a somewhat longer distance than that observed for the reduced tuna cytochrome c (3.02 Å) and the *Rsp. rubrum* molecule (3.46 Å).

In Figure 7(b), (c) and (d), close-up views of the heme binding pockets are shown for the cytochrome c proteins isolated from tuna, *Rsp. rubrum* and *P. denitrificans*, respectively. These should be compared to the close-up view of the *Rb. capsulatus* protein shown in Figure 7(a). X-ray co-ordinates for the tuna and *Rsp. rubrum* molecules were obtained from the Brookhaven Protein Data Bank (Bernstein \ et al., 1977), while the X-ray co-ordinates for the *P. denitrificans* were determined in this laboratory. As can be seen, the local environment around the buried heme propionate on the “right” side of the binding pocket is very similar for the tuna, *Rb. capsulatus* and *P. denitrificans* proteins. All three have tryptophan, tyrosine and arginine residues and one conserved water molecule within hydrogen bonding distance of the carboxylate oxygen atoms. In the case of the *Rsp. rubrum* cytochrome, however, His42 structurally replaces the arginine residue seen in the other proteins. The hydrogen bond to the heme propionate provided by the conserved solvent molecule in the other cytochromes is replaced in the *Rsp. rubrum* protein by the hydrogen of the amide nitrogen of His42.

From the structural determination of the *Rb. capsulatus* cytochrome c\textsubscript{2} described here, as well as from the refined *P. denitrificans* cytochrome model, it is now apparent that the typically higher oxidation–reduction potentials of the bacterial cytochrome c\textsubscript{2} proteins, compared to the eukaryotic molecules, cannot be attributed simply to a more hydrophobic heme binding pocket which lacks buried solvent molecules (Salemm\ et al., 1973b). As described above, there is a water molecule located on the “left” side of the heme binding pocket in the eukaryotic cytochromes, which had not been observed previously in the bacterial proteins. In the
Figure 8. Hydrogen bonding pattern around the conserved water molecule located on the “left” side of the heme binding pocket. (a) A close-up view of the conserved water molecule, seen in the eukaryotic cytochrome c proteins but not previously observed in the bacterial cytochromes, is shown in the *R. capsulatus* heme binding pocket. The position of the water molecule is indicated by an open circle with potential hydrogen bonds indicated by dashed lines. Distances to the relevant amino acid residues are also indicated. (b) The superposition of the *R. capsulatus* molecule (filled bonds) onto the *P. denitrificans* protein (dashed lines) in the vicinity of the conserved water molecule is shown. The numbering corresponds to the *Rb. capsulatus* cytochrome. Corresponding amino acid residues in the *P. denitrificans* protein are Tyr79, Met100 and Thr88. The solvent molecule closest to the HOH label is the one found in the *Rb. capsulatus* protein. X-ray co-ordinates for this figure were determined in this laboratory. As can be seen, like the cytochrome from *Rb. capsulatus* and the eukaryotic cytochrome c proteins, the *P. denitrificans* protein also has this evolutionarily conserved solvent molecule. (c) The superposition of the cytochromes from *Rb. capsulatus* (filled bonds) and *Rsp. rubrum* (dashed bonds) is shown in the vicinity of the conserved water molecule. The numbering again corresponds to the *Rb. capsulatus* protein with homologous amino acid residues in the *Rep. rubrum* molecule being Tyr70, Met91 and Ser89. X-ray co-ordinates for the *Rep. rubrum* molecule were obtained from the Brookhaven Protein Data Bank. As can be seen the hydroxyl group of Tyr52 (not labeled) in the *Rep. rubrum* structure occupies the same general position as the conserved water molecule seen in all the other cytochrome c proteins.

tuna cytochrome c, the water molecule is within hydrogen bonding distance of the hydroxyl groups of Tyr67 and Thr78 and the carbonyl oxygen of Asn52 (Takano & Dickerson, 1981a,b; X-ray co-ordinates from the Brookhaven Protein Data Bank (Bernstein et al., 1977)). This hydrogen bonding pattern is likewise observed in the yeast iso-1-cytochrome c (Louie & Brayer, 1990) and in the horse heart cytochrome c (Bushnell et al., 1990). As shown in Figure 8(a) and (b), this solvent molecule experiences a similar environment in the *Rb. capsulatus* and *P. denitrificans* proteins. It is within hydrogen bonding distance of the hydroxyl groups of Tyr75 and Thr94 in the *Rb. capsulatus* protein and Tyr79 and Thr98 in the *P. denitrificans* cytochrome. However, whereas in the tuna cytochrome this water molecule is 3-56 Å from the carboxylate oxygen of the heme propionate group, in the *Rb. capsulatus* protein it is 3-0 Å and in the *P. denitrificans* molecule it is 2-8 Å. Also, Asn52 in the tuna structure is replaced by Ile57 in the *Rb. capsulatus* molecule and Ile69 in *P. denitrificans* cytochrome. In the bacterial cytochrome c₂ from *Rsp. rubrum* this solvent molecule is replaced by the hydroxyl group of Tyr52 as shown in Figure 8(c). Interestingly, the cytochrome c₂ from *Rb. capsulatus*, although having very similar oxidation-reduction potential to that of *Rsp. rubrum* protein (350 mV versus 340 mV), exhibits a hydrogen bonding pattern on the “left” side of the heme pocket more closely related to the cytochrome c proteins from tuna and *P. denitrificans*, which have oxidation-reduction potentials of +260 and +250 mV, respectively.

There has been enormous effort within recent years directed towards understanding the modulation of oxidation-reduction potentials and the determinants of electron transfer in c-type cytochromes. Qualitatively, factors now thought to control the redox potential of the cytochrome c proteins include local dielectric effects arising from the amount of heme exposed to the solvent, the presence of either hydrophobic or hydrophilic residues near the heme, orientation of the fifth and sixth ligands relative to the heme plane, the extent of hydrogen bonding to the heme ligands and the heme propionates, and solvent structure in the heme binding pocket (Cusanovich et al., 1987). Clearly, the determinants of redox potential are highly complex, interrelated and undoubtedly subtle. For example, it is not at all apparent, by comparing the three-dimensional structures of the heme binding pockets in Figure 7(a), (c) and (d) why the redox potentials for the *Rb. capsulatus* and the *P. denitrificans* cytochromes should differ by 120 mV while the potentials for *Rb. capsulatus* and *Rsp. rubrum* differ by only 30 mV. Fortunately, with the advent of site-directed mutagenesis, it is
now possible to test various hypotheses concerning the modulation of oxidation-reduction potentials. In the elegant crystallographic study of a site-directed mutant protein of yeast iso-1-cytochrome c by Louie & Brayer (1990) it has been shown that replacement of Phe82 with a glycine residue results in the introduction of a number of polar groups into the heme binding pocket. The more hydrophilic environment may be, in fact, responsible for the decrease in the oxidation-reduction potential of this mutant protein.

The overall goal of our structural work is to investigate those factors believed to influence the electron transfer kinetics and redox potentials of the *Rh. capsulatus* c2 and other proteins in the c2 family. The *Rh. capsulatus* molecular structure described here represents the first stage of this analysis. X-ray data corresponding to a nominal resolution of 1.8 Å are currently being collected so that the structure may be refined to as high a resolution as possible. A variety of site-directed mutants of the *Rh. capsulatus* protein have also been constructed, crystallized and the determination of their three-dimensional structures is underway. We anticipate that the high resolution structures of these mutant proteins, along with the parallel studies of Louie & Brayer in eukaryotic cytochrome c systems, will provide us with unique insights into the determinants of oxidation-reduction potentials and electron transfer rates for cytochrome c proteins in general.

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