Crystal Structure of a Complex Between Electron Transfer Partners, Cytochrome c Peroxidase and Cytochrome c

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The crystal structure of a 1:1 complex between yeast cytochrome c peroxidase and yeast iso-1-cytochrome c was determined at 2.3 Å resolution. This structure reveals a possible electron transfer pathway unlike any previously proposed for this extensively studied redox pair. The shortest straight line between the two hemes closely follows the peroxidase backbone chain of residues Ala^{194}, Ala^{193}, Gly^{192}, and finally Trp^{191}, the indole ring of which is perpendicular to, and in van der Waals contact with, the peroxidase heme. The crystal structure at 2.8 Å of a complex between yeast cytochrome c peroxidase and horse heart cytochrome c was also determined. Although crystals of the two complexes (one with cytochrome c from yeast and the other with cytochrome c from horse) grew under very different conditions and belong to different space groups, the two complex structures are closely similar, suggesting that cytochrome c interacts with its redox partners in a highly specific manner.

Long-range electron transfer reactions involving cytochromes (a class of heme-containing proteins) are prevalent in energy-producing biological processes such as photosynthesis and respiration. Respiration, in particular, is the process by which dioxygen is ultimately reduced to water, producing energy-rich adenosine triphosphate (ATP) along the way. The machinery of the respiratory electron transport chain is located in the inner mitochondrial membrane of eukaryotic cells or in the plasma membrane of respiring bacteria. In the penultimate stage of respiration, cytochrome c, a small (12 kilodaltons) water-soluble electron-carrier protein, mediates the flow of electrons between two large multisubunit membrane-bound enzymes, cytochrome reductase and cytochrome oxidase. In 1925, Keilin discovered the brightly colored cytochromes (*1*) and subsequently elucidated the role of cytochrome c in the respiratory electron transport chain (*2*). Ever since then the questions of how cytochrome c interacts with its partners and, more generally, how long-range, outer sphere electron transfer occurs in biological systems, have been lively areas of research (*3*, *4*).

High-resolution crystal structures of cytochromes c from various species have been determined (*5*–*8*), but because crystallizing large membrane-bound proteins is difficult, the crystal structures of cytochrome reductase and cytochrome oxidase remain unknown. Yeast cytochrome c peroxidase (CCP), in contrast, is a biological redox partner of cytochrome c for which high-resolution crystal structures are available (*9*, *10*). Although the biological function of CCP is still obscure, the complex formed between cytochrome c peroxidase and cytochrome c (CCP:cc) (*11*) serves as a simple model for the much more elaborate electron transfer complexes of the respiratory electron transport chain. The following equation represents the overall reduction of an alkyl hydroperoxide by ferrocytochrome c, as catalyzed by CCP

$$2\ \text{cytochrome c}\ (Fe^{2+}) + ROOH + 2H^{+} \rightarrow$$

$$2\ \text{cytochrome c}\ (Fe^{3+}) + ROH + H_2O$$

In the catalytic cycle, CCP undergoes a two-electron oxidation by peroxide to form a radical-containing intermediate. This intermediate, usually termed compound I, is reduced back to the resting state by two ferrocytochrome c molecules in two consecutive one-electron steps (*12*).

Even though overall sequence similarity between mitochondrial cytochromes c and bacterial cytochrome c_2 was considered slight at the time, when the crystal structure of cytochrome c_2 was solved it revealed a ring of positively charged surface residues encircling the heme crevice similar to that observed previously in the tuna, horse, and bonito cytochrome c structures. This result, together with earlier studies showing that polycations compete with cytochrome c for binding to cytochrome reductase and cytochrome oxidase (*13*), represent only a few of many experimental observations leading to proposals that interactions between cytochrome c and its redox partners are predominantly electrostatic (*14*). Thus, an apparent ring of negatively charged residues surrounding the heme crevice in CCP seemed to provide the perfect docking site in models of CCP complexed with tuna cytochrome c [CCP:cc(T)] (*11*, *15*, *16*) and with yeast cytochrome c [CCP:cc(Y)] (*11*, *17*). In efforts to validate these models, numerous mutagenesis experiments were designed to target to amino acid residues in both CCP (*18*, *19*) and cytochrome c (*20*), but the results were often difficult to reconcile with the proposed models. We now present a crystallographic structure of a complex involving eukaryotic cytochrome c which differs significantly from previously proposed models. We believe that this structure represents a specific electron transfer complex.

Crystallization and preliminary diffraction studies. Yeast iso-1-cytochrome c (type VIII B) and horse heart cytochrome c (type VI) were purchased (Sigma) and used without further purification. CCP was expressed in *Escherichia coli*, purified, and stored as described (*21*).

CCP:cc(Y) complex crystals were grown at room temperature in sitting drops prepared by mixing 10 μl of a reservoir solution (150 mM NaCl, 12 to 15 percent polyethylene glycol 3350) with 10 μl of the protein solution. This protein solution was an equimolar mixture of CCP (in 50 mM KH_2PO_4, pH 7.0) and yeast iso-1-cytochrome c [in fresh 40 mM dithiothreitol (DTT)] which was subjected to three cycles of washing and ultrafiltration (Centricon-10 Microconcentrator, Amicon) with unbuffered 40 mM DTT as the washing solution. The final protein sample consisted of 1 mM CCP:cc(Y) complex (*22*) in 40 mM DTT and possibly trace amounts of potassium phosphate buffer. Complex crystals could be obtained and were shown to be isomorphous in salt concentrations ranging from 5 mM NaCl to 300 mM NaCl. Crystals grown at physiological salt concentrations (150 to 200 mM NaCl) were consistently the best, becoming as large as 1.2 by 1.2 by 0.3 mm in about 2 weeks. These crystals belong to space group $P2_1$ with unit cell parameters a = 88.4, b = 118.6, and c = 46.1 Å, and β = 104.6°. There are two CCP:cc(Y) complexes per asymmetric unit related by a noncrystallographic twofold axis. After the x-ray data were collected, spectroscopic measurements on dissolved crystals (5 mM KH_2PO_4, pH 7.0) indicated that the cytochrome c was fully oxidized.

Crystals of the CCP:cc(H) (*11*) complex were grown at room temperature in sitting drops prepared by mixing 20 μl of reservoir solution (18 to 22 percent methylpentanediol) with 20 μl of the protein solution. This protein solution, a 2:1 molar ratio of CCP (in 50 mM KH_2PO_4, pH 7.0) and

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horse heart cytochrome c (in distilled water), was washed and ultrafiltered (Centricon-10 microconcentrator) with 5 mM KH_2PO_4, pH 7.0, for at least three washing cycles. The reasons for using a 2:1 CCP:cc(H) molar ratio rather than 1:1 are discussed below. The final protein sample consisted of 1.5 mM CCP:cc(H) (*22*) in 5 mM KH_2PO_4, pH 7.0. Small tetragonal crystals appeared after 3 or 4 weeks, with the largest crystals growing to 1.5 by 1.0 by 0.75 mm in about 3 months. The crystals belong to space group $P4_32_12$ with unit cell parameters $a = b = 105.3$ Å, $c = 186.2$ Å. The asymmetric unit contains the following: two CCP molecules, one ordered horse cytochrome c molecule, and a second, partially occupied, disordered cytochrome c site. The cytochrome c in these crystals was fully oxidized.

In contrast to the CCP:cc(Y) complex, crystals of the CCP:cc(H) complex apparently do not grow over a wide range of salt concentrations, but only at 5 mM phosphate buffer or below. This observation is consistent with kinetic studies showing that the CCP:cc(H) complex is much more sensitive to variations in salt concentration than the CCP:cc(Y) complex (*23, 24*).

Many investigators have tried to crystallize the CCP:cc complex; Yonetani, who prepared the first highly purified samples of CCP, recalls trying to grow complex crystals as early as 1967 (*25*). For this reason, and because these crystals will probably be of continuing interest as an experimental system, we present a brief discussion of their idiosyncrasies.

One of the difficulties in finding crystallization conditions for the CCP:cc(Y) complex arose from conflicting requirements with respect to the presence of a disulfide-reducing agent. A reducing agent must be included in order to keep yeast iso-1-cytochrome c from dimerizing by way of an intermolecular disulfide bridge (*26*). Indeed, the crystallization of yeast iso-1-cytochrome c by itself calls for the presence of 40 mM DTT (*8*). However, reducing agents may undergo unwanted side reactions with CCP, reducing the heme iron from its normal Fe(III) resting state to the Fe(II) state. In this reduced form, CCP behaves somewhat like cytochrome oxidase in that it is capable of catalyzing the reduction of dioxygen by ferrocytochrome c (*27*). In any event, after numerous failed attempts with DTT-free crystallization solutions, we tried including 40 mM DTT despite the possible side reactions (*28*). Crystals of the complex were eventually obtained. Subsequently we discovered that the presence of either DTT or β-mercaptoethanol (BME) allows the crystals to grow, but only if these reagents are used at relatively high concentrations. At concentrations of either reducing agent that are lower than 30 mM, only amorphous aggregates or CCP crystals alone will form (*29*).

A much different problem arose in the crystallization of the CCP:cc(H) complex. Poulos *et al.* (*30*) had earlier reported on an attempted crystal structure determination of the CCP:cc(H) complex in 1987. From their results they concluded that, although cytochrome c molecules are present in the crystals, they are so disordered as to be unobservable, probably occupying spaces between the ordered CCP molecules. We were able to obtain CCP:cc(H) crystals under similar conditions; although our CCP derives from a different source (*21*), the space group, unit cell parameters, and diffraction limit for our crystals are the same as those reported by Poulos *et al.*, suggesting that there is not much difference between their crystals and those we describe here. We therefore set out to alter the crystal growth conditions so that the horse cytochrome c molecules would be more ordered—or at least sufficiently ordered to allow crystallographic visualization.

One way in which we altered the crystal growth conditions was to change the ratio of CCP to cytochrome c. Using a modified version of the polyacrylamide gel electrophoresis (PAGE) experiment described by Poulos *et al.* (*30, 31*), we observed that the CCP:cc(H) molar ratio in our crystals consistently exceeded 1:1. In fact, for every two molecules of CCP, there were never more than one and a half molecules of cytochrome c, even when the crystals were grown from a protein sample containing a large excess of cytochrome c (1:7, molar). An explanation for this odd CCP:cc(H) ratio emerged with the structure solution, as discussed below. By varying the CCP:cc(H) ratios in our crystallization attempts, we discovered that crystals grown at 2:1 ratios were much larger and better formed than those grown at 1:1, but there was still no improvement in their diffraction limit (without further treatment, as discussed below), suggesting that the degree cytochrome c disorder was probably the same. Nevertheless, we were able to solve the structure with these crystals and to visualize the interaction between CCP and horse cytochrome c in the complex.

We are unable to pinpoint exactly why we can see the cytochrome c molecule (or molecules), whereas they were not previously observed (*30*). More specifically, we do not yet know whether it is because the cytochromes c in our 2:1 crystals are better ordered than in the 1:1 crystals.

In our attempts to obtain more highly ordered CCP:cc(H) crystals, we noticed that their diffraction limits could be improved if, after the usual crystal mounting procedures, the crystals were left in their sealed capillary tubes for extended periods of time before data collection. Because better diffraction was usually accompanied by a notable decrease in the volume of the unit cell, we concluded that these crystals had become partially "dried out" (*32*). The CCP:cc(H) structure that we present was refined with x-ray data from a 2:1 crystal that had been mounted and left in its sealed capillary tube for 3 weeks before the x-ray data were collected. Whereas a typical freshly mounted "wet" CCP:cc(H) crystal yielded a 3.4 Å data set at best, this partially dried crystal gave a 2.8 Å data set (Table 1). In extreme cases, we observed CCP:cc(H) crystals diffracting to as high as 2.3 Å resolution after they had been left in their sealed capillary tubes for as long as 4 months.

Even though the partially dried CCP:cc(H) crystals appear to be more ordered than the "wet" crystals, a 3.4 Å data set collected on a wet CCP:cc(H) crystal nevertheless led to a perfectly solvable complex structure in which the cytochrome c molecule could be visualized crystallographically (*32*).

Structure determination and refinement. Data collection and refinement statistics for both the CCP:cc(Y) and the CCP:cc(H) complexes are listed in Table 1. Structure solutions utilized the refined molecular coordinates of cloned CCP (*10, 21*), available from the Brookhaven Protein Data Bank, as well as the refined coordinates of yeast iso-1-cytochrome c and horse heart cytochrome c (*7, 8, 33*).

The CCP:cc(Y) structure solution and refinement was carried out as follows. The molecular replacement program package MERLOT (*34*), version 2.3, gave very clear rotation and translation solutions for two CCP molecules in the asymmetric unit. Yeast cytochrome c, however, is only one-third the size of CCP, and similar attempts to locate the cytochrome c molecules failed. The program PROLSQ (*35*) was then used to refine the CCP partial structure, and $F_o - F_c$, α_c, difference maps revealed clear additional electron density corresponding to two cytochrome c molecules in the asymmetric unit. An anomalous difference Fourier map, in which only the two CCP molecules had been used to calculate the phases, confirmed that the highest two peaks on the $F_o - F_c$ map did correspond to two iron atoms—those of the cytochrome c hemes. The graphics program TOM, run on an Iris 4D/340 graphics work station, was used to manually position two complete yeast iso-1-cytochrome c molecules. Their placement was facilitated by obvious electron density for the hemes, and many (but not all) side chains could already be seen at this stage. The full complex structure was then subjected to rigid body refinement with the program XPLOR (*36*),

allowing only the two cytochrome c molecules to move as independent rigid bodies at first, then later allowing all four molecules (two CCP and two cytochrome c) to do so. Final refinement was carried out with PROLSQ accompanied by periodic manual intervention and placement of water molecules. All reflections from the maximum resolution (Table 1) to infinity were included in all refinement cycles, and a solvent correction was applied according to Bolin *et al.* (*37*) to allow the best fit between calculated and observed low-order reflections. Although no restraints were applied to maintain the noncrystallographic twofold axis relating the two copies of the complex in the asymmetric unit, this pseudosymmetry was well preserved during refinement and the two complex structures remained virtually identical.

The structure solution for the CCP:cc(H) complex was carried out in exactly the same manner as described above for the CCP:cc(Y) complex with one important exception: despite the successful positioning of two CCP molecules with MERLOT, only one cytochrome c could be oriented unambiguously in the asymmetric unit with the aid of $F_o - F_c$ maps. Another distinction between the CCP:cc(H) and CCP:cc(Y) structures is that the two CCP molecules in the CCP:cc(H) asymmetric unit are not related by a noncrystallographic twofold axis. Once again, the highest peak on the difference map corresponded to the cytochrome c heme iron, which was confirmed, as before, by an anomalous difference Fourier map. After refinement, some residual $F_o - F_c$ density remained in the open space between the one cytochrome c and two CCP molecules. This density was attributed to a partially occupied, disordered second cytochrome c molecule. A partially occupied second cytochrome c site would explain the odd ratio of two molecules of CCP for every one and a half molecules of cytochrome c observed in the CCP:cc(H) crystals. Extensive use of $F_o - F_c$ omit maps confirmed the positions of every amino acid side chain that we specifically describe.

The CCP:cc(Y) structure. An α-carbon representation of the refined crystal structure of the complex between yeast cytochrome c peroxidase and yeast iso-1-cytochrome c is shown in Fig. 1A. Inspection of the complex interface (Fig. 1C) reveals that the closest approach distance is a weak intermolecular hydrogen bond (3.3 Å) between Glu^{290} of CCP and Asn^{70} of cytochrome c (*38*). The cytochrome c molecule is positioned so that it could form a few charge-mediated intermolecular hydrogen bonds with CCP if certain side chain adjustments can take place. One potential interaction, for instance, could occur between Lys^{73} of cytochrome c and Glu^{290} of CCP, another between Lys^{87} of cytochrome c and Asp^{34} of CCP. However, the distances between these side chains (Table 2) and their geometries, all of which has been confirmed by omit maps (Fig. 2A), indicate that these hydrogen bonds do not form in this crystal structure. The question as to whether these hydrogen bonds exist in the CCP:cc(Y) structure at lower salt concentrations may be answered by refinement of the 3.0 Å structure of a complex crystal grown at 50 mM NaCl as opposed to 150 mM NaCl (*32*). As discussed above, isomorphous crystals of the CCP:cc(Y) complex could be obtained under salt concentrations ranging as low as 5 mM NaCl, but crystals grown at less than 50 mM NaCl were not large enough for high-resolution x-ray diffraction studies.

With only one weak intermolecular hydrogen bond evident, it appears that hydrophobic and van der Waals interactions are the predominant forces holding the CCP:cc(Y) complex together under these ionic strength conditions. In contrast is the formation, in the CCP:cc(H) complex, of three intermolecular hydrogen bonds, two of which are charge-mediated (Fig. 1D and Table 3). Crystals of the CCP:cc(H) complex were grown at a much lower salt concentration, and therefore, stronger charge-charge interactions between CCP and cytochrome c may be expected for this complex structure. However, we caution against thinking of our CCP:cc(Y) structure as the "high salt" complex and our CCP:cc(H) structure as the "low salt" complex. As discussed above, the kinetic behavior of the CCP:cc(Y) complex under varying ionic strength conditions differs from that of the CCP:cc(H) complex. As a result, we view the study of the CCP:cc complexes as a puzzle consisting of four distinct pieces: the CCP:cc(Y) complex under (1) low salt and (2) high salt conditions, and the CCP:cc(H) complex under (3) low salt and (4) high salt conditions. We have not yet obtained crystals of the CCP:cc(H) complex under high salt condi-

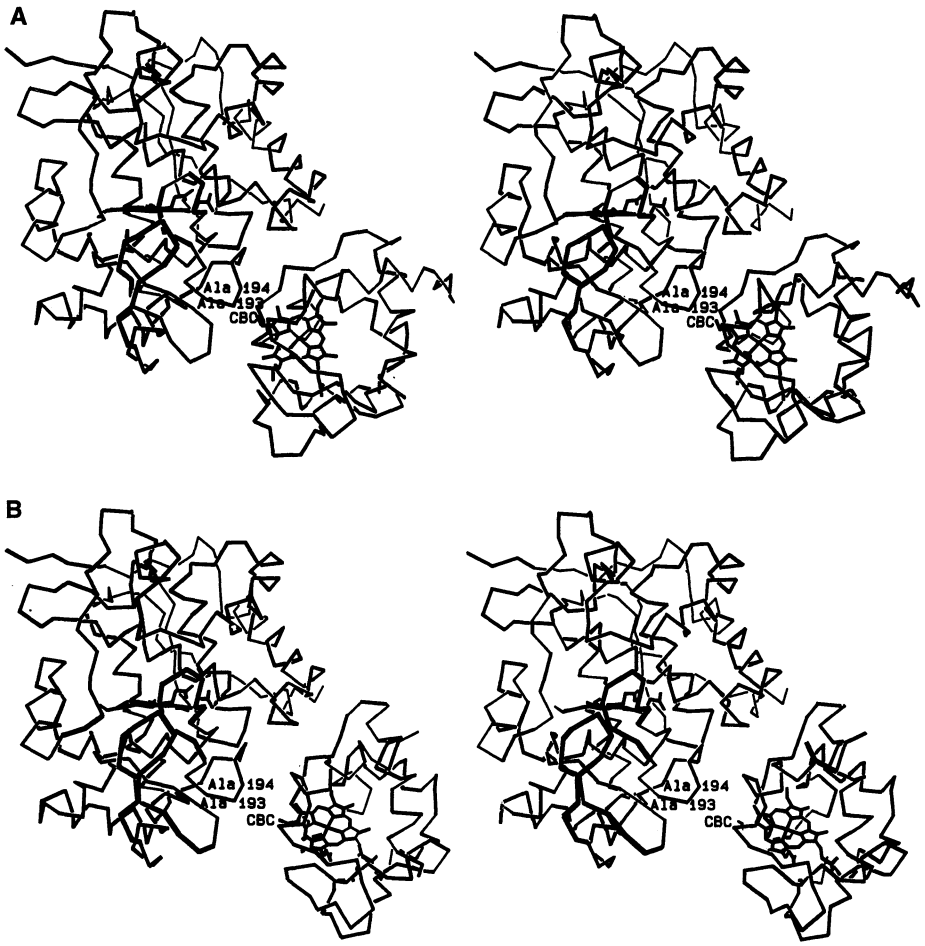
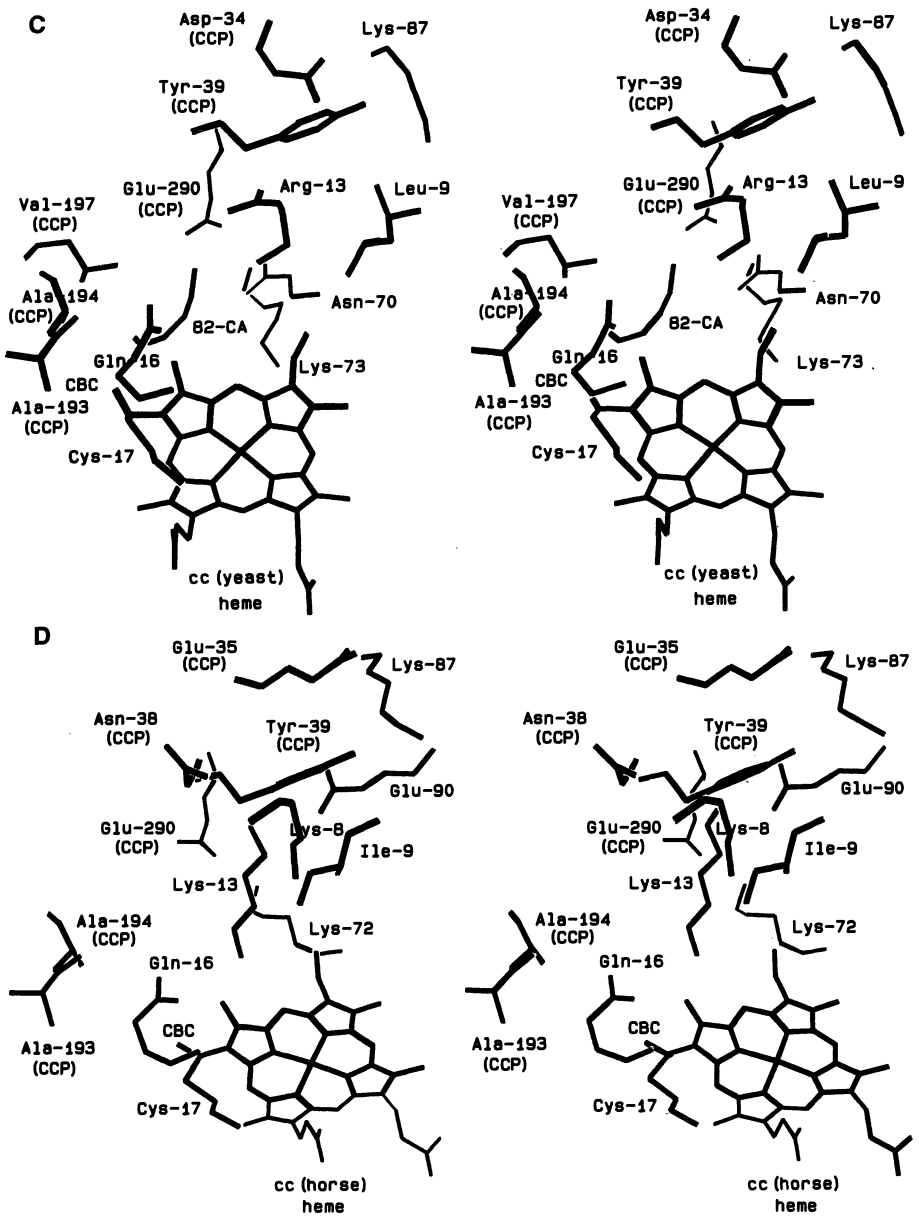


Fig. 1. α-Carbon representations of (above) (**A**) CCP:cc(Y) and (**B**) CCP:cc(H) complex structures, and (facing page) (**C** and **D**) close-up views of their interfacial regions from the same perspective. The other molecules in the asymmetric unit [one full complex for CCP:cc(Y) and a second CCP molecule for CCP:cc(H)] are not shown. In (C and D) all residues not specifically marked (CCP) belong to cytochrome c. In (C), Val^{197} of CCP is in van der Waals contact with main chain segment 81-N to 83-O of yeast cytochrome c but only the 82-CA (the α-carbon of Phe^{82}) is labeled.



tions, and therefore, piece 4, mentioned above, is still missing from our puzzle.

Especially noteworthy in the CCP:cc(Y) structure is the short chain of amino acid residues in CCP, Trp^{191}, Gly^{192}, Ala^{193}, Ala^{194}, which appears to connect the cytochrome c heme with the CCP heme (Fig. 3A). The peroxidase heme is in van der Waals contact with the indole ring of Trp^{191}, which is in turn the site of an intermediate radical (*39–41*). There are two lines of kinetic evidence supporting the involvement of Trp^{191} in electron transfer. One is that when Trp^{191} is replaced by phenylalanine the steady-state rate of the CCP catalyzed reaction decreases from about 1000 s^{-1} to about 1 s^{-1}, while the intracomplex electron transfer rate becomes so small as to be unobservable on the 1-second time scale of the experiment (*42*). The other is the finding by Millett and co-workers that an electron is transferred directly to the radical site in CCP complexes with both yeast (*23*) and horse heart (*24*) cytochromes c.

The shortest through-bond pathway from the Trp^{191} indole ring to the surface of the CCP molecule follows the backbone of the chain segment Trp^{191}, Gly^{192}, and Ala^{193}. We suspect that this may well be the major pathway for electron transfer.

The other end of this heme-connecting chain segment offers an intriguing and surprising result; a short hydrophobic loop consisting of only two CCP residues, Ala^{193} and Ala^{194}, serves as a docking site for the exposed methyl group, CBC, of the cytochrome c heme (*43*). The peptide bond between Ala^{193} and Ala^{194}, with its conjugated pi orbital pointing directly at the CBC methyl group, may also serve some function in the electron transfer event. We do not know whether an electron is transferred specifically from the CBC methyl group. Another possibility, for instance, may be direct electron transfer from the thioether linkage (between Cys^{14} and CAC of the cytochrome c heme) to the methyl group of Ala^{193} (Table 2). In any case, any specific electron transfer suggested by this structure would logically include the exposed corner (pyrrole ring C) of the cytochrome c heme.

Other experimental results have implicated pyrrole ring C of the cytochrome c heme as participating in electron transfer. In proton nuclear magnetic resonance (NMR) studies of cytochromes c and their complexes with CCP, for instance, one of the largest complex-induced resonance shifts occurs at the heme methyl group CMC resonance (*44*). Although apparently not directly in contact with CCP in our structure, CMC is the other methyl group attached to pyrrole ring C of the cytochrome c heme.

Also supporting the idea that pyrrole ring C takes part in electron transfer is the crystal structure of *p*-cresol methylhydroxylase (PCMH) (*45*). PCMH is a bacterial flavocytochrome composed of two subunits; one subunit binds a flavin cofactor, FAD, and the other resembles a bacterial subclass of cytochrome c. PCMH is the only other interprotein electron transfer complex involving a c-type cytochrome for which a crystal structure is known. Because of its proximity to the flavin cofactor, pyrrole ring C of the cytochrome heme is once again implicated as taking part in electron transfer. This is a significant similarity to our structure since the cytochrome subunit in PCMH does not possess a characteristic preponderance of positively charged residues encircling the heme crevice (*45*). Other comparisons, such as whether pyrrole ring C of the cytochrome heme similarly interacts with an Ala-Ala segment of the flavin subunit, cannot be made because the flavin subunit of the PCMH structure is not highly resolved.

Perhaps also of significance in comparing our complex with the structure of PCMH is the relative orientation of their respective redox centers. Most heme-to-heme or flavin-to-heme electron transfer models to date have the planes of these prosthetic groups aligned in a coplanar fashion. Thus, it was not surprising when the crystal structure of yet another flavocytochrome, flavocytochrome b_2, revealed that the isoalloxazine ring of the FMN-containing subunit is nearly coplanar with the b-type heme of the cytochrome subunit (*46*). In PCMH, however, the flavin and the heme planes are not coplanar, but instead make an angle of about 65° to each

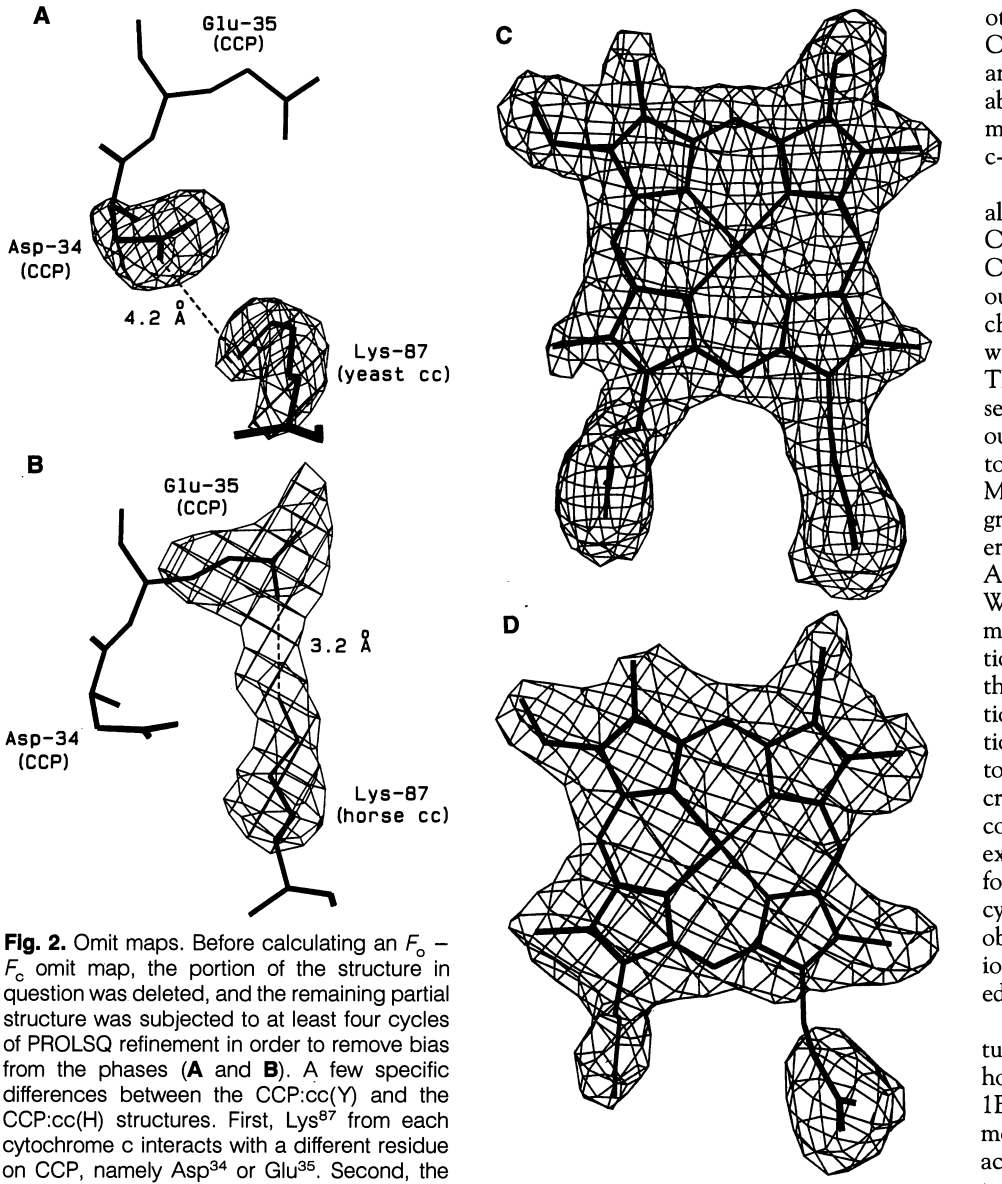


Fig. 2. Omit maps. Before calculating an $F_o - F_c$ omit map, the portion of the structure in question was deleted, and the remaining partial structure was subjected to at least four cycles of PROLSQ refinement in order to remove bias from the phases (**A** and **B**). A few specific differences between the CCP:cc(Y) and the CCP:cc(H) structures. First, Lys^{87} from each cytochrome c interacts with a different residue on CCP, namely Asp^{34} or Glu^{35}. Second, the omit map in (B) reveals a clear intermolecular hydrogen bond in the CCP:cc(H) structure that is not seen in the CCP:cc(Y) structure (A). The average temperature factor for the Lys^{87} side chain was 48 Å^2 and 82 Å^2 for the yeast and the horse structures, respectively. The average temperature factors for the yeast and the horse cytochrome c hemes (**C** and **D**) were 26 and 49 Å^2, respectively.

other (*45*). In the structure of the CCP:cc(Y) complex the two heme planes are also not coplanar, making an angle of about 60° to each other. Could this be a magic angle for electron transfer involving c-type cytochromes?

There were no significant conformational changes for the CCP molecule in our CCP:cc(Y) complex as compared to free CCP (*29*). The yeast cytochrome c part of our complex, in contrast, has its Gln^{16} side chain folded back to form a hydrogen bond with its own backbone amide nitrogen. This is the only significant difference observed between the yeast cytochrome c in our complex structure and yeast cytochrome c solved by Louie *et al.* (*8*). Movement of the Gln^{16} side chain allows greater access to pyrrole ring C, since otherwise the side chain would overlap the Ala^{193}, Ala^{194} loop of CCP in the complex. We believe, however, that this difference may not be a response to complex formation, but rather a consequence of growing the crystals at physiological salt concentrations or lower. A similar Gln^{16} conformation difference is seen in the horse cytochrome c of the CCP:cc(H) complex, crystallized under very low ionic strength conditions, even though in this case an extended Gln^{16} would not affect complex formation in any way. All mitochondrial cytochrome c crystal structures to date were obtained with crystals grown at very high ionic strengths, usually from nearly saturated ammonium sulfate.

The CCP:cc(H) structure. The structure of the complex between CCP and horse heart cytochrome c is shown in Fig. 1B. As already mentioned, a second CCP molecule in the asymmetric unit is not accompanied by an ordered, bound cytochrome c. Our best approximation places the second cytochrome c near the carboxyl terminus (Leu^{294}) of the second CCP. However, this is probably not a biologically

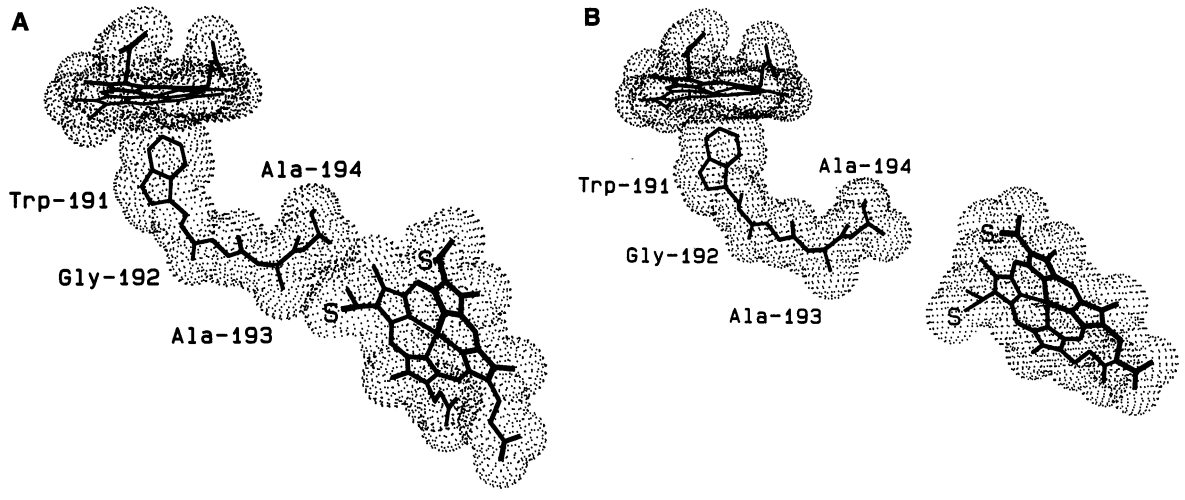


Fig. 3. The proposed electron transfer pathway, including van der Waals surfaces, from the cytochrome c heme, shown on the right of each diagram, to the CCP heme. (**A** and **B**) Representations of the CCP:cc(Y) and the CCP:cc(H) complexes, respectively. The cysteine sulfur atoms (S), covalently bonded to the cytochrome c hemes, are included. The CCP heme propionates point outward toward the viewer.

significant second binding site for cytochrome c as the distance from Leu^{294} to the CCP heme is about 25 Å. If we look at the second CCP molecule, we see that negatively charged residues Asp^{34}, Glu^{35}, and Glu^{290} interact with positively charged lysine side chains on the backside of the first CCP molecule. All three of these CCP residues take part, to some extent, in charge-charge interactions with cytochrome c lysine residues in either the CCP:cc(Y) complex [where Asp^{34} and Glu^{290} are poised to form intermolecular hydrogen bonds (Table 2)] or in the CCP:cc(H) complex [where Glu^{35} and Glu^{290} form definite intermolecular hydrogen bonds (Table 3)]. Therefore, in a way, this accidental CCP:CCP interface in the asymmetric unit resembles the CCP:cc interface.

The most noticeable distinction between the CCP:cc(H) and the CCP:cc(Y) complexes is a slight relative rotation-translation of the cytochrome c molecule (Fig. 1). Considering that they exhibit such different kinetic behavior in electron transfer reactions with CCP (*23*, *24*) it is not surprising that horse and yeast cytochromes c should bind to CCP somewhat differently. One reason may simply be that the CCP:cc(H) crystal was grown under conditions approaching zero ionic strength, whereas the CCP:cc(Y) crystal was grown in the presence of 150 mM NaCl. However, because crystals of the CCP:cc(Y) complex remain isomorphous throughout a large range of salt concentration, to as low as 5 mM NaCl, the differences in the CCP:cc(H) and CCP:cc(Y) structures are most likely due mainly to species-specific amino acid differences at the complex interface. For instance, Lys^{72} of horse cytochrome c forms a charge-charge hydrogen bond with Glu^{290} of CCP (Fig. 1D and Table 3). In yeast cytochrome c, however, Lys^{72} is trimethylated and therefore incapable of forming a hydrogen bond. Although we are not certain how much of an effect this has on the interaction between yeast cytochrome c and CCP, the fact remains that Lys^{73}, not Lys^{72}, is poised to form an intermolecular hydrogen bond with Glu^{290} in the CCP:cc(Y) structure (Fig. 1C and Table 2). Possibly another important difference is that Lys^{13} of horse cytochrome c, which is buried in the complex interface, forms an intramolecular hydrogen bond with Glu^{90} (Fig. 1D). In yeast cytochrome c residues number 13 and 90 are arginine and aspartic acid, respectively, and there is no intramolecular hydrogen bond between them.

If the CCP:cc(H) structure had been the only structure that we solved, we would have found it difficult to argue for an electron transfer pathway like that proposed for the CCP:cc(Y) complex. The closest approach between the heme methyl group CBC of horse heart cytochrome c and the Ala^{193}, Ala^{194} loop of CCP is about 7 Å (Fig. 3B and Table 3). However, the intermolecular hydrogen bonds in the CCP:cc(H) structure are clustered in one area, namely the top left-half of the cytochrome c molecule as viewed in Fig. 1B. Moreover, two out of three of these hydrogen bonds involve charge-charge interactions and may therefore be susceptible to weakening at higher ionic strength. Thus, with increasing salt concentration these hydrogen bonds may break, allowing the cytochrome c molecule to rotate slightly and bring its bottom half, where the heme is located, closer to the Ala^{193}, Ala^{194} loop of CCP. The existence of a second, slightly different binding geometry which can more readily accommodate electron transfer agrees well with kinetic studies showing biphasic behavior in rates of electron trans-

Table 1. Data collection and refinement statistics. X-ray diffraction data were collected on a multiwire area detector (San Diego Multiwire Systems) with monochromatized CuKα radiation (Rigaku rotating anode x-ray generator). During refinement an overall B factor was applied to the entire complex structure until the R factor had dropped below 30 percent. At that point, individual isotropic B factors were refined along with the positional parameters for each atom. The horse heart cytochrome c molecule in the refined CCP:cc(H) structure has many relatively high individual B factors, particularly the bottom right portion of the horse cytochrome c molecule in (Fig. 1B), which consists of residues 38 to 62. The individual B factors in this chain segment are 90 $Å^2$ or higher. However, this part of the molecule lies opposite the intermolecular contact with CCP, so it is expected to have a higher degree of disorder. The lowest B factors for the cytochrome c molecules averaged 26 $Å^2$ and 49 $Å^2$ for the yeast and horse hemes, respectively; the highest averaged 120 $Å^2$ (that is, essentially completely disordered) for some of the COOH-terminal and NH_2-terminal side chains in both cytochrome c structures. Regardless of temperature factors, all atoms were included in both complex models during the refinement process.

Complex	Data collection				R_{sym}†	Refinement			
	Maximum resolution (Å)	⟨1/σ⟩*	Unique reflections	Completeness (%)		Final *R*‡	Non-hydrogen atoms§	rms¶	
								Bond	Angle
CCP:cc(Y)	2.3	3.2	37,845	93	0.044	0.172	7088	0.021	2.3°
CCP:cc(H)	2.8	2.2	24,671	93	0.055	0.179	6012	0.020	2.7°

*⟨I/σ⟩ is the average ratio of observed intensity to sigma at the maximum resolution. †$R_{sym} = (\Sigma|I_{obs} - I_{avg}|/\Sigma I_{avg})$. ‡Final $R = (\Sigma||F_o| - |F_c||/\Sigma|F_o|)$, including all data. §The number of non-hydrogen atoms includes 489 and 332 water oxygens for the CCP:cc(Y) and the CCP:cc(H) asymmetric units, respectively. Only those water molecules that could potentially form good hydrogen bonds with nearby protein atoms or with other water molecules were modeled into the structure, and all water oxygens having individual isotropic B factors of more than 100 $Å^2$ were periodically removed from the atomic coordinate file during refinement. ¶The root-mean-square (rms) (bond) and rms (angle) are the rms deviations of the bond lengths and bond angles in the final structures from the ideal values.

Table 2. Intermolecular distances of interest in the CCP:cc(Y) structure, including all intermolecular contacts of 4 Å or less. Only the average of two distances measured for both complexes in the asymmetric unit is listed.

CCP	Yeast iso-1-cytochrome c	Distance (Å)
Cytochrome c heme and surrounding area		
Ala^{193}, CB	Heme, CBC	4.1
Ala^{193}, C	Heme, CBC	4.2
Ala^{194}, N	Heme, CBC	4.0
Ala^{194}, CB	Heme, CBC	4.0
Ala^{193}, CB	Cys^{17}, SG	3.9
Ala^{193}, O	Gln^{16}, NE2	3.6
Ala^{193}, O	Gln^{16}, CG	3.7
Heme, Fe	Heme, Fe	26.5
Potential hydrogen bonds		
Asp^{34}, OD1	Lys^{87}, NZ	4.2
Glu^{290}, OE1	Asn^{70}, ND2	3.3
Glu^{290}, OE1	Lys^{73}, NZ	4.4
Van der Waals interactions		
Arg^{31}, NH2	Lys^{86}, CG	3.4
Tyr^{39}, CZ	Leu^{9}, CD1	3.8
Tyr^{39}, CB	Arg^{13}, CD	3.7
Val^{197}, CG1	Ala^{81}, O	3.7
Val^{197}, CG1	Ala^{81}, C	3.8
Val^{197}, CG1	Phe^{82}, N	3.9
Val^{197}, CG1	Phe^{82}, CA	3.9
Val^{197}, CG1	Phe^{82}, C	3.7
Val^{197}, CG1	Gly^{83}, N	3.7
Gln^{120}, NE2	Gly^{83}, CA	3.9

Table 3. Intermolecular distances of interest in the CCP:cc(H) structure, including all intermolecular contacts of 4 Å or less.

CCP	Horse heart cytochrome c	Distance (Å)
Cytochrome c heme and surrounding area		
Ala^{193}, CB	Heme, CBC	9.7
Ala^{194}, CB	Heme, CBC	7.0
Heme, Fe	Heme, Fe	30.0
Potential hydrogen bonds		
Glu^{35}, OE2	Lys^{87}, NZ	3.2
Asn^{38}, OD1	Lys^{8}, NZ	2.9
Glu^{290}, OE1	Lys^{72}, NZ	3.3
Van der Waals interactions		
Tyr^{39}, CZ	Ile^{9}, CD1	3.8
Tyr^{39}, CZ	Ile^{9}, CG1	3.5
Asp^{34}, OD2	Glu^{90}, OE2	—*

*See (*48*).

fer between horse heart cytochrome c and CCP (*24*). Biphasic kinetics were not observed in similar experiments with the CCP:cc(Y) complex (*23*). Our CCP:cc(H) structure most likely resembles the binding geometry responsible for the biphasic kinetics observed in the CCP:cc(H) experiments. In agreement with the idea that the cytochrome c heme can make better contact with the Ala^{193}, Ala^{194} loop at higher ionic strength, the rate constant of the slow phase increases with increasing ionic strength, despite weaker binding between CCP and horse cytochrome c (*24*).

Just as in the case of the CCP:cc(Y) complex, CCP does not undergo any large conformational changes in the CCP:cc(H) complex. But while the yeast cytochrome c in the CCP:cc(Y) structure shows only one significant side chain conformational change (at Gln^{16}), there are four or five such changes in the case of horse heart cytochrome c. For instance, as compared with the uncomplexed horse heart cytochrome c structure solved by Bushnell *et al.* (*7*), the side chains of both Lys^{13} and Glu^{90} have moved significantly in order to form the strong intramolecular hydrogen bond (2.8 Å) mentioned earlier. This hydrogen bond does not exist in the previously reported structure (*7*). We argue that these changes are probably not complex-induced but are due to the fact that the complex crystals were grown at near-zero ionic strength as opposed to near-saturated ammonium sulfate. This idea is supported by recent resonance Raman studies showing that neither horse heart cytochrome c nor yeast cytochrome c undergoes significant conformational changes upon complex formation with CCP (*47*).

Although horse heart cytochrome c is not the physiological redox partner of yeast CCP, the CCP:cc(H) complex may nevertheless serve as a good model, in some respects, for the complex between mammalian cytochrome c and mammalian cytochrome oxidase or reductase. Of special interest in this regard are the chemical modification studies of Margoliash and coworkers (*49*), which revealed that lysines 8, 13, 72, and 87 of horse heart cytochrome c are involved in complex formation, to varying degrees, with cytochrome oxidase. In our CCP:cc(H) complex structure, three out of four of these residues make intermolecular hydrogen bonds with CCP, and the fourth, Lys^{13}, is buried within the complex interface. In fact, if we were to draw a schematic map of the interfacial residues of horse heart cytochrome c based solely on our complex structure, it would not look much different from [figure 1 in (*49*)]—published nearly 15 years ago as the inferred binding surface of horse heart cytochrome c in its complex with cytochrome oxidase. Oddly enough, a slightly different cytochrome c binding surface was deduced by Margoliash and coworkers for the CCP:cc(H) complex [figure 7 in (*50*)], and it does not agree quite as well with our CCP:cc(H) complex structure. As suggested by those investigators, the difference may simply be due to the fact that different kinetic parameters were employed for the two enzymes to estimate the inhibitory effects of the cytochrome c modifications—V_{max} for the peroxidase versus K_m for the oxidase. Perhaps if our CCP:cc(H) crystals could be grown at slightly higher salt concentrations [for example, 20 mM, the concentration of phosphate buffer at which the reaction between CCP and cytochrome c reaches the maximum velocity, V_{max}, according to Margoliash (*50*)], the CCP:cc(H) interface we see could differ in a way that would allow the cytochrome c heme better access to the Ala^{193}, Ala^{194} loop of CCP, as proposed earlier, and the agreement with [figure 7 in (*50*)] should be improved.

Single-site binding rather than multiple-site binding. We have described the crystal structures of cytochrome c peroxidase complexed with two different eukaryotic cytochromes c. Although the CCP:cc(Y) complex structure should receive more consideration than the CCP:cc(H) structure because yeast cytochrome c is the true physiological redox partner of yeast CCP, and the CCP:cc(Y) crystals were grown at physiological salt concentrations, both complexes offer important insights. Our assumption is that, in the case of both CCP:cc(Y) and CCP:cc(H), the most stable complex between CCP and cytochrome c in solution was the one which crystallized. The question of whether this complex represents the geometry required for successful electron transfer is then open to debate. However, we have pointed to already existing evidence supporting two main ideas: (i) our CCP:cc(Y) structure does, indeed, represent a true electron transfer complex and (ii) our CCP:cc(H) structure, though very similar to the CCP:cc(Y) structure, represents a geometry which is not optimal for electron transfer. Furthermore, evidence also exists supporting our proposal that, under higher ionic strength conditions, the binding geometry for CCP:cc(H) changes to form an optimal electron transfer complex, one which may more closely resemble the CCP:cc(Y) structure.

A question arises as to whether there are other possible geometric arrangements of these redox partners that could also lead to successful electron transfer events—perhaps a great many others. Opinion on this question has varied. Some experimental results have been presented in favor of the idea that CCP and cytochrome c form a highly specific 1:1 electron transfer complex (*51–53*). However, results from Brownian dynamics simulations (*54*) and from kinetic studies with metal-substituted CCP (*55*) have led to arguments in favor of the existence of many electron transfer–competent binding sites for cytochrome c on CCP (*56*).

We believe that a highly specific electron transfer complex is formed between CCP and cytochrome c, and that the slight differences which exist between our CCP:cc(Y) and CCP:cc(H) structures cannot properly be considered as evidence that CCP possesses multiple binding sites for cytochrome c. Although the cytochrome c of one species may bind to CCP in a slightly different orientation from the cytochrome c of another species, this does not necessarily mean that one particular cytochrome c binds in many different orientations. Also, in the case of CCP:cc(H), we suggest that a highly specific 1:1 complex exists under high salt conditions as well as under low salt conditions. The fact that these two complexes (high salt and low salt) probably differ slightly does not, in our opinion, constitute "multiple binding."

Ironically, a commonly cited argument in favor of the multiple binding site model is that the cytochrome c molecules have been reported to be totally disordered in a previous x-ray study on the CCP:cc(H) crystals (*30*, *56*). By the same line of reasoning, our crystal structure solutions of two geometrically similar complexes of CCP with cytochromes from yeast or horse provide additional evidence that a highly specific 1:1 complex is formed between CCP and cytochrome c.

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22. The final protein concentrations of all complex samples were estimated from the total amount of CCP measured initially (before the addition of an appropriate amount of cytochrome c) and the final volume of the complex sample after washing in the microconcentrator. Usually, the protein samples had to be diluted with an estimated amount of washing solution to obtain the desired final concentration. Analysis of the eluants collected during washing and ultrafiltration indicated that no detectable amounts of CCP or cytochrome c were lost through the membrane of the microconcentrator.
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29. It was quite common for CCP crystals to grow instead of or alongside the desired CCP:cc(Y) crystals throughout the crystallization experiments. The 2.2 Å structure of a CCP crystal which grew under the same crystallization conditions as the CCP:cc(Y) crystals was solved in order to make an accurate comparison of complexed versus uncomplexed CCP. This new CCP structure belongs to the same space group as previously solved CCP structures (*9, 10*), but it has different unit cell parameters and it has a different crystal packing pattern.
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