Crystal Structures at Atomic Resolution Reveal the Novel Concept of "Electron-harvesting" as a Role for the Small Tetraheme Cytochrome c*

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The genus Shewanella produces a unique small tetraheme cytochrome c that is implicated in the iron oxide respiration pathway. It is similar in heme content and redox potential to the well known cytochromes c₅₅ but related in structure to the cytochrome c domain of soluble fumarate reductases from Shewanella sp. We report the crystal structure of the small tetraheme cytochrome c from Shewanella oneidensis MR-1 in two crystal forms and two redox states. The overall fold and heme core are surprisingly different from the soluble fumarate reductase structures. The high resolution obtained for an oxidized orthorhombic crystal (0.97 Å) revealed several flexible regions. Comparison of the six monomers in the oxidized monoclinic space group (1.55 Å) indicates flexibility in the C-terminal region containing heme IV. The reduced orthorhombic crystal structure (1.02 Å) revealed subtle differences in the position of several residues, resulting in increased solvent accessibility of hemes and the withdrawal of a positive charge from the molecular surface. The packing between monomers indicates that intermolecular electron transfer between any heme pair is possible. This suggests there is no unique site of electron transfer on the surface of the protein and that electron transfer partners may interact with any of the hemes, a process termed "electron-harvesting." This optimizes the efficiency of intermolecular electron transfer by maximizing chances of productive collision with redox partners.

Recent structural studies on multiheme cytochromes c have revealed new folds that surprisingly seem to incorporate a common "core" of heme groups. It has indeed been observed that the spatial organization of the heme planes is remarkably similar in cytochromes unrelated in function, size, or fold. Examples include hydroxylamine oxidoreductase (1), the pentaheme c₅₅₂ nitrite reductase (2), the diheme cytochromes c NapB (3) and Split-Soret (4), soluble fumarate reductases (5, 6), and the tetraheme cytochrome c₅₅₄ from Nitrosomonas europae (7). This conserved orientation of heme groups could be due to a common function: transport of electrons over large distances from the initial electron-accepting heme group to the active site redox cofactor. Obviously these series of communicating hemes, in effect "molecular wires," need to conduct electrons quickly, resulting in the requirement that interheme distances be within 14 Å to avoid electron transport across the wire becoming the rate-limiting step (8). However, the question remains whether the "conserved" heme arrangement is due to stringent heme-heme packing restrictions or to a more delicate fine tuning of electron transfer by optimization of this observed heme motif for as yet unidentified reasons (9). Clearly the observation that two evolutionary related multiheme cytochromes c have a distinct heme core arrangement would favor the first explanation. One such couple of related redox enzymes carrying distinct heme cores are the soluble tetraheme flavocytochrome c fumarate reductase (SFR)¹ and the small tetraheme cytochrome c (STC), both isolated from Shewanella sp. The recently solved SFR crystal structures (5, 6) all reveal a new arrangement of heme groups in comparison to the "classic" Desulfovibrio tetraheme cytochromes c₅₅. The different position of the axial heme ligands along the polypeptide chain in SFR in comparison to the cytochromes c₅₅ is clearly the main determinant for the observed semilinear heme motif versus the classical c₅₅ heme core pattern. While the first three heme groups in SFR are surrounded by polypeptide from the cytochrome c domain, the C-terminal heme group resides largely in a cavity made by the flavin binding catalytic domain. As mentioned, Shewanella sp. produce under anaerobic conditions a small tetraheme cytochrome c that is highly similar to the cytochrome c₅₅ domain of SFR (10). Genetic studies have indicated this cytochrome to play a role in the reduction of Fe(III) oxides, an unusual property of Shewanella sp., rather than in fumarate respiration (11). The STC is the smallest tetraheme protein isolated to date and consequently has the lowest amino acid to heme ratio observed. Notwithstanding the significant homology with the cytochrome c domain of SFR and the obvious conformational restrictions, a significant rearrangement of the heme core is postulated to allow incorporation of the C-termi-

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1 The abbreviations used are: SFR, soluble fumarate reductase; STC, small tetraheme cytochrome c; Bicine, N,N-bis(2-hydroxyethyl)glycine; r.m.s.d., root mean square deviation.
n al heme in the hydrophobic core. Recent preliminary NMR studies on the small tetraheme cytochrome c isolated from Shewanella frigidimarina have indeed revealed considerable differences in the heme core with respect to the observed orientation in SFR (12).

The present work reports the crystal structure of, and redox-dependent conformational changes in, the small tetraheme cytochrome c from S. oneidensis MR-1. Both oxidized and reduced crystal structures were determined at atomic resolution (0.97 and 1.02 Å, respectively), providing the highest resolution data obtained for a multiheme cytochrome c to date. In addition, a 1.55-Å structure of a different space group is reported. Comparison of these structures reveals unexpected flexibility in both protein and heme core, notwithstanding the high heme to amino acid ratio observed. Significant differences with the SFR cytochrome domain, in addition to the observed flexibility, indicate that the common “heme core” in multiheme proteins is a consequence of the limited number of ways bis-histidine-coordinated heme groups can be packed within direct contact rather than of an evolutionary pressure to maximize certain electronic effects between porphyrin planes. Analysis of the intermolecular contacts made in both crystal forms suggests a promiscuous “electron-harvesting” role for this protein in contrast to a classic predetermined electron path along a “heme wire” with specific points for entry and delivery and the associated need for specific interaction sites with electron donor and acceptor, respectively. This strategy increases the efficiency of electron transfer by increasing the chances of productive collision between redox partners and might well prove to be a new and general robust engineering principle in multiheme redox proteins.

**EXPERIMENTAL PROCEDURES**

**Crystallization and Data Collection**—The protein was purified as described by Tsapin et al. (13). Crystals of the oxidized protein were grown using the hanging drop vapor diffusion technique. The reservoir solution contained 3.5–3.8 M ammonium sulfate, 0.1 M Bicine (pH 8.5–9.2). The drops were formed using equal volumes of protein solution at 30 mg/ml and reservoir solution. At 21 °C, long very thin needles appeared within days. On rare occasions a second crystal form arose after the needles had ceased to grow. These new crystals grew in three dimensions to an average size of 0.05 × 0.05 × 0.05 mm. The solution used as cryoprotectant for flash cooling these crystals at liquid nitrogen temperatures was the original reservoir solution supplemented with 15% (v/v) glycerol for the needle-shaped crystals, while the second crystal form was flash-cooled in the reservoir solution, which contained ammonium sulfate near saturation. A full data set was collected on a single, oxidized needle at ID13, European Synchrotron Radiation Facility (Grenoble, France) taking advantage of the special microfocusing set-up. Different sections along the longitudinal section of the crystal were exposed to avoid excessive radiation damage due to the intense beam. The crystal diffracted to 1.55 Å and proved to be a P21 space group with unit cell a = 93.69 Å, b = 34.01 Å, c = 108.51 Å, and β = 110.49°. Data were processed and scaled with the HKL package programs DENZO and SCALEPACK (14). Data were collected on the second crystal form at X11, Deutsches Elektronen Synchrotron (Hamburg, Germany). These crystals diffracted up to 0.97 Å and proved to be a P212121 space group with unit cell a = 92.86 Å, b = 48.14 Å, c = 58.34 Å. These contained one molecule in the asymmetric unit as indicated by solvent content calculations. Complete data sets to atomic resolution were collected on an air-oxidized crystal and on a dithionite-reduced crystal. The latter was prepared by soaking a crystal for several minutes in reservoir solution supplemented with a large surplus of fresh dithionite crystals. Change in color of the crystal from dark red to a homogeneous pink-red color indicated full reduction, after which the crystal was quickly flash-cooled in anhydrous nitrogen.

**Structure Determination and Refinement**—All attempts to solve the structure of the P21 space group using the cytochrome c domain of the soluble fumarate reductase failed despite prolonged efforts. The structure elucidation of the P212121 crystal form was possible due to the atomic resolution obtained. A weak molecular replacement solution (using the program AmoRe; Ref. 15) using only a fragment of the SFR cytochrome c domain (containing hemes II and III and surrounding regions) was used to generate an initial phase set. In combination with the available anomalous data, these phases quickly led to the identification of the four iron sites, giving confirmation of the correctness of the initial molecular replacement solution as well as the position of all four irons. ARP-WARP (16) in auto-building mode was used to build and refine the initial structure. This resulted in an almost complete tracing of the polypeptide chain. After refinement, the resultant model was used to solve the P21 space group crystal structure by molecular replacement using the program AmoRe, finding all six molecules within the asymmetric unit. All structures were refined in initial stages using the program REFMAC (17) from CCP4 suite. After the Rw values dropped below 20, the program SHELEX (18) was used. While the P212121 structures were refined with anisotropic B-factors for all atoms and acceptors, respectively. This strategy increases the efficiency of electron transfer by increasing the chances of productive collision between redox partners and might well prove to be a new and general robust engineering principle in multiheme redox proteins.

**RESULTS AND DISCUSSION**

**Oxidized Orthorhombic STC Crystal Structure**—The final model for the oxidized, orthorhombic form of the S. oneidensis STC contains all 91 amino acids of the sequence (with the exception of the N-terminal residue), four hemes, and 294 water molecules. In addition, two sulfate ions were detected. The overall fold of an STC monomer is very different from any of the known tetraheme cytochrome c folds (Fig. 1). The protein is folded in a series of α-helices connected by extended loop regions and has no regular β-strand structure. The tertiary structure is elongated and resembles an opened vault encompassing the four hemes. In addition to three helical regions (A: Lys41–Met45; B: His42–His52; and C: Val57–Cys61), three α-helices can be discerned: (A: Lys4–Glu11), (B: Gly28–Gly40), and (H3: Thr84–Lys90). None of these helices interacts with one another and, none are separated by interspersed heme porphyrin planes, the exception being a weak hydrophobic packing between H3 and the helical 310 B region, both lining the heme IV pocket. The helices are separated by large loop regions in

| Structure determination | Oxidized | Reduced | Oxidized |
|-------------------------|---------|---------|---------|
| **Diffraction data statistics** | | | |
| Space group | P212121 | P212121 | P212121 |
| Resolution (Å) | 10.97 | 10.97 | 10.97 |
| Observations | 295,545 | 275,007 | 197,538 |
| Unique | 51,882 | 47,690 | 83,476 |
| Completeness (%) | 94.1 | 96.3 | 92.1 |
| Rmerge(%) | 13.3 | 14.8 | 17.8 |
| Temperature (K) | 107 | 107 | 107 |
| Refinement statistics | | | |
| Space group | P212121 | P212121 | P212121 |
| Resolution (Å) | 10.97 | 10.97 | 10.97 |
| No. of molecules in asymmetric unit | 1 | 1 | 6 |
| Rwork(|I|/|I|) | 15.1 | 16.5 | 22.4 |
| Rmerge(|I|/|I|) | 0.011 | 0.012 | 0.014 |
| r.m.s.d. in bond lengths (Å) | 2.10 | 2.13 | 2.7 |

**TABLE I**

**Oxidized STC**

The overall fold of an STC monomer is very different from any of the known tetraheme cytochrome c folds (Fig. 1). The protein is folded in a series of α-helices connected by extended loop regions and has no regular β-strand structure. The tertiary structure is elongated and resembles an opened vault encompassing the four hemes. In addition to three helical regions (A: Lys41–Met45; B: His42–His52; and C: Val57–Cys61), three α-helices can be discerned: (A: Lys4–Glu11), (B: Gly28–Gly40), and (H3: Thr84–Lys90). None of these helices interacts with one another and, none are separated by interspersed heme porphyrin planes, the exception being a weak hydrophobic packing between H3 and the helical 310 B region, both lining the heme IV pocket. The helices are separated by large loop regions in
which three hairpin bends can be discerned: Gly$^{14}$–Glu$^{16}$, Lys$^{20}$–Thr$^{23}$, and Asn$^{68}$–Gln$^{71}$. The N-terminal helix I provides the distal axial His$^{5}$ ligand to heme II and is wedged between the two flexible stretches of polypeptide observed in the structure, e.g. Ala$^{47}$–Gly$^{14}$ immediately following in the sequence and the Asp$^{56}$–Gly$^{70}$ loop that packs against the N-terminal section of H1. The side chains of H1 residues involved in this packing and in creating part of the hydrophobic distal heme I pocket, Leu$^{5}$ and Phe$^{8}$, both exhibit multiple conformations, probably in response to the multiple conformations adopted by the Asp$^{56}$–Gly$^{70}$ stretch. Immediately following the rigid His$^{9}$ heme ligand, the Ala$^{10}$–Gly$^{14}$ polypeptide stretch exhibits two clear conformations significantly different in the backbone position of Glu$^{11}$ (the largest difference being 2.7 Å). The higher electron density clearly indicates one conformation to be preferentially occupied, and the respective ratio of occupancy was refined to 0.64/0.36 (Fig. 2). This particular stretch has no direct contact with any heme group and seems to lack any direct structural function. The subsequent segment of polypeptide provides the first CXXCH heme binding sequence and forms most of the hydrophobic proximal heme I pocket. While several residues are observed to occupy two distinct conformations, the backbone is clearly occupying a single position. The following secondary structure element, H2, is packed against the first CXXCH heme binding residues. This helix ends with the second heme binding motif, and the polypeptide chain proceeds with the segment donating the distal heme III ligand His$^{69}$. This segment provides the majority of residues that line the distal heme III cavity, while residues Ala$^{47}$ and Val$^{48}$ pack with the C-terminal helix H3. The polypeptide chain proceeds with the $3_{10}$B helix after His$^{49}$ to provide the heme III binding motif. The $3_{10}$B helix itself contains His$^{62}$ as distal ligand to heme IV in addition to the few hydrophobic residues lining the heme IV distal cavity. Only a few residues separate the proximal heme I ligand His$^{65}$ from the distal heme III ligand His$^{69}$. The short distance between both residues His$^{49}$ and His$^{52}$ and residues His$^{62}$ and His$^{65}$ clearly imposes strict conformational restrictions on the local backbone conformation and the heme–heme packing. This is similar but clearly different from the e$_{3}$ tetraheme family where two consecutive histidines impose the local backbone organization and heme orientation. In contrast to the stringent conformational restrictions imposed on the preceding region, the Asp$^{56}$–Gly$^{70}$ stretch following His$^{65}$ clearly occupies two distinct conformations (with 80 and 20% occupancy) and is packed against the flexible residues Leu$^{5}$ and Phe$^{8}$ from H1. Again this region of the polypeptide chain is not in direct contact with any of the heme groups, although it is packed against other protein secondary elements. The first rigid amino acids after the last residue occupying a dual backbone conformation precede Pro$^{73}$ that stacks to the proximal heme III side. At this residue the polypeptide chain for the first time approaches the proximal side of heme IV and provides the heme IV binding residues in addition to residues lining the proximal pocket. The last element of secondary structure is H3 that packs lightly with an edge of heme IV.

When comparing the available sequences for STC and SFR isolated from Shewanella sp. it becomes clear that overall most conserved residues are involved in covalently binding the porphyrin groups and ligating the iron atoms (Fig. 3). With the exception of the heme-ligating residues, surprisingly few residues are conserved throughout this family. Both Leu$^{5}$ and Leu$^{14}$ are involved in lining heme groups, while both Gly$^{22}$ and Gly$^{44}$ are important in folding. Glu$^{31}$ is unique among the strictly conserved residues in that it is not involved in direct heme contact but forms a hydrogen bond to the distal His$^{19}$ of heme I, a contact conserved in the SFR crystal structures. Most surface charges conserved among the STCs are the negatively charged amino acids that are more or less uniformly positioned over the protein surface (Fig. 4). The surface of the protein is therefore predominantly negatively charged with no obvious heterogeneity that might indicate binding sites for redox partners. All four hemes are highly solvent-accessible, hemes I and IV from one side of the molecule and hemes II and III from the opposite side. Of the very few positively charged residues that are conserved, Arg$^{83}$ is involved in ionic interaction with heme IV propionate A, and Lys$^{72}$ is the only conserved positive charge on the surface in addition to the N terminus. The latter is also found to have a unique role as described below.

**Heme Core Structure**—The four bis-Histidine-ligated hemes are packed in pairs against each other. While hemes I–II and hemes III–IV exhibit a perpendicular orientation of heme planes, the planes of the central hemes II–III are stacked in a parallel fashion. Each heme has distinctive features, however, as can be seen from Table II. The N-terminal heme I is the most solvent-exposed heme, the distal side only covered by His$^{65}$, Val$^{64}$, and Ser$^{8}$, while the remaining distal heme surface and Cys$^{15}$ are completely solvent-exposed. More remarkable is the observation that pyrrole group D and the attached propionate group occupy two distinct conformations, the minor conformation (32% occupancy) being rotated by 15° from the main conformation and the pyrrole nitrogen atom being the pivotal point. The D propionate group either forms a single hydrogen bond to the backbone oxygen of conserved residue Ser$^{25}$ in the main conformation or is disordered in the minor conformation. This unprecedented flexibility in the heme porphyrin plane itself is no doubt due to the extremely high solvent accessibility of this particular heme group and is detected due to the high resolution obtained. The His ligands are oriented parallel to each other, the proximal His$^{19}$ is hydrogen-bonded to the charged and conserved Glu$^{31}$, while the distal His$^{65}$ is hydrogen-bonded to a positionally ill-defined water molecule. Given the high solvent accessibility and close interaction with the negatively charged Glu$^{31}$, this redox center is most likely to have one of the lowest reduction potentials of the four heme...
groups. This conclusion does not correspond with the order of heme oxidation determined by NMR for the STC from the related *S. frigidimarina* for which it was observed that the hemes oxidize in the order IV-II-I-III (12). It is possible that the redox potentials of the *S. oneidensis* MR1 STC are different, changing the order in which hemes oxidize to start with heme I. Other explanations for this discrepancy may be found in more subtle effects that counteract the influence of the high solvent accessibility on the redox potential.

Heme II is slightly less solvent-exposed than heme I, the porphyrin plane being distinctively buckled. Propionate D is hydrogen-bonded to propionate A, which in turn interacts with solvent atoms, indicating that at least one propionate is an uncharged species (Fig. 2). This is the first observation of heme propionates hydrogen bonding at basic pH and is very similar to the hydrogen bonding of heme propionates in the crystal structure of the diheme NapB cytochrome *c* from *Haemophilus influenzae* (3). The His ligands are oriented perpendicular to
with each other (mean r.m.s.d., 0.31 Å).

...Heme III leads to the conclusion that heme III His interacting with the oxygen backbone of Val 48, and the propionate D interacts with Asp46, which in turn interacts with both the sixth ligand His49 and the nitrogen backbone atom of Val48. Propionate A is slightly less defined and interacts with solvent molecules. The His ligands are oriented parallel, the angle of the His 49 imidazole plane with the porphyrin plane being almost perfectly flat. Propionate A in-

one another, the fifth ligand His39 interacting with a water molecule, while the sixth ligand His48 is hydrogen-bonded to the backbone oxygen of the conserved Leu49.

Heme III is clearly the least solvent-exposed of all four hemes with both cysteines well buried within the protein matrix. Propionate D is hydrogen-bonded to Asp46, which in turn interacts with both the sixth ligand His49 and the nitrogen backbone atom of Val48. Propionate A is slightly less defined and interacts with solvent molecules. The His ligands are oriented parallel, the angle of the His49 imidazole plane with the porphyrin group being offset roughly 30° from the normal perpendicular orientation. This deviation in ligation geometry might be due to the close contact of the imidazole plane with the conserved Met46 side chain sulfur atom. The low solvent accessibility of heme III leads to the conclusion that heme III has the highest potential despite the interaction of His49 with Asp46. This corresponds well with the conclusions drawn on the basis of NMR-monitored redox titrations of the S. frigidimarina NCIM400 STC (12).

The C-terminal heme IV is relatively solvent-exposed, the porphyrin plane being almost perfectly flat. Propionate A interacts with the conserved Arg83 that is stacked against the proximal His79, while propionate D interacts with solvent molecules. The His ligands are oriented perpendicular with distal His79 interacting with the oxygen backbone of Val48, and the proximal His79 is hydrogen-bonded to a water molecule.

Oxidized STC Monoclinic Crystal Structure.—The oxidized P21 structure contains six monomers in the asymmetric unit that each exhibit slight structural differences with the obtained atomic model in the oxidized orthorhombic crystal form and with each other (mean r.m.s.d., 0.31 Å for backbone atoms). An overlay of the Ca trace of the seven monomers indicates several regions that have significantly higher flexibility and B-factors than the average deviation in position or B-factor (Fig. 5). Besides the N-terminal residues, these include the Ala10–Gly30 loop that was identified in the P212121 structure as having multiple backbone conformations, the Ser25–Gly30 loop, which is only weakly interacting with other parts of the structure, and the entire C-terminal fragment from Pro79 onward. The latter fragment is separated from the main part of the structure by the heme IV plane and is therefore only restricted in conformation with respect to the remainder of the protein by the covalent attachment at Pro73 and the ligation between heme IV and the distal His52. It clearly can be seen that the entire region, including the porphyrin group of heme IV, has considerable rotational freedom with Pro73 and the His52-iron ligation serving as hinge points. The maximal difference between monomers for identical backbone atoms is 2.0 Å and for the porphyrin atoms is 1.6 Å. Since the axis of rotation is along the His52-iron ligation and therefore almost parallel to the heme III plane, the contact between heme III and IV is practically uninfluenced by this movement. Besides several multiple con-

Reduced STC Orthorhombic Crystal Structure.—The reduced orthorhombic crystal structure is very similar to the oxidized structure with no obvious shifts in the geometry of the four-heme cluster. However, several subtle but significant changes can be observed at this high resolution. The most dramatic difference occurs at the surface near the exposed heme II and III edges. The side chain of Lys72 rearranges upon reduction from a solvent-exposed conformation back into the protein core and makes a hydrogen bond to propionate A of heme II, thereby expelling two water molecules from the immediate vicinity of heme III and His62 (Fig. 6). As a consequence of the side chain rearrangement, the backbone positions of both Lys72 and adjacent conserved Pro73 (one of the pivotal points of the rotational movement of the C-terminal domain) shift toward the interior of the protein, bringing the side chain of Pro73 slightly closer to the heme III plane. Another effect at the N-terminal side of Lys72 is the preferential population of the conformation closest to heme II of both the flexible Asp66–Gln71 region and the closely associated residues Leu5 and Phe8. As a consequence of the shift in backbone position of Gln71, the water-ligating His62 has shifted position to form an additional hydrogen bond to the backbone oxygen of Gln71. As Lys72 occupies a solvent-exposed position in all seven oxidized monomers, this reorganization is clearly a consequence of the reduction of the cytochrome. Reduction therefore results in the withdrawal of the only conserved positive charge on the STC surface, possibly assisting complex formation with positively charged electron acceptors. Since Lys72 is interacting with the hydrogen-bonded propionates of heme II upon reduction, a redox-Bohr effect might well be present in this molecule. Near heme IV, the side chain of Asn55 that is between the conserved Gly44 and Leu48 behaves in a manner opposite to the Lys72 side chain. The side chain is shifting to a more solvent-exposed position, strengthening the hydrogen bond with the backbone oxygen of Pro53 (Fig. 6). The side chain of Pro51 occupies two distinct conformations in the reduced structure but only populates the one closest to heme IV in the oxidized structure. In addition, there is a slight backbone reorganization in this region that brings both Pro51 and conserved Leu48 slightly further away from heme IV. The N terminus of a crystallographically related molecule packs close to this region, and a consequence of the Asn55 rearrangement is the repositioning of the N terminus of the protein, which

| TABLE II | Heme characteristics |
|----------|---------------------|
|          | Heme I | Heme II | Heme III | Heme IV |
| Solvent-accessible area (Å²) | 286 | 245 | 134 | 250 |
| Heme-heme distance* (Å) | 9.86 | 7.85 | 14.23 | 13.96 |
| Heme I | 14.23 | 3.89 | 8.65 | 7.74 |
| Heme II | 7.85 | 8.65 | 11.83 | 9.78 |
| Heme III | 13.96 | 7.74 | 9.78 | 10.15 |

* Distances listed are for the minimum distance between atoms that are part of the aromatic ring system for interprotein heme-heme contacts.
thereby becomes more ordered, leading to a clear definition of the first residue. This change is thus due to crystal packing and not to reduction of the protein. In summary, it is clear that upon reduction, the interaction of the protein with each of the four hemes is changed by subtle repositioning of a few residues.

Similar studies on tetraheme cytochrome $c_3$ molecules have

**FIG. 5. Overlay of seven oxidized STC monomers.** Both the $C_u$ trace and heme groups are rendered in thin lines.

**FIG. 6. Structural changes upon reduction.** Residues significantly shifted in position upon reduction have been represented in both states, colored blue for the reduced and red for the oxidized conformation. The final $2F_o - F_c$ map of reduced (in blue) and oxidized (in red) structures are superposed and contoured at 2s. (a) stereoview of the region around Lys$^{72}$. Heme III is colored blue-gray, while part of heme II and His$^{82}$ are represented in atom color code. (b) stereoview of the region around Asn$^{55}$. Heme IV and associated residues are colored blue-gray.
revealed redox-dependent changes of a similar magnitude (20). In all cases studied, it appears that reduction-induced differences are unique to the protein, and general aspects have yet to emerge. However, the fact that one of the changes observed in STC is triggered by the movement of a conserved charge (Lys72 or Arg72) indicates that this might well prove to be a general property of members of this family.

Comparison with SFR—A superposition of the STC structure with the crystal structure of the SFR from S. frigidimarina NCIMB400 (6) (39% identity) immediately reveals why molecular replacement would only work with the heme II-heme III-containing fragment (Fig. 7). While both these porphyrin groups and the surrounding polypeptide chain occupy very similar positions, heme group IV and the associated protein region are very different in conformation with respect to the corresponding SFR region. A superposition of the tetraheme cytochrome c domains using DALI (21) gave a Z score of 7.5 with an r.m.s.d. of 2.6 Å for 78 Ca atoms, while superposition of the N-terminal region containing hemes I–III gave a Z score of 6.9 with an r.m.s.d. of 1.6 Å for 63 Ca atoms. From the latter superposition it is evident that the orientation of heme I is different in the two cytochromes c. The contact area with heme II, Cys17, and the covalently linked vinyl of pyrrol C is mostly conserved in position and serves as a hinge point for the rotation superposing heme I of STC on heme I of SFR. This apparent rotational freedom of heme I in the two proteins is similar to the rotational freedom of heme IV with respect to heme III in STC and is likely a consequence of the perpendicular orientation of both hemes. It is clear from simple packing considerations that such perpendicular positioning of bis-His-ligated hemes results in less conformational restriction versus the parallel orientation as observed for hemes II and III where optimal hydrophobic packing between hemes can be reached in a very limited set of positions. We propose this restricted possibility of packing bis-His-ligated hemes in close contact to be the predominant reason for the observation of a conserved heme core in several unrelated multiheme cytochromes c. It is highly likely that, during the initial stages of folding in these proteins, the heme core samples a series of metastable states in which the bis-His-ligated hemes are in close hydrophobic contact. These states will be formed by a series of heme pairs having either a parallel or perpendicular orientation. The consecutive folding and correct ligation of all hemes effectively will select one of these metastable states, giving rise to the conserved heme cores. In contrast, in cases where the folding is completely determined by the protein, it is more likely that heme-heme interactions are weak and the porphyrin planes are maximally separated as observed for the tetraheme cytochrome c that interacts with the photosynthetic reaction center (22). The influences of the protein on the observed heme pattern can clearly be seen by examining the difference in position of heme IV in STC and SFR. While the polypeptide chain traces are similar from the N terminus up to Asp66, the C-terminal region is highly dissimilar. This is a consequence of the latter region strongly interacting with the FAD binding domain in SFR, which results in a nearly 180° difference with STC between the
direction of the chain for both the C-terminal stretch and the region immediately after Ser<sup>66</sup>. As mentioned previously, this results in the lining of heme IV by several FAD domain residues and the close contact of heme IV with the FAD cofactor. This heme IV plane of STC makes a 60° angle with the corresponding heme plane of SFR, positioning it closer to the heme core with respect to SFR. Similar to the discussed heme I-heme II interface, the contact area between hemes IV and III is again largely conserved and serves as a hinge point for the rotation superposing STC heme IV on SFR heme IV. Given the high mobility of the C-terminal region in STC and the small restrictions on the contact between hemes III and IV, one can envisage how during evolution the ancestral SFR was created from a simple fusion between an STC and the catalytic domain followed by optimization of the FAD-heme IV contact by subtle repositioning of heme IV.

**Intermolecular Contacts Reveal an Electron-harvesting Role**—When analyzing the intermolecular contacts formed in between crystallographic symmetry-related and non-crystallographic symmetry-related molecules in both the P<sub>2</sub><sub>1</sub> and P<sub>2</sub><sub>1</sub>2<sub>1</sub>2<sub>1</sub> space groups, it becomes immediately clear that the STC molecules can pack together in a wide range of mutual orientations, mostly using the exposed heme edges to provide hydrophobic contact points (Fig. 8). The closest distance each porphyrin plane can approach another porphyrin plane is within 14 Å in each possible intermolecular heme pair. Distances involving heme pairs with heme III are, on average, longer due to the lower solvent accessibility of heme III. This observation indicates that intermolecular electron transfer between any of the heme pairs should be possible on a subsecond time scale (8). Furthermore, we might speculate that this protein has a role different from the “standard” electron-transferring redox proteins where specific sites for electron acceptors and donors have been identified. Redox partners of the STC have an almost equal chance to make a productive collision with any of the four hemes given the fact that the surface charge distribution on STC is nearly homogeneous and all hemes (with slight exception of heme III) are solvent-exposed. Therefore, any of the hemes can accept electrons that immediately can be transferred by fast intramolecular transfer to any other heme. Such a property would clearly increase the chance, and therefore rate, of intermolecular electron transfer between STC and its redox partners. A similar conclusion can be drawn for the tetraheme cytochrome c domain of the soluble *Shewanella* fumarate reductases (5, 6). While fumarate reduction needs two electrons, this enzyme also has four hemes of which the three N-terminal hemes are very similar to the STC hemes I–II. We therefore propose this domain in SFR to have an electron-harvesting role, maximizing the chance of a productive collision with the electron donor species by exposing a series of closely contacting hemes at the surface of the molecule. Electrons can therefore pass from the redox partner to each individual heme and consecutively to the FAD. This is clearly reminiscent of the light-harvesting complexes where a series of interacting chlorophyl groups (equivalent to hemes I–III) harvests light and passes it on to the central special chlorophyll pair (equivalent to heme IV) in the associated reaction center (23). The fact that all heme groups are in close contact makes electron transfer from the redox partner to each individual heme and consecutively to the FAD feasible regardless of the initial potential difference. In case of a “hopping mechanism,” even electrons that initially might need to tunnel uphill from the redox partner to the initial heme group are immediately passed along the “molecular wire” through to the active site redox cofactor and finally to
the substrate. Alternatively, electrons may even be passed directly on to the active site with no formal reduction of any of the heme cofactors given the fact that electrons might not necessarily reside on any of the heme cofactors but tunnel more directly to the FAD. Such a superexchange mechanism has been recently shown to occur under certain conditions for fumarate reductases (24). In both cases there will be less stringent requirements for the fine tuning of the redox potential of each of the hemes, rendering such designs more robust against mutations. This electron-harvesting behavior might well be a general property of multiheme proteins and a new robust engineering principle in multicenter redox proteins. Electron-harvesting could indeed explain the mechanism of metal oxide reduction by outer membrane cytochromes c in Shewanella sp. since metal oxide particles are on average many times larger than the bacterium and provide a non-homogeneous surface to any redox protein that needs to interact with them. Therefore, the bacterium is faced with the problem of recognition and efficient electron transfer to its substrate. Many of the extracellular cytochromes c thought to form part of the metal oxide-reducing enzyme complexes contain 10 or more heme groups, and one can envisage their structures to involve several arrays of closely spaced, solvent-accessible heme groups. Such structures would have the maximal chance of productively interacting with the heterogeneous metal oxide surfaces, providing an elegant solution to the problem. The fact that the STC has been implicated in iron oxide respiration in Shewanella sp. indicates that this strategy might be an integral part of the entire iron respiration electron transfer pathway.

Conclusions—We have determined the crystal structure of the small tetraheme cytochrome c to atomic resolution in two crystal forms and two redox states. The structures reveal a surprising amount of flexibility in both heme groups and heme-heme interactions. While the overall structure remains unchanged upon reduction, two side chains adopt different conformations with local shifts in the backbone conformation as a consequence. Both shifts result in slightly different interactions with the heme porphyrin planes, decreasing the solvent accessibility of the heme core, while the single conserved positive charge is withdrawn from the protein surface. Comparison with the related SFRs indicates a different folding for the C-terminal region containing heme IV and further strengthens the idea that, during initial folding of multiheme redox proteins, the bis-His-ligated heme core samples a series of related metastable states of which one is selected by the folding protein. Analysis of intermolecular contacts between STC monomers reveals close contacts between all possible heme pairs. We postulate the protein to have an electron-harvesting role rather than the more classical directed electron transport role. The positioning of several closely interacting redox centers on the surface of the protein clearly maximizes the chance of a productive collision with redox partners and therefore increases the rate of intermolecular transfer. Such a new and robust engineering principle of multicenter redox proteins clearly applies to the soluble fumarate reductases and could well explain the mechanism of reduction of metal oxide particles by Shewanella sp. Multiheme cytochromes c, bristling on the surface of the bacterium, are proposed to interact with the heterogeneous surface of metal oxides. Clearly the lack of specific interaction sites on the surface of the electron acceptor can be counterbalanced by the presence of multiple donor sites through exposure of a series of closely spaced hemes at the surface of the ferric reductase.

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