Structure of Complex III with Bound Cytochrome c in Reduced State and Definition of a Minimal Core Interface for Electron Transfer*\(^\text{[5]}\)

Received for publication, December 12, 2007, and in revised form, March 27, 2008. Published, JBC Papers in Press, April 4, 2008, DOI 10.1074/jbc.M710126200

Suzanne R. N. Solmaz\(^\text{1,1}\) and Carola Hunte\(^\text{4,2}\)

From the \(^*\)Department of Molecular Membrane Biology, Max Planck Institute of Biophysics, 60438 Frankfurt am Main and \(^\text{§}\)Cluster of Excellence Macromolecular Complexes, Max Planck Institute of Biophysics, 60539 Frankfurt am Main, Germany

In cellular respiration, cytochrome c transfers electrons from cytochrome bc\(_1\) complex (complex III) to cytochrome c oxidase by transiently binding to the membrane proteins. Here, we report the structure of isof orm-1 cytochrome c bound to cytochrome bc\(_1\) complex at 1.9 Å resolution in reduced state. The dimer structure is asymmetric. Monovalent cytochrome c binding is correlated with conformational changes of the Rieske head domain and subunit QC6pQ and with a higher number of interfacial water molecules bound to cytochrome c. Pronounced hydration and a “mobility mismatch” at the interface with disordered charged residues on the cytochrome c side are favorable for transient binding. Within the hydrophobic interface, a minimal core was identified by comparison with the novel structure of the complex with bound isoform-2 cytochrome c. Four core interactions encircle the heme cofactors surrounded by variable interactions. The core interface may be a feature to gain specificity for formation of the reactive complex.

Electron transfer processes are essential for all living organisms. Most energy equivalents in eukaryotic cells are generated by the mitochondrial respiratory chain. In cellular respiration, the soluble protein cytochrome c (cyt c)\(^3\) transports electrons from the cytochrome bc\(_1\) complex (cyt bc\(_1\)) to cytochrome c oxidase (1). The interaction of cyt bc\(_1\) and cyt c is transient and amazingly efficient, enabling turnover rates higher than 100/s (2, 3). The mitochondrial cyt bc\(_1\) is a homodimeric multisubunit integral membrane protein complex with a molecular mass close to 500 kDa. The enzyme catalyzes the electron transfer from ubiquinol to cyt c coupled to the net translocation of protons over the membrane (4). A key feature of the mechanism is the large scale domain movement of the Rieske protein by 20 Å, which facilitates electron transfer from oxidation of ubiquinol at center P to subunit cyt c\(_1\) (5). Cyt c docks onto the latter subunit and takes up the electron. An x-ray structure of yeast cyt bc\(_1\) with cyt c and an antibody fragment bound has been previously determined at 2.97 Å resolution (3). A single cyt c molecule is bound to the homodimeric complex. Direct and specific interactions of the electron transfer complex visualized in the x-ray structure are mediated by non-polar forces and a cation-π interaction with charged residues positioned peripherally to the interface. These interactions appear to be the dominant features of transient electron transfer complexes and are also observed for the interface of the yeast cyt c peroxidase-cyt c (6) and the bacterial reaction center-cyt c\(_2\) complexes (7). In general, a two-step model for formation of transient electron transfer complexes is today strongly supported. A short-lived, dynamic encounter complex steered by long-range electrostatic interactions precedes a dominant well defined bound state based mainly on non-polar interactions (8, 9). However, electron transport proteins such as cyt c do have several structurally unrelated reaction partners, and it is not understood how the high degree of specificity for the reaction partners is achieved in combination with the necessary weak binding and whether redox state-dependent alterations are required to facilitate association and dissociation processes. No large structural changes are known to explain these requirements. These features most likely have their molecular causes in subtle structural differences, including specific contributions of water molecules.

Here, the structure of yeast cyt bc\(_1\) with its substrate cyt c and an Fv fragment bound was determined at 1.9 Å resolution in reduced state. It is the highest resolution for a cyt bc\(_1\) structure so far, and it allows the accurate description of the complex interface, focusing especially on the role of electrostatic and water-mediated interactions as well as on differences related to the monovalent binding mode of cyt c. A second structure of cyt bc\(_1\) with isoform-2 cyt c was determined, and comparison of the structures resulted in the identification of common binding interactions, the core interface, important for specificity of binding.

EXPERIMENTAL PROCEDURES

Preparation of cyt bc\(_1\)-cyt c Crystals—The ternary complex of cyt bc\(_1\), Fv fragment, and isoform-1 (iso-1) cyt c was prepared as
described in Ref. 3 with the following modifications. To reduce nuclei formation, sucrose was added (1 M) to the buffer of the cyt bc\textsubscript{1}F\text{v} fragment complex prior to concentrating the protein to 30 mg/ml. The derivatized yeast cyt c was added in a molar ratio of 2.6/cyt bc\textsubscript{1} dimer. An ultracentrifugation step (40 min at 106,000 \times g) was introduced. 2 \mu l of the ternary complex were mixed with 1 \mu l of precipitant in a microbatch setup under paraffin oil. The crystallization buffer was 1 M sucrose, 10% DMSO, 20 mM Tris, pH 7.5, 66 mM NaCl, 1.67% polyethylene glycol 4000, 0.05% n-undecyl-\beta-D-maltopyranoside, and 1 \mu M stigmatellin (final concentrations). Cyt bc\textsubscript{1} is highly active in this buffer if the inhibitor stigmatellin is omitted. Crystal grew up to 0.4 mm within 6 weeks to 6 months at 4 °C. Crystals were soaked for 5 min in crystallization buffer with 1 mM ascorbate, 80 mM NaCl, and 5% polyethylene glycol 4000 prior to flash-freezing in liquid nitrogen. The reduced state of the c-type hemes was spectroscopically confirmed (supplemental Fig. S5A). Isoform-2 (iso-2) cyt c was prepared as described (2); the crystals were prepared as for iso-1 cyt c, but ascorbate was omitted.

Structure Determination and Refinement—Diffraction data were collected from single crystals at 100 K at the European Synchrotron Facility (Grenoble, France) at beam line ID23EH1 for the high resolution structure (MarmosacCCD Q225 detector) and at beam line ID14EH1 for the structure with iso-2 cyt c bound (ADSC CCD Q4 detector). Data were processed and scaled with XDS (10). The structures were solved by molecular replacement as described in Ref. 3. For iso-2 cyt c, Protein Data Bank accession code 1YEA (11) was used as a starting model. The models were refined in CNS (12) and by manual remodeling in program O (13). The two monomers were refined independently without non-crystallographic symmetry restraints. The final statistics are listed in supplemental Table S1.

Hydrogen bonds and non-bonded atom contacts were analyzed with the programs LIGPLOT (14) and CNS. All binding pairs were checked for well defined electron density. Least-squares superimpositions were performed in LSQMAN, ESCET (25), or CNS as indicated. Root mean square deviations were calculated with CNS. For the crystal contact analysis, a model of cyt c bound at the empty binding site was created by superimposition of the two monomers via cyt b with the program O. Figures were created with MolScript (15), Raster3D (16), and PyMOL (42).

RESULTS

Structure of cyt bc\textsubscript{1} with Bound cyt c in Reduced State at 1.9 Å Resolution—The electron transfer complex of cyt c bound to cyt bc\textsubscript{1} from the yeast Saccharomyces cerevisiae was crystallized with the antibody fragment F\textit{v} \textsubscript{18E11}, and the structure was determined at 1.9 Å resolution ($R_{free}$ = 26.2%, $R_{cryst}$ = 24.5%, see supplemental Table S1). Optimized crystallization conditions and cryo-cooling for data collection provided the basis for the improved quality as compared with the previously published structure of this complex at 2.97 Å resolution, for which data collection was carried out at 277 K (3). In agreement with the initial structure, only one molecule cyt c is bound to the homodimeric cyt bc\textsubscript{1}. As a result of the higher resolution, 1648 water, 13 lipid, and 2 detergent molecules were included in the model and refined (Fig. 1). A mixed redox state has to be assumed for the initial structure, as the complex was used as isolated with cyt c\textsubscript{1} reduction below 25% and oxidized cyt c (3). For the high resolution structure, a defined redox state was achieved by soaking the crystals in ascorbate solution prior to flash cooling and data collection at 100 K. Ascorbate reduces cyt c and the high potential chain subunits cyt c\textsubscript{1} and the Rieske protein (17, 18). The reduced state of the crystals was spectroscopically confirmed (supplemental Fig. S5, A and B).

The high resolution allows an accurate description of the complex interface present between cyt c and subunit cyt c\textsubscript{1} (Fig. 2A, Table 1). At the center of the interface, heme c\textsubscript{1} and heme c are in close contact, with 4.1 Å distance only between two carbon atoms in the respective thioether-bonded substituents of the tetrpyrrol rings. Nine pairs of interacting atoms surround the heme clefts separated by 3–4 Å, permitting hydrophobic interactions (Table 1, Fig. 3, A and B). Four contacts including the cation–\pi interaction Phe-230/Arg-19 of cyt c\textsubscript{1}/cyt c are the same as in the 2.97 Å structure, for which only a total of five interacting pairs was observed (Table 1). An energy-based analysis with the program CaPTURE (19) indicates that the Phe-230/Arg-19 pair contributes a strong cation–\pi interaction with a calculated interaction energy of $E = -7.07$ kcal/mol. Residues Ala-103/Ala-87, Ala-103/M3L78 and Met-233/Gly-89 of cyt c\textsubscript{1}/cyt c mediate hydrophobic interactions with the respective carbon atoms in van der Waals distance (M3L: trimethyllysin). The contribution of the modified amino acid residue trimethyllysin (M3L78) of cyt c is noteworthy. In addition, Phe-230/Thr-18, Ala-168/Val-34, Gln-170/Gln-22, and Gln-170/Lys-33 of cyt c\textsubscript{1}/cyt c form van der Waals contacts.

FIGURE 1. In the 1.9 Å resolution structure, one molecule cyt c (green) binds to one of the two subunits cyt c\textsubscript{1} (pink) of the cyt bc\textsubscript{1} dimer (light gray). The protein was crystallized with F\textit{v} fragments (dark gray). Water molecules are shown in cyan. Lipid and detergent molecules are colored yellow with oxygen atoms in red. Carbon and oxygen atoms of heme groups and stigmatellin are in black and red, respectively. Yellow horizontal lines indicate the relative position of the membrane.
A Minimal Core Interface for Electron Transfer

![Image: The interface of cyt c (green) and cyt c (pink) at high resolution.](Image)

**FIGURE 2.** The interface of cyt c (green) and cyt c (pink) at high resolution. Hemes, black; water molecules, cyan. A, 2Fo – Fc electron density map at the interface, contoured at 1σ and drawn as a blue mesh. B, stereo view of the core interface. The minimal core interface (dotted lines) is defined by four residue pairs (labeled) that identified in three structures of the electron transfer complex. The contact of the heme CBC atoms is shown as a dotted line.

**TABLE 1**

| Interactions in the cyt bc1, cyt c interface and heme-to-heme distances for three structures of the electron transfer complex with either iso-1 cyt c or iso-2 cyt c |
|---|
| Atom-to-atom distances are listed for residue pairs of cyt c, and the respective cyt c isoform. Close contacts are mainly non-polar. They are grouped as core interface when the pairs are present in all structures or otherwise as variable interactions. Long-range electrostatic interactions are listed separately. d, distance; M3L, trimethyllysine. For several charged residues, the electron density of the side chain (*) or charged atom ($) was not well defined, indicating high mobility of the side chain. Therefore, Glu-201/Lys-17 (§) was considered as weak "long-range" interaction. |

| | cyt bc1, iso-1 cyt c | cyt bc1, iso-1 cyt c | cyt bc1, iso-2 cyt c |
|---|---|---|---|
| Heme-to-heme distances | | | |
| Heme c, CBC | Heme c CBC | Heme c CBC | Heme c CBC |
| Heme c, FE | Heme c FE | Heme c FE | Heme c FE |
| Edge-to-edge | | | |

The interface is not built from modules of closely interacting residues (20), but the individual contacts are dispersed over the small interface and are separated from each other by distances longer than 4 Å. Interacting residues close enough for hydrogen bonds or salt bridges were not observed in the interface.

Water Molecules at the Interface—For the first time, water molecules at the cyt bc1, cyt c interface were analyzed. The ring of non-polar interactions around the heme clefts does not seal off the interface from the aqueous environment. The hydration of the interface was analyzed for residue pairs from...
cyt c₁/cyt c with an interatom distance of less than 7 Å. 30 water molecules bind within this distance constraint to either surface by hydrogen bonds. The interface is well hydrated, and water molecules are either in hydrogen bond distance to cyt c or to cyt c₁ (Fig. 2, A and B, and Fig. 3, D and E). Eight water molecules mediate a hydrogen bond either to cyt c₁ or cyt c and are in van der Waals contact distance to atoms of the respective interaction partner. Two water molecules mediate an interaction by hydrogen bonds between the two interaction partners, although the interaction is weak due to the extended bond length: water molecule X1708 is bound to Thr-18 of cyt c (distance 2.9 Å) and to the backbone carbonyl oxygen atom of Arg-227 of cyt c₁ (distance 3.2 Å); water molecule X1845 is bound to the main chain nitrogen atom of Ala-226 of cyt c₁ (distance 3 Å) and the Gln-22 OE1 atom of cyt c (distance 3.3 Å).

Extending the analysis of the interface to a cutoff distance of 10 Å between cyt c₁ and cyt c showed that there are no interactions mediated by two water molecules.

Considerable rearrangement of water molecules is observed upon binding of cyt c. The simultaneous presence of an occupied and a free cyt c binding site in the cyt bc₁ dimer permitted the comparison of water molecule positions between the two sites in the same crystal structure (Fig. 3F). 9 water positions are the same in both sites. 5 and 10 water molecules are only present on the free and occupied surface, respectively. The latter include the two water molecules with hydrogen bonds to both cyt c₁ and cyt c. Overall, more crystallographically defined water molecules are located on the surface of cyt c₁ with bound cyt c.

The Electrostatic Interactions Form a Semicircle around the Non-polar Interface—The non-polar interface is surrounded by oppositely charged residue pairs, which are not in close contact.
A Minimal Core Interface for Electron Transfer

For the analysis of long-range electrostatic interactions, a cutoff distance of 4–9.6 Å was chosen. The latter is known as Debye length between single-charged ions at an ionic strength of 100 mM (21). Above this distance, the charge of an ion is shielded by counter ions of the solvent. Eight oppositely charged residue pairs were identified in the structure; they are separated by distances of 4.7–9.2 Å (Table 1). Three of five of the identified cyt c residues have been shown by selective labeling to be important for binding of horse heart cyt c (homologous yeast residues are in parentheses): Lys13 (Arg-19), Lys-86 (Lys-92), and Lys-87 (Lys-93) (22). Whereas the selected residues from cyt c1 are overall well defined by the electron density map, most of the positively charged side chains of cyt c appear to be flexible, indicating that the long-range electrostatic interactions are weak (Table 1). Analysis of the hydration shell clearly showed that there are no electrostatic interactions mediated by one or two bridging water molecules. The charged residue pairs are clustered at one side of the interface and form a semicircle around it (Fig. 3, A–C).

Structure of cyt bc1 with Isoform-2 cyt c Bound—Yeast cyt c exists in two isoforms that share 83% sequence identity (supplemental Fig. S6). Depending on growth conditions, yeast cells contain typically 95% iso-1 and 5% iso-2 (23). Both the high resolution and the previous structure (3) contain iso-1 cyt c. To challenge the specificity of the binding interaction and the monovalent binding mode, we crystallized the complex of yeast cyt bc1 and iso-2 cyt c with the antibody fragment Fv_{BRE1}. The crystals were directly used for x-ray diffraction data collection at cryo conditions without any redox treatment, and the structure was determined at 2.5 Å resolution (Rfree = 25.6%, Rcryst = 22.5%, see supplemental Table S1). As for the complexes with iso-1 cyt c, only one molecule of iso-2 cyt c is bound. A slight shift within the error limits was observed for the position of iso-2 cyt c. The heme c1 to heme c distances of the electron transfer complex are nearly identical to the complexes with iso-1 cyt c bound. Six residue pairs of the non-polar interface are the same for iso-2 and iso-1 cyt c: Ala-103/Ala-91 (87), Ala-103/M3L82 (78), Ala168/Val38 (34), Phe-230/Thr22 (18), Phe230/Arg-23 (19), and Met-233/Ala-93 (Gly-89) of cyt c2/iso-2 cyt c (iso-1 cyt c) (Table 1). This includes the cation–π interaction and the trimethyllysine of cyt c. Only one of these residues differs in iso-2; Ala-93 is substituted with glycine in iso-1 cyt c (Gly-89). Seven additional contacts, including two close (<4 Å) electrostatic interactions between oppositely charged residue pairs (Glu-201/Lys-21) were identified; all involve residues that are conserved between the two isoforms.

The coinciding monovalent binding mode and the highly similar interfaces could be enforced by protein-protein interactions between neighboring molecules in the crystal lattice. Therefore, crystal contact analysis was carried out using models in which cyt c binding to the second monomer was generated by superimposition.

In the occupied binding site of the high resolution structure, atoms of His-45 from iso-1 cyt c are in contact with subunit QCR2p of the neighboring molecule. The strongest interaction is a polar hydrogen bond between His-45 NE2 of cyt c and Leu-368 OT2 of QCR2p with a distance of 2.9 Å. At the second binding site, the nearest atom of the neighboring molecule would be 7 Å apart from cyt c. If both sites were occupied, the two cyt c molecules would be separated by a minimal distance of 18 Å. In conclusion, no steric hindrance impedes the binding at the second binding site.

Furthermore, His-49 of iso-2 cyt c (homologous to His-45 of iso-1 cyt c) does not mediate any crystal contacts. In the respective structure, the only contacts present are van der Waals contacts between Asn-66 from cyt c and the neighboring cyt bc1 at the occupied binding site. There is no indication for steric hindrance at the modeled (empty) binding site, but Asn-66 of cyt c could potentially form a polar hydrogen bond with Asp-366 of cyt bc1 that could stabilize cyt c at the empty binding site. Taken together, the contacts of cyt c to neighboring molecules in the crystal lattice are very weak and are mediated by different residues in the two structures. This strongly indicates that the interface, which has a larger contact area, dominates and specifies the binding interaction.

The Core Interface Is Defined by Interactions Occurring in Three cyt bc1/cyt c Structures—Three structures of the electron transfer complex have now been determined, the structures with iso-1 cyt c at 1.9 Å and 2.97 Å resolution and with iso-2 cyt c. The structures differ in crystallization conditions, redox state, data collection temperature, and cyt c amino acid sequence. However, when comparing the respective interfaces (see Table 1), four interacting residue pairs of cyt c1/cyt c were identified in all three structures: Ala-103/Ala-87, Ala-168/Val-34, Phe-230/Arg-19, and Phe-230/Thr-18. These pairs are apparently important for the stabilization. They define an area around the heme cleft, which we term the core interface (Fig. 2B). Besides the core interface, the high resolution structure and the structure with iso-2 cyt c bound share two more interacting pairs (Ala-103/M3L78 and Met-233/Gly-89 of cyt c1/cyt c). The 2.97 Å resolution structure and the structure with iso-2 cyt c share one additional interaction (Met-233/Arg-19 of cyt c1/cyt c). Two of the variable interactions appear only in the high resolution structure, and five pairs appear only in the structure with iso-2 cyt c (Table 1). The core interface interactions (orange in Fig. 3, A and B) are all located in close vicinity to the heme clefts whereas the variable interactions are farther away from the hemes and surround the core interface (pale yellow in Fig. 3, A and B).

The heme-to-heme distances are consistent between the three structures. The edge-to-edge distances of the porphyrin rings that govern the electron transfer rates range from 9.0 to 9.3 Å (Table 1). The resulting calculated electron transfer rates (3, 24) are very fast and in the range of 1.0 × 10^6 to 2.6 × 10^7 s⁻¹, affected by subtle differences in edge-edge distance and packing density. A comparison of the long-range electrostatic interactions for the three complex structures shows that, with few exceptions, the same residues are involved (Table 1). Asp-231, Asp-232, Glu-99, and Glu-235 of cyt c1 and Lys-92 and Lys-93 of cyt c mediate long-range electrostatic interactions in all three structures. The other residue pairs mediate long-range electrostatic interactions in two out of three structures.

Comparison of cyt bc1 Monomers with Free and Occupied cyt c Binding Sites Reveals Rigid Body Movement of the Rieske Pro-
tein Head Domain—The resolution of 1.9 Å and the independent refinement of the two monomers allowed a detailed comparison of monomer A and B with empty and occupied binding site, respectively. As previously observed (3), the occupancy of the Q site with ubiquinone was higher for the monomer with bound cyt c. However, the overall occupancy was too low at both binding sites to refine ubiquinone. No significant structural differences were observed for the structures of the two ubiquinone binding sites at center N. Also, the two binding sites for cyt c do not show significant structural differences, indicating that the cyt c binding site does not rearrange upon substrate binding.

The B-factors, and thus also the coordinate error, showed very large differences for the different regions of the dimer, imposing limitations for assigning the significance of conformational changes. Thus, the monomers were compared with the program ESCET (25), which uses the diffraction precision index DPI (26) that includes B-factors in the error model. For the analysis, a sigma cutoff of two was used. Three rigid bodies were identified (Fig. 4A). The major part of the monomer structure is equivalent between the two monomers and forms the blue rigid body. The second rigid body contains residues of subunit QCR6p (74–104 and 141–174, green in Fig. 4A) and is likely to have a different conformation. QCR6p shows overall increased per-residue root mean square deviation if the monomers are superimposed by the catalytic subunits. The highly acidic subunit has been suggested to guide cyt c docking to cyt c1 (27). The third rigid body (yellow, Fig. 4A) changes its conformation significantly. It includes the residues of the Rieske head domain (residues 89–158, 168–0176, and 184–208) and also the attached antibody Fv fragment. The iron-sulfur cluster-bearing tip of the Rieske protein (residues 159–167 and 177–183) remains in place locked in this conformation by the inhibitor stigmatellin.

The significance of these results was confirmed by least-squares superimpositions. A superimposition of the monomers via the blue rigid body is displayed in Fig. 4B. For the superimposition of the monomers via subunit cyt b, which belongs to the blue rigid body, a shift with root mean square deviation of 0.81 Å was calculated for the Rieske head domain (residues 94–205). The superimposition of the Rieske head domain itself has a root mean square deviation of 0.42 Å. If the latter is interpreted as the noise and the superimposition via subunit cyt b as signal for the rigid body domain movement, the signal is 2-fold above the noise, which is significant.

It cannot be excluded that the conformational change of the Rieske head domain and the attached Fv fragment is caused by crystal contacts that are mediated by residues 123–126 at the C terminus of the Fv heavy chain (orange in Fig. 4B). However, a comparison of monomer B (with cyt c bound) with cyt c-free monomer A and with a cyt bc1 structure without cyt c bound (3) showed that the crystal contacts of the Fv fragments are in all cases very similar. This indicates that the observed differences are a genuine feature of the complex and that the crystal lattice accommodates the asymmetric complex rather than enforces it.

DISCUSSION

In this work, the high resolution structure of the electron transfer complex of cyt bc1 with cyt c bound was determined in its reduced state at 1.9 Å resolution. Notably, the complex does not dissociate upon reduction of the crystal, as indicated by the same monovalent binding mode and a very similar interface when compared with the structure of the complex in mixed, mostly oxidized state (3).

The mobile electron carrier cyt c has to react transiently with multiple redox partners in its reduced as well as in its oxidized state. One may assume that the binding interactions for cyt c do not or only slightly change between the redox states. Direct binding measurements indicate that at physiological ionic strength oxidized and reduced cyt c binds to cyt bc1 with very similar affinity, whereas at low ionic strength ferriicytochrome c binds more tightly than the reduced protein (28). Crystallization of the cyt bc1–cyt c complexes in this and the previous (3) study was performed close to physiological ionic strength. Furthermore, only minor structural differences are observed for cyt c alone in reduced and oxidized state (29). An NMR study showed comparable chemical shift changes for the interaction of cyt552 with the bacterial soluble CuA domain of the cyt c oxidase in fully reduced and fully oxidized state, indicating that the interaction does not depend on the redox states of the electron transfer partners (30). Molecular dynamics simulations with the photosynthetic reaction center and cyt c, also indicated that the differences between the redox states are subtle. A structured cluster of water molecules was observed in the

A Minimal Core Interface for Electron Transfer

![Figure 4. Conformational changes between monomer A and B with cyt c bound. A, three rigid bodies are mapped on the structure of monomer B (blue, green, yellow) and overlaid with a transparent surface presentation of the dimer surface and cofactors (hemes and stigmatellin in black). The blue rigid body has essentially the same structure for both monomers. The yellow rigid body (Rieske head domain and Fv fragments) changes the conformation in the monomer with cyt c bound. The green rigid body, regions of subunit QCR6p, might change its conformation. B, closeup of the conformational change of the Rieske head domain. The structures of monomer A and B are superimposed via the blue rigid body. The yellow rigid body is colored cyan for monomer A and yellow for monomer B to highlight differences. The crystal contacts mediated by the C terminus of the Fv heavy chain are shown in orange.](image-url)
A Minimal Core Interface for Electron Transfer

reduced cyt c system, whereas fluctuations of water and residues at the interface increased upon oxidation (31). The authors suggested that the higher mobility may mediate the undocking process. The oxidized complex was found less stable than the reduced by ~6 kcal/mol effective free binding energy.

Cyt c binds to the homodimeric cyt bc₁ in a monovalent mode in each of the three complex structures. The cyt c binding site with and without cyt c shows no differences in the protein structure at high resolution. Interestingly, the cyt bc₁ complex dimer is asymmetric in the high resolution structure. Monovalent cyt c binding is correlated with a conformational change of the Rieske head domain and subunit QCR6p in one monomer. The highly acidic subunit QCR6p has been shown to aid cyt c binding (32). Deletion of this subunit from the cyt bc₁ complex decreases the turnover number dramatically at physiological ionic strengths (33). A role of this subunit in preorientation of cyt c has been suggested (3). The Rieske protein physically cross-links the two functional units of the intertwined dimer and has been suggested as a regulatory element in the mechanistic alternating site model, in which only one monomer of cyt bc₁ is active at a time (34). Cyt c binding may be affected by or may itself influence equilibration of electrons within or between cyt bc₁ monomers. Pre-steady-state kinetics analysis showed that cyt c binding to cyt bc₁ variants Y132F and E272D, both substitutions in subunit cyt b, altered kinetics of center N and activated ubiquinol oxidation (35). Further studies are required to analyze in solution the binding mode of cyt c to cyt bc₁ and potential regulatory long-range interactions.

The cyt bc₁/cyt c complex has a small hydrophobic interface surrounded by charged residues, typical for transient electron transfer complexes and in agreement with the two-step model of complex formation. The presence of salt bridges could not be addressed in the initial structure due to limited resolution (3). In the high resolution structure, it is clear that the oppositely charged residue pairs are separated by 4.7–9.2 Å and that they do not form stable salt bridges but provide long-range Coulombic interactions. These interactions appear to be of low specificity, as the positively charged interacting side chains of cyt bc₁ show no differences in the protein structure at high resolution. Interestingly, the interacting charged residue pairs form a semicircle on cyt c1 and cyt c. In contrast to a complete circle, this arrangement orients the electric dipoles of the interacting partners to the same direction, a feature that should have an impact on their relative orientation. This is a common, but yet not described, feature among the three electron transfer complexes cyt bc₁/cyt c, reaction center-cyt c₂, and yeast cyt c peroxidase-cyt c. This observation suggests that the electrostatic component of the interactions already creates a defined orientation that provides the basis for a single well defined bound state for efficient electron transfer.

Hydrogen bonds are not favorable for transient complexes due to the energetically unfavorable desolation process (36). Inter-protein hydrogen bonds are not observed for the cyt bc₁/cyt c interface and only two water molecules mediate a hydrogen bond interaction between cyt c1 and cyt c. The contribution of hydrogen bonds to the binding interaction is even lower than in comparable complexes. The interface of yeast cyt c peroxidase-cyt c (6) displays only one intermolecular bond; the interface of the reaction center-cyt c₂ (7) displays three, none of them mediated by charged residues. In the structure of the reaction center-cyt c₂, seven interface water molecules make hydrogen bonds to both proteins, compared with three waters in the structure of the cyt c peroxidase-cyt c with a site-specific cross-link (38). This suggests that the cyt bc₁/cyt c interaction is especially weak, as should be expected for a fully reduced complex.

A further indication for the weak cyt bc₁/cyt c interaction is the high solvation of the interface, which contains 1.5 times more water molecules than an average protein-protein interface (39). In reverse, high affinity interactions are usually characterized by a watertight seal around a set of energetically favorable interactions (27). It should be noted that cyt c binding results in a higher number of ordered water molecules on the cyt c₁ surface. Eight water molecules are bound by a hydrogen bond to either cyt c or cyt c₁ and by a van der Waals contact to the second binding partner. In high affinity complexes, most of the interface waters make hydrogen bonds to both binding partners (39). Interestingly, more water molecules are associated with the cyt c₁ side of the interface than with that of cyt c. This coincides with the higher mobility of the positively charged interacting side chains on the cyt c side, which is unfavorable for structured water molecules. The observed mobility mismatch comprising interfacial water molecules and interacting charged residue side chains appears to be well suited for a transient interaction and may aid the undocking process of cyt c.

Many concepts for inter-protein electron transfer have been developed from the interaction of the photosynthetic reaction center with cyt c₂. A key difference between this complex and cyt bc₁ is the edge-to-edge distance. Fast physiological electron transfer is observed for distances up to ~14 Å (24). For the reaction center-cyt c₂ complex, an edge-to-edge distance of 14 Å is reached in the bound state. For the cyt bc₁/cyt c complex, the edge-to-edge distance in the bound state is 9 Å. In principal, electron transfer could already occur while cyt c is approaching the binding site. One may ask whether cyt bc₁ forms multiple productive complexes with cyt c or whether a mechanism exists that prevents less efficient complexes and favors formation of a defined single complex.

Despite all indications for a weak interaction, the now available three structures of the cyt bc₁/cyt c electron transfer complex clearly show a highly specific, congruent interface even in respect to the different cyt c isoforms. Four residue pairs
including the cation-π interaction were identified in all three structures, suggesting that they play an important role in the stabilization of the complex. These residue pairs define the minimal core interface. Mutational analysis is in progress to probe whether these residues are so-called interaction hot spots, meaning that they contribute more to the binding energy than others.

Cyt c residues homologous to the core interface residues mediate interactions in structures of other electron transfer complexes. Examples are (homologous yeast residues in parentheses) Arg-19 and Ala-87 in the complex of cyt c peroxidase and cyt c from yeast (6), Val-26 (Val-34) and Ala-79 (Ala-87) in the complex of cyt c with the CuA domain of cyt c oxidase (30), Gln-14 (Arg-19) and Thr-36 (Val-34) in the cyt c2 reaction center complex (7), as well as Thr-18 in the complex of yeast cyt c with the cyt f domain of the cyt bf complex (40). The analogous cation-π interaction of the reaction center-cyt c complex has been observed as the most stable contact in molecular dynamic simulations (31, 41). The core interface appears to be a central feature of the interaction of cyt c with many, structurally not related, binding partners.

Comparison of the three cyt bc1-cyt c structures shows that in addition to the core interface in close vicinity to the heme clefts, variable hydrophobic interactions are formed peripheral to the core. The variable interactions are combined with small shifts of the cyt c position, while the heme-heme distance and the geometry are kept constant. The specific interactions of the newly identified core interface may create sufficient specificity for the transient interaction, being a minimal site that ensures a geometry suited for electron transfer. Comparison with high resolution structures of the electron transfer complex in fully oxidized state as well as with electron donor and acceptor in different redox states are in progress to allow precise discrimination of redox-specific binding interactions.

Acknowledgments—We thank Sofia Hollschwander for excellent technical assistance and Sebastian Richers and Raimond Ravelli for discussions. We acknowledge the beam time and the assistance of the personnel at beam lines ID23-1 and ID14-EH1 at the European Synchrotron Facility, Grenoble, France.

REFERENCES
1. Saraste, M. (1999) Science 283, 1488–1493
2. Hunte, C., Solniz, S., and Lange, C. (2002) Biochim. Biophys. Acta-Bioenerg. 1555, 21–28
3. Lange, C., and Hunte, C. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 2800–2805
4. Berry, E. A., Guergova-Kuras, M., Huang, L. S., and Crofts, A. R. (2000) Annu. Rev. Biochem. 69, 1005–1075
5. Zhang, Z. L., Huang, L. S., Shulmeister, V. M., Chi, Y. I., Kim, K. K., Hung, L. W., Crofts, A. R., Berry, E. A., and Kim, S. H. (1998) Nature 392, 677–684
6. Pelletier, H., and Kraut, J. (1992) Science 258, 1748–1755
7. Axelrod, H. L., Abresch, E. C., Okamura, M. Y., Yeh, A. P., Rees, D. C., and Feher, G. (2002) J. Mol. Biol. 319, 501–515
8. Ubbink, M., Ejdebäck, M., Karlsson, B. G., and Bendall, D. S. (1998) Structure 6, 323–335
9. Volkov, A. N., Worrall, J. A. R., Holtzmann, E., and Ubbink, M. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 18945–18950
10. Kabasch, W. (1993) J. Appl. Crystallogr. 26, 795–800
11. Murphy, M. E. P., Nall, B. T., and Brayer, G. D. (1992) J. Mol. Biol. 227, 160–176
12. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, Pt. 5, 905–921
13. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. A 47, Pt. 2, 110–119
14. Wallace, A. C., Laskowski, R. A., and Thornton, J. M. (1995) Protein Eng. 8, 127–134
15. Kaura, P. (1991) J. Appl. Crystallogr. 24, 946–950
16. Merritt, A. E., and Murphy, M. E. P. (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 869–873
17. Vanneste, W. H. (1966) Biochim. Biophys. Acta 113, 175–178
18. Yu, C. A., Yu, L., and King, T. E. (1974) J. Biol. Chem. 249, 4905–4910
19. Gallivan, J. P., and Dougherty, D. A. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 9459–9464
20. Reichmann, D., Rahat, O., Albeck, S., Meged, R., Dym, O., and Schreiber, G. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 57–62
21. Wedler, G. (2004) Lehrbuch der Physikalischen Chemie 5th Ed., Wiley-VCH, Weinheim, Germany
22. Speck, S. H., Ferguson-Miller, S., Osheroff, N., and Margoliash, E. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 155–159
23. Lai, T. M., Pietras, D. F., and Sherman, F. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 4475–4479
24. Page, C. C., Moser, C. C., Chen, X. Y., and Dutton, P. L. (1999) Nature 402, 47–52
25. Schneider, T. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 12 Part 1, 2269–2275
26. Cruickshank, D. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 583–601
27. Bogan, A. A., and Thorn, K. S. (1998) J. Mol. Biol. 280, 1–9
28. Speck, S. H., and Margoliash, E. (1984) J. Biol. Chem. 259, 1064–1072
29. Banci, L., Bertini, I., Huber, J. G., Spyroulias, G. A., and Turano, P. (1999) J. Biol. Inorg. Chem. 4, 21–31
30. Wienk, H., Maneg, O., Pirstovsek, P., Lucke, C., Lohr, F., Ludwig, B., and Ruterjans, H. (2003) Biochemistry 42, 6005–6012
31. Autenrieth, F., Tajkhorshid, E., Schulten, K., and Luthey-Schulten, Z. (2004) J. Phys. Chem. B 108, 20376–20387
32. Kim, C. H., and Donnelly, J. E. (1983) J. Biol. Chem. 258, 13543–13551
33. Schoppik, P. J., Hemrika, W., Reyen, J. M., Grivell, L. A., and Berden, J. A. (1988) Eur. J. Biochem. 173, 115–122
34. Trumpower, B. L. (2002) Biochim. Biophys. Acta 1555, 166–173
35. Wenz, T., Covan, R., Hellwig, P., MacMillan, F., Meunier, B., Trumpower, B. L., and Hunte, C. (2007) J. Biol. Chem. 282, 3977–3988
36. Miyashita, O., Onuchic, J. N., and Okamura, M. Y. (2003) Biochemistry 42, 11651–11660
37. Miyashita, O., Onuchic, J. N., and Okamura, M. Y. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 16174–16179
38. Guo, M., Bhaskar, B., Li, H., Barrows, T. P., and Poulos, T. L. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 5940–5945
39. Lo Conte, L., Chothia, C., and Janin, J. (1999) J. Mol. Biol. 285, 2177–2198
40. Crowley, P. B., Rabe, K. S., Worrall, J. A. R., Canters, G. W., and Ubbink, M. (2002) ChemBioChem. 3, 526–533
41. Pogorelov, T. V., Autenrieth, F., Roberts, E., and Ludhey-Schulten, Z. A. (2007) J. Phys. Chem. B 111, 618–634
42. DeLano, W. L. (2002) The PyMOL Molecular Graphics System, DeLano Scientific, Palo Alto, CA