Definition of the Interaction Domain for Cytochrome c on Cytochrome c Oxidase

I. BIOCHEMICAL, SPECTRAL, AND KINETIC CHARACTERIZATION OF SURFACE MUTANTS IN SUBUNIT II OF RHODOBACTER SPHAEROIDES CYTOCHROME aa₃

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To determine the interaction site for cytochrome c (Cc) on cytochrome c oxidase (CcO), a number of conserved carboxylic residues in subunit II of Rhodobacter sphaeroides CcO were mutated to neutral forms. A highly conserved tryptophan, Trp143, was also mutated to phenylalanine and alanine. Spectroscopic and metal analyses of the surface carboxyl mutants revealed no overall structural changes. The double mutants D188Q/E152N and D195N/E152N exhibit similar steady-state kinetic behavior as wild-type oxidase with horse Cc and R. sphaeroides CcO, showing that these residues are not involved in Cc binding. The single mutants E148Q, E157Q, Asp214N, and Glu254N have decreased activities and increased Km values, indicating they contribute to the CcCcO interface. However, their reactions with horse and R. sphaeroides Cc are different, as expected from the different distribution of surface lysines on these cytochromes c. Mutations at Trp143 severely inhibit activity without changing the Km for Cc or disturbing the adjacent Cu₄ center. From these data, we identify a Cc binding area on CcO with Trp143 and Asp214 close to the electron acceptor from Cc (8–10), and with the crystal structure of bovine CcO subunit II, Asp112 (Asp151 in R. sphaeroides CcO), Glu198 (Glu254 in R. sphaeroides), and Glu198 (Glu254 in R. sphaeroides), were found to be protected from modification by Cc binding. Cross-linking studies also identified a specific cross-link between Lys13 of horse Cc and His162 (His217 in R. sphaeroides), a Cu₄ ligand in the same region (7). The general location identified by these modifications is consistent with time-resolved electron transfer kinetic studies, which show that Cu₄ in this domain is the initial electron acceptor from Cc (8–10), and with the crystal structures of CcO from both bovine and Paracoccus (11–13), since both reveal a concentration of negatively charged residues near the Cu₄ site in subunit II.

Now, with the availability of bacterial CcO with a high degree of homology to the mammalian enzyme, a more incisive analysis of the precise binding domain is possible. Using Paracoccus denitrificans CcO, site-directed mutagenesis studies have implicated several residues in subunit II, Glu142, Glu148, Glu157, Asp195, Asp214, and Glu254 (R. sphaeroides numbering), and one residue in subunit I (Asp257, not conserved in R. sphaeroides) as important contributors (14–16).

In some of the studies mentioned above (5, 14, 15), Glu254 and Asp259 were identified as part of the interaction domain with Cc. However, the subsequently published crystal structures clearly show that both these residues are buried inside the protein as ligands of Cu₄ and magnesium (11, 12). These results highlight the long-standing difficulty in unambiguously interpreting the complex kinetics of the CcO reaction with Cc (17) and the importance of applying a variety of methods to a variety of systems to obtain a clear understanding.

Here we report our studies on Cc interaction with R. sphaeroides CcO using mutational, biochemical, spectral, and kinetic techniques. A number of conserved carboxyl residues in complex IV (CcO). Picking up and delivering electrons requires Cc to rapidly and specifically dock with at least two different proteins in the concentrated protein milieu of the intermembrane space of the mitochondrion. In addition to these demanding docking requirements, a newly discovered role for Cc in signaling cell death in the cytoplasm (1) emphasizes the importance of defining the chemistry of its interactions with diverse biological partners.

Electron transfer between Cc and CcO is thought to involve the formation of one or more CcCcO complexes guided by electrostatic interactions. However, the nature, number, kinetic characteristics, and physiological significance of the complex(es) have yet to be established. Extensive chemical modification studies identified a group of lysine residues on the surface of Cc that is responsible for its interaction with CcO (2, 3). The region on CcO that provides the Cc docking site has also been investigated. In chemical modification/protection and monoclonal antibody studies (4–6), several carboxyls in the soluble domain of bovine CcO subunit II, Asp112 (Asp151 in Rhodobacter sphaeroides), Glu198 (Glu254 in R. sphaeroides), and Glu198 (Glu254 in R. sphaeroides), were found to be protected from modification by Cc binding. Cross-linking studies also identified a specific cross-link between Lys13 of horse Cc and His162 (His217 in R. sphaeroides), a Cu₄ ligand in the same region (7). The general location identified by these modifications is consistent with time-resolved electron transfer kinetic studies, which show that Cu₄ in this domain is the initial electron acceptor from Cc (8–10), and with the crystal structures of CcO from both bovine and Paracoccus (11–13), since both reveal a concentration of negatively charged residues near the Cu₄ site in subunit II.

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the subunit II soluble domain of CcO from \( R. \) \( sphaeroides \) have been mutated to neutral forms. These residues include the highly conserved Glu^{148} \( \) Asp^{151}, Glu^{152}, Glu^{157}, Asp^{195}, Asp^{214}, Asp^{229}, and Glu^{254}. A pair of residues, Asp^{188}/Glu^{189}, which are only present in an extra loop in \( R. \) \( sphaeroides \) and \( P. \) \( denitrificans \), have also been mutated. Besides these carboxyl residues, a stretch of aromatic residues is also conserved in the subunit II C-terminal domain of CcO and even in quinol oxidases (18). Among these residues, Trp \(^{443} \) has been mutated to phenylalanine and alanine. Extensive analysis of these mutants reported in this and the accompanying paper (19) defines the CcCc protein interface and identifies the importance of its chemistry in controlling electron transfer within it.

**EXPERIMENTAL PROCEDURES**

**Materials**—Horse heart Cc (Sigma type VI) was purchased from Sigma and was purified on a carboxymethyl-cellulose column before use (20). All of the mutated oxidases were constructed using the mutagenesis systems described (21). The mutated oxidases were overexpressed and purified using nickel-nitrilotriacetic acid affinity column chromatography as described by Zhen et al. (22).

**Purification of \( R. \) \( sphaeroides \) Cc**—\( R. \) \( sphaeroides \) Cc or Cc2 after two runs on DEAE-cellulose column was washed with 20 mM Tris, pH 7.0, 25 mM NaCl, and Cc2 was eluted with 50 mM NaCl in the same buffer. The purity of Cc2 can be improved by running the sample through a second DEAE-cellulose column. The purified reduced Cc2 overexpression strain, pC2P404.1 (23).

**Electron Paramagnetic Resonance (EPR) Spectroscopy**—Measurements were performed as described by Hosler et al. (26), with the exception that the samples were in 10 mM Tris, 40 mM KCl, pH 8.0, 0.1% LM. The sample preparations for high magnesium/low manganese content, or low magnesium/high manganese content oxidases were as described by Hosler et al. (27). The EPR spectra were recorded using a Bruker EP300E spectrometer.

**Metal Analyses**—Metal analyses were done using inductively coupled plasma emission spectroscopy (ICP) at the chemical analysis laboratory in the University of Georgia and total reflection x-ray fluorescence spectrometry (TXRF) at the physics department of the University of Göteborg, Sweden. The sample concentrations were in the range of 30–70 μM.

**Results**

**Design and Purification of Mutants**

To test the involvement of carboxyl residues in subunit II of CcO in Cc binding, these acidic residues were mutated to their corresponding neutral amide forms, creating E148Q, D151N/E157Q, D151E, D156E, D156N, D188N/E189Q, D195N, D214N, and D229N. The locations of these residues in subunit II of CcO are shown in Fig. 1. In E254A, the residue was changed to alanine. Although Asp^{188} and Glu^{189} are not conserved, they have been suggested to be involved in Cc binding (28). In the subunit II soluble domain, there is a set of highly conserved aromatic residues (Trp^{143}, Tyr^{147}) that were originally postulated to play a role in electron transfer from Cu_{A} to heme a (29). Trp^{143}, shown in the crystal structures (11, 13) to be in close proximity to the Cu_{A} site, has been implicated in transferring electrons from Cc to Cu_{A} (30). In this study, Trp^{143} was mutated to phenylalanine and alanine, creating W143F and W143A, respectively. Another mutant, Y144A/W145A, was created as well.
The backbone of subunit II is shown as a ribbon with all of the residues involved in this study and the Cu A ligands highlighted. The copper (green) and magnesium (red) atoms are shown as spheres. Coordinates are from the P. denitrificans structure (1ar1pdb) with the numbering from R. sphaeroides CcO.

All of the mutants were successfully overexpressed using the system previously described (22). The expression levels measured by the cytochrome a/b ratio as shown in Zhen et al. (22) ranged from 0.5 to 1.0. These enzymes were purified using nickel-nitrilotriacetic acid affinity column (see “Experimental Procedures”). Some properties of these mutants are summarized in Table I. Among these mutants, Y144A/W145A has a very low expression level in the cell membrane with a cytochrome a/b ratio of 0.15 and a significantly shifted a-peak in the visible spectrum, indicating structural alteration of the heme environments in this mutant. Such widespread structural changes make it difficult to interpret any localized effects of removing the aromatic side chains, and this double mutant was not further analyzed.

Although previously implicated in Cc binding (5, 15), residues Glu254 and Asp229 are revealed in the x-ray structures to be internally located forming ligands to Cuα or magnesium or both, so they are unlikely candidates for Cc binding. Indeed, D229N and E254A are slightly altered in their EPR spectra of the Cuα site (Fig. 2) and cause loss of the magnesium/manganese site (Fig. 3). Further, due to destabilization of the subunit I/II interface, the nickel-nitrilotriacetic acid-purified forms of these two internal carboxyl mutants contain a significant amount of subunit I alone, giving rise to the altered heme spectra. Such widespread structural alterations again make it difficult to draw any specific conclusions. Previous mutagenesis (14, 15) studies concluding that these residues contribute to the Cc interaction can probably be explained by these secondary effects on structure. In addition, chemical modification/protection evidence (5) for their role is probably due to their location at the water-containing subunit I/II interface.

This paper will focus on the characterization of all of the surface carboxyl mutants, including E148Q, D151N/E152Q, E157Q, D188N/E189Q, D195N, D214N, and the tryptophan mutants W143F and W143A, to determine their structural integrity and measure their functional effects.

**Spectral Characterization**

**Optical Spectra**

In the reduced form, wild-type CcO from R. sphaeroides has characteristic heme absorbances at 445 and 606 nm for the Soret and α-peaks, respectively, with a Soret to α-peak ratio \( (A_{445-499}/A_{606-630}) \) of 5.6. All of the surface carboxyl mutants, W143A, and W143F have these wild-type spectral characteristics with a similar Soret to α-peak ratio (Fig. 4, Table I), indicating that the heme centers in these mutants were not disturbed. In contrast, the two internal mutants, D229N and E254A, display disturbed heme centers, indicated by the blue shift of the visible spectra (Fig. 4).

Carbon monoxide binds to the heme a₃-CuB binuclear center with distinctive and quantifiable spectral characteristics that provide a sensitive probe of the structural integrity of this center. All of the surface carboxyl mutants, W143A, and W143F, have CO-binding properties identical to that of wild-type enzyme (Table I), showing that the binuclear centers in these mutants are intact.

In the oxidized form, bovine CcO has a broad absorption peak at the near-infrared region centered at 830 nm, which has been assigned to Cuα (31). A similar absorption band is also observed in CcO from R. sphaeroides with a similar extinction coefficient of 2.0 m⁻¹ cm⁻¹ for the oxidized minus reduced spectra (Fig. 5) (32). The peak position of R. sphaeroides CcO is at 850 rather than at 830 nm, suggesting the electronic structures of the bovine and R. sphaeroides Cuα centers are slightly different. Nevertheless, the term of “830-nm band” is retained for simplicity. The near-infrared spectra for all of the surface carboxyl mutants, W143A, and W143F are very similar to that of the wild type, indicating that the Cuα centers in these mutants are not altered (Fig. 5). Lower extinction coefficients are measured for D229N and E254A (data not shown), due to structural alterations and the loss of subunit II in a portion of the samples.

**Metal Content**

The metal contents of wild-type and mutant forms of CcO were measured using either ICP or TXRF techniques or using both (Table I). For wild-type oxidase, the copper/iron ratio measured by ICP is 1.51, which is in agreement with the fact that there are three copper and two iron atoms per oxidase molecule resolved by crystal structures. The copper/iron ratio of wild-type oxidase measured by TXRF was slightly lower than the theoretical value of 1.5. However, for all of the surface carboxyl mutants, W143A, and W143F, the copper/iron ratios measured using either technique are comparable with that of the wild-type oxidase, indicating no loss of copper from the Cuα center. Again, the lower copper/iron ratios measured for D229N and E254A are probably due to the loss of subunit II, since the Cuα centers of these two mutants showed only slightly altered EPR spectra (Fig. 2).

**EPR Spectra**

In the X-band EPR spectrum of R. sphaeroides CcO, the signals at \( g = 2.85, 2.31, \) and 1.62 are due to the low spin heme a, and those at \( g = 2.00, 2.19 \) are from Cuα (26). The heme and Cuα EPR spectra of all of the surface carboxyl mutants, W143A, and W143F are similar to that of the wild-type (Fig. 2), in agreement with the optical spectral analysis indicating that the heme and Cuα centers in these mutants were not disturbed. In D229N and E254A, although the amplitude of heme a signals at \( g = 2.85, 2.31, \) and 1.62 positions are decreased, the Cuα spectra are similar to wild type, except for the slightly decreased signals at \( g = 2.19 \), suggesting that the Cuα centers in...
these two mutants are not greatly altered.

In bacterial CcO, the magnesium can be replaced during growth by manganese (27) without changing the structure or function of the enzyme. The distinct EPR hyperfine spectrum of the manganese atom provides a sensitive probe of the integrity of the subunit I/II interface. The manganese EPR spectra of all of the surface carboxyl mutants, W143A, and W143F are similar to that of wild-type (Fig. 3), indicating no structural alteration at the interface in these mutants. In contrast, both D229N and E254A fail to bind manganese, consistent with the fact that both Asp229 and Glu254 are ligands of the magnesium/manganese site (33–35).

The sum total of these spectral analyses shows that there is no global change in the structure of any of the surface mutants of subunit II of cytochrome oxidase. The alteration of activity associated with these mutants can thus be attributed to localized effects of the altered side chains.

Steady-state Kinetics of Reaction with Cc

Comparison of Activities of Mutant Oxidases at High Ionic Strength

The turnover numbers for the wild-type oxidase, measured in 50 mM KH2PO4, pH 6.5, and in the presence of lipid, is 1700 s⁻¹ with horse Cc. Under these conditions, the turnover numbers measured for all of the mutants vary significantly, ranging
All of the enzymes were purified using nickel-nitrilotriacetic acid resin and were reduced by dithionite in 100 mM Tris, 40 mM KCl, pH 8.0, 0.1% LM. The ferricyanide was the fully oxidized oxidases, ferricyanide was added to 300 μM for the oxidized, reduced, and difference (oxidized minus reduced) spectra of wild-type CcO. The enzyme concentrations were normalized in this figure. To prepare the fully oxidized oxidases, ferricyanide was added to 300 μM of oxidase in 100 mM Tris, pH 8.0, 40 mM KCl, 0.1% LM. The ferricyanide was removed by running the samples through Sephadex G-10 spin columns, and the oxidized spectrum was recorded. To record the reduced spectrum, dithionite was added to the cuvette.

![Near-infrared spectra of wild-type and mutant R. sphaeroides CcO.](image)

**Fig. 5.** Near-infrared spectra of wild-type and mutant *R. sphaeroides* CcO. All of the enzymes were purified using nickel-nitrilotriacetic acid resin and were reduced by dithionite in 100 mM Tris, 40 mM KCl, pH 7.0, 0.1% LM.

From 1% to nearly 100% of wild-type activity. Among them, W143A has the lowest activity (Table I), and the more conservative mutation in this position, W143F, is almost equally inhibited. Among the surface carboxyl mutants interacting with horse Cc, D214N has the lowest turnover number, retaining about 24% of the wild-type activity, while the activity of D188N/E189Q is equivalent to that of wild type. E157Q has about one-third and E148Q, D195N, and D151N/E152Q have about two-thirds of the wild-type activity. The varying levels of activities observed for these mutants appear to reflect different contributions to the binding of horse Cc and the nature of the resultant complex. Rates of association/dissociation of Cc, as well as the intrinsic electron transfer rate (19), will contribute to the turnover numbers for the two internal mutants, D229N and E254A, are 400 s⁻¹ and 600 s⁻¹, respectively. These changes cannot be attributed to the same cause, since their metal centers and stability are affected, although their $K_m$ values remain like wild-type (Table II).

The turnovers of the wild type and the mutants have also been measured with 30 μM *R. sphaeroides* Cc, under the same conditions as with horse Cc. The turnover number for wild-type oxidase with Cc, is about 270 s⁻¹, significantly lower than with horse Cc. However, the percentages of wild-type activities retained by D188N/E189Q, D151N/E152Q, E157Q, D229N, E254A, W143A, and W143F, when reacting with Cc, follow a similar pattern of decreased activity as with horse Cc (Table I). E148Q retains a slightly higher percentage of wild-type activity with *R. sphaeroides* Cc, than with horse Cc.

The activity of W143A and W143F with *R. sphaeroides* Cc is also severely inhibited, showing 2–7% wild-type activity, in the same range as horse Cc. It is therefore likely that both horse Cc and *R. sphaeroides* Cc use the same residue, Trp¹⁴³, as an important part of the electron conduit to CcO.

In the case of D214N and D195N, a major difference is observed in their relative reactivities with the two different cytochromes. With horse Cc, D214N and D195N retain about 24 and 71% of wild-type activity, respectively. In contrast, when reacting with *R. sphaeroides* Cc, they have 67 and 19% of wild-type activity, respectively. The reversal of relative activities of these two mutant oxidases reacting with the two cytochromes probably reflects differential effects of the mutants on both $V_{max}$ and $K_m$ values with the two cytochromes, consistent with the different distribution of lysines on their interaction surfaces and suggesting a specific complementation of these two carboxyls (see accompanying paper (37)). These differences are also reflected in the ion strength dependence of maximal activity assay (see below).

**Ionic Strength Dependence of Activity**

The turnover numbers of wild-type and mutant CcO have been studied at different ion strengths by measuring the oxidation of reduced Cc in a stopped-flow spectrophotometer in the absence of additional reducing agents. In this assay, the overall reaction can be described by the following scheme.

$$
Cc^{II} + \text{CcO} \rightleftharpoons \text{Cc}^{III} \text{CcO} \rightleftharpoons \text{Cc}^{II} \text{CcO} \rightleftharpoons \text{Cc}^{III} \text{CcO} \rightleftharpoons \text{Cc}^{II} + \text{CcO}
$$

**Scheme 2**

First, reduced Cc (CcIII) associates with CcO to form a complex, within which electron transfer takes place. Dissociation of the oxidized Cc (CcII) from CcO is required before another reduced Cc can bind and transfer electrons in the multiple turnover assay.

**Reaction with Horse Cc**—In the assays containing 20 μM Cc, 5 mM Tris, pH 7.0, and different ion strengths of NaCl, the turnover number for wild-type oxidase with horse Cc increases from 590 s⁻¹ at 5 mM ionic strength to a maximum of 980 s⁻¹ at 75 mM ionic strength and then decreases to 290 s⁻¹ at 155 mM ionic strength (Fig. 6).

For all of the mutants measured with horse Cc, similar bell-shaped curves are observed as for wild-type oxidase, but the ionic strengths at which the maximum activities are achieved are different, as are the maximal activities attained.
TABLE II

| Enzymes          | R. sphaeroides Cc2 | Horse Cc |
|------------------|--------------------|----------|
|                  | \( K_{a1} \) \( \mu M \) | \( k_{a1} \) \( s^{-1} \) | \( K_{a2} \) \( \mu M \) | \( k_{a2} \) \( s^{-1} \) | \( K_{b1} \) \( \mu M \) | \( k_{b1} \) \( s^{-1} \) | \( K_{b2} \) \( \mu M \) | \( k_{b2} \) \( s^{-1} \) |
| Wild type        | 0.35 ± 0.09        | 144 ± 20 | 6.71 ± 0.99 | 435 ± 7 | 0.04 ± 0.04 | 109 ± 56 | 0.85 ± 0.20 | 469 ± 8 |
| D188N/E189Q      | 0.39 ± 0.05        | 145 ± 10 | 9.56 ± 1.32 | 392 ± 7 | 0.02 ± 0.01 | 80 ± 19 | 1.00 ± 0.11 | 445 ± 12 |
| D151N/E152Q      | 0.44 ± 0.11        | 149 ± 21 | 8.62 ± 1.63 | 415 ± 9 | 0.05 ± 0.04 | 100 ± 54 | 0.72 ± 0.11 | 457 ± 5 |
| E148Q            | 0.75 ± 0.21        | 192 ± 38 | 7.13 ± 1.46 | 475 ± 9 | 0.10 ± 0.03 | 219 ± 44 | 1.19 ± 0.21 | 582 ± 7 |
| D195N            | 1.64 ± 0.08        | 105 ± 19 | 13.11 ± 2.07 | 300 ± 6 | 0.12 ± 0.04 | 220 ± 40 | 2.32 ± 0.75 | 499 ± 12 |
| E157Q            | 1.19 ± 0.25        | 141 ± 23 | 11.26 ± 2.59 | 335 ± 8 | 0.11 ± 0.04 | 217 ± 44 | 1.85 ± 0.48 | 525 ± 9 |
| D214N            | 0.43 ± 0.18        | 79 ± 20  | 6.85 ± 1.54 | 263 ± 6 | 0.02 ± 0.01 | 53 ± 10 | 0.73 ± 0.08 | 261 ± 2 |
| E254A            | 0.29 ± 0.06        | 34 ± 4   | 7.54 ± 1.38 | 88 ± 2 | 0.03 ± 0.01 | 45 ± 3 | 1.84 ± 0.37 | 84 ± 1 |
| W143F            | 1.00 ± 0.25        | 44 ± 8   | 10.0 ± 1.54 | 80 ± 3 | -            | -     | -            | -     |

**Fig. 6.** Ionic strength dependence of maximal activity of wild-type and mutant R. sphaeroides CcO with horse Cc (A) and R. sphaeroides Cc2 (B) as substrates. The turnover numbers (TN, representing molecular activity) were measured spectrophotometrically using a stopped-flow apparatus by following the oxidation of Cc at 550 nm in 10 mM Tris acetate, pH 7.0, 0.1% LM, with 20 \( \mu M \) reduced Cc.

The peaks of activities for D214N, D195N, and E148Q are at 55 \( \mu M \) ionic strength, and the peak for E157Q is at 45 \( \mu M \) ionic strength (Fig. 6). The peak activities for D188N/E189Q and D151N/E152Q occur at the same ionic strength as for wild-type. At low ionic strength, the dissociation of Cc is probably rate-limiting (36, 19), while at very high ionic strength, the electrostatically mediated association rates are markedly reduced. The ionic strength at which the peak of activity is achieved therefore represents the balance between increasing dissociation rates and decreasing association rates and is a measure of the binding strength of Cc to CcO. The shift of the peak of activity to lower ionic strengths for the mutants is a measure of the binding strength of Cc to CcO. The shift of the peak of activity to lower ionic strengths for the mutants is a measure of the binding strength of Cc to CcO.

**Comparison of horse Cc and R. sphaeroides Cc2 as Substrates for Wild-type R. sphaeroides Oxidase**

**R. sphaeroides Cc2**, which is induced under photosynthetic conditions and is unlikely to be the native substrate for CcO (41), is missing several of the highly conserved lysines on the front face that are likely to be important in the interaction with oxidase (Fig. 8). As a result, the binding affinity of wild-type oxidase with R. sphaeroides Cc2 is much weaker than with horse Cc. As shown in Table II, the high affinity phase with horse Cc displays a smaller \( K_a \) value (0.04 \( \mu M \)) than the corresponding one with R. sphaeroides Cc2 (0.35 \( \mu M \)). This suggests that horse Cc indeed binds more tightly than R. sphaeroides Cc2 to R. sphaeroides CcO, as indicated by the lower peak activities of wild-type oxidase with horse Cc. In the case of D195N, decreased binding affinity with R. sphaeroides Cc2 is observed, with a peak of activity at or below the lowest ionic strength tested, indicating that Asp214 is strongly involved in binding R. sphaeroides Cc2, consistent with the activity assay in Table I.
interaction domain for cytochrome c on cytochrome oxidase

The results obtained with horse Cc as substrate were similar to those for R. sphaeroides Cc2. However, with the C. albicans Cc, the 

Km values for the high affinity phases obtained with R. sphaeroides Cc2 are similar to those for C. albicans Cc (Table II). These results suggest that the binding affinity with R. sphaeroides Cc2 is higher than that of wild-type oxidase (Table II). These results are in agreement with the ionic strength-dependent activity assay with R. sphaeroides Cc2 (Fig. 6). The direct binding assay (19) is also consistent with no binding change for R. sphaeroides Cc2 with D214N but significant change with horse Cc, as also seen for the ionic strength dependence in Fig. 6. The anomalous behavior of the D214N mutant in different assays may relate to the strong hydrogen bonding interaction of Asp214 with His127 and Glu142 in the native structure (37), which is probably altered and weakened in the asparagine mutant so that the position of the side chain is variable.

**Comparison of Mutant Oxidase Reactions with R. sphaeroides Cc2**

The 

Km values of the high affinity phases obtained with R. sphaeroides Cc2 for D188N/E189Q and D151N/E152Q are comparable with those for wild-type oxidase, while the values for E148Q, D195N, and E157Q are 2–4-fold higher than those of wild-type oxidase (Table II). These results again suggest that Asp188, Glu189, Asp151, and Glu152 do not contribute to binding Cc, while Glu214, Asp185, and Glu157 are important contributors to the electrostatic aspect of binding to R. sphaeroides Cc2. Although the mutation in E254A altered the heme centers and inhibited the overall activity of the enzyme, it apparently has no effect on the binding of R. sphaeroides Cc2, which is consistent with the internal location of residue Glu214. The results obtained with horse Cc as substrate were similar to those for R. sphaeroides Cc2, but with larger errors due to the very low 

Km values (Table II).

In the assay of W143F with horse Cc (Table II), the 

Km of the high affinity phase is unaltered, suggesting no change in binding. The same result was found in a direct binding assay (19). But with R. sphaeroides Cc2, the 

Km value of the high affinity phase is significantly larger than that of the wild-type oxidase, suggesting a change in binding affinity in contradic-
tion of the results from the other two assays (Table II). This difference is probably due to the complexity of the TMPD/ascorbate steady-state kinetics assay as discussed above; the 

Km values do not always relate to the binding constants, em-
phasis the importance of confirmation by direct binding assay (19).

In the assays with both horse Cc and R. sphaeroides Cc2, the 

Km value obtained for the high and low affinity phases for D214N are generally comparable with that of wild-type oxida-

The 

Km values of the high and low affinity phases for D214N are generally comparable with that of wild-type oxidase, suggesting that the binding affinity with R. sphaeroides and horse Cc did not change. The result is in agreement with the ionic strength-dependent activity assay with R. sphaeroides Cc2 (Fig. 6). The direct binding assay (19) is also consistent with no binding change for R. sphaeroides Cc2 with D214N but significant change with horse Cc, as also seen for the ionic strength dependence in Fig. 6. The anomalous behavior of the D214N mutant in different assays may relate to the strong hydrogen bonding interaction of Asp214 with His127 and Glu142 in the native structure (37), which is probably altered and weakened in the asparagine mutant so that the position of the side chain is variable.

**Discussion**

Cc interaction with various redox partners—Cc, as a versatile redox protein, can react with a number of partners including cytochrome bc1, cytochrome b5, sulfite oxidase, cytochrome c peroxidase (CcP), CcO, plastocyanin, and a variety of small molecules (43). Its nonredox role in the signaling pathway of programmed cell death is relatively unexplored (1), but the redox interactions of Cc have been studied thoroughly using a variety of techniques, in attempts to understand the mechanism of rapid efficient electron transfer between proteins.

Early chemical modification studies suggested a group of lysine residues, Lys8, Lys13, Lys27, Lys72, and Lys86/87, located on the upper half of the front surface surrounding the heme edge of horse Cc (Fig. 8), as part of the binding interface between Cc and CcO (2, 3). Further support for this conclusion was derived from chemical protection experiments (44). A similar surface domain of Cc was also identified as participating in its interaction with other redox partners (45–47).

Among the physiological redox partners, the interaction of Cc with CcP is the best characterized. Besides extensive biochemical and spectroscopic studies in this system (48), the crystal structures of CcCcP complexes have been resolved, revealing that the same lysine residues identified by chemical modification are responsible for the interaction (49). Furthermore, mutagenesis and spectroscopic studies have established that the interface resolved in the crystal structures represents the kinetically competent interaction in solution (50).

Although it is generally accepted that Cc uses these lysine residues in its interactions with most of its redox partners, the corresponding binding domain on CcO and the chemistry of the resulting interface is still not well defined. In the present study, a number of carboxyl residues in subunit II of CcO, as well as a centrally located tryptophan, were mutated, purified, fully characterized, and studied with respect to their kinetics of reaction with horse Cc and R. sphaeroides Cc2. On the basis of these studies and with the guidance of the crystal structures of CcO, a Cc binding site is proposed (Fig. 8). In the accompanying papers, time-resolved kinetics of electron transfer (19) and a systematic computational docking analysis (37) substantiate, extend, and refine our understanding of the nature of the docking interaction.

The data are in agreement with many of the results obtained by Ludwig and co-workers using the P. denitrificans CcO (16, 30) but significantly extend these findings by providing the basis for interpreting the kinetics of interaction of Cc with CcO as a function of ionic strength and for defining the detailed chemistry of the protein/protein interface involved in...
Asp151 and Glu152 are on the Cc552 (52); 2) although they are present in the membrane-bound form, residues that are involved in this study are highlighted in color. In this model, residues Asp188 and Glu189 are on the back of the molecule, and Asp185 and Glu186 are on the upper right corner. In both horse Cc and R. sphaeroides Cc2, the lysine residues proposed to be involved in binding to CcO are shown in color, and all of the other surface lysines are shown in black. The residues in CcO and Cc that are proposed to interact with each other are highlighted in the same color.

electron transfer.

Effect of Cc Variants, Horse Cc and R. sphaeroides Cc2, on the Kinetics of Reaction with R. sphaeroides CcO—In R. sphaeroides, the physiological substrate for CcO is not yet established, but some studies suggest that the membrane-bound Cc552 may be the direct electron donor in vivo (41, 51, 52), whereas other studies have suggested that the soluble Cc2 is the natural substrate (53, 54). However, horse Cc has been found to transfer electrons efficiently to R. sphaeroides oxidase, and it has been used in most of the kinetic studies. R. sphaeroides Cc2 also reacts with R. sphaeroides CcO but less efficiently.

Both horse heart Cc (1hrc.pdb) and R. sphaeroides Cc2 (1cx-a.pdb) have been crystallized, and although their overall structures are similar, these two proteins differ in many respects, including the following: 1) the highly conserved Lys13 and Lys17 in horse Cc are not present in the R. sphaeroides Cc2 (Fig. 8), although they are present in the membrane-bound form, Cc552 (52); 2) R. sphaeroides Cc2 is more acidic than horse Cc, and the distribution of the positive charges is different (horse Cc has a positive surface potential centered at the upper left of the front face, whereas in R. sphaeroides Cc2, it is more diffusely distributed on the whole front face (55)) (also see Fig. 1 in the accompanying paper (37)); and 3) R. sphaeroides Cc2 has a high redox potential of 356 mV versus 260 mV for horse Cc (24).

Because of these differences, these two natural variants of Cc would be expected to react differently with R. sphaeroides CcO, as is also seen in the CcP complexes with horse and yeast Cc (49).

The turnover number of wild-type CcO with 30 μM horse Cc is about 6-fold higher than with R. sphaeroides Cc2 (Table I), as confirmed in the ionic strength dependence study (Fig. 6). The apparent affinity for horse Cc is also much higher, as shown in the steady-state kinetic assay (Table II) and the direct binding assay (19). Nevertheless, with either horse Cc or R. sphaeroides Cc2, the turnover number for W143F was equally inhibited, suggesting that the two different cytochromes use the same residue as the electron conduit to CcO, which places the two cytochromes on roughly the same binding domain on CcO but with slightly different orientations and guiding electrostatics.

Among the carbonyl mutants, the mutations in D188N/E189Q and D151N/E152Q have almost no effect on the overall activity and the binding strength with either horse Cc or R. sphaeroides Cc2, suggesting that residues Asp188, Glu189, Asp151, and Glu152 are not involved in binding either cytochrome and that docking is not determined by overall surface charge.

The $K_m$ values of E148Q, E157Q, and D195N for both the cytochromes are all decreased, but the interaction of D195N with R. sphaeroides Cc2 is significantly more affected than that with horse Cc, as confirmed by direct binding assays (19). Together, these results suggest that residue Asp185, although involved in binding both cytochromes, contributes more to the formation and electron transfer capacity of the R. sphaeroides Cc2/CcO complex. The mutation in D214N also has different effects on the formation of the complexes with the two cytochromes, indicated by the different ionic strength dependences (Fig. 6) and overall activities (Table I). The mutation in D214N has less effect on the R. sphaeroides Cc2 than on the horse Cc reaction with oxidase. Since the conserved Lys15 and Lys72 are missing in R. sphaeroides Cc2, this suggests that Asp214 in CcO may interact with one of them, most likely Lys72 in horse Cc (Fig. 8). The lack of the Lys72/Asp214 interaction (and Lys15) in the R. sphaeroides Cc2/CcO complex could tilt the complex so that Asp185 in CcO assumes a more important role in a predicted interaction with Lys8 (see also accompanying paper (37)).

Effect of Surface Carboxyl Mutants on the Kinetics of Cc Oxidation—The rate of oxidation of Cc by mutant forms of R. sphaeroides oxidase was compared with wild-type under a variety of assay conditions. In all studies, the double mutants D151N/E152Q and D188N/E189Q behave similarly to wild-type oxidase, indicating that these four carboxyl residues are not involved in Cc binding. Furthermore, these mutants establish the fact that overall charge is not the controlling factor in the reaction.

$E148Q$, $E157Q$, D195N, and D214N all showed decreased activity and weaker Cc binding strength as evidenced by $K_m$ values and ionic strength dependence of activity, indicating their involvement in Cc binding. However, their individual effects on Cc oxidation are qualitatively and quantitatively different, again emphasizing that overall charge, even at the reaction interface, is not the simple determinant of activity.

The mutation in D214N has the largest effect on the turnover rate with horse Cc, while Glu157 has its major effect on Cc binding (also confirmed in direct binding assays (19)). Both E148Q and D195N have moderate effects on Cc binding and electron transfer activity.

The large decrease in the turnover of D214N with horse Cc (24% of wild type) is similar to that seen in studies on the...
isolated Cuₐ domain of \textit{P. denitrificans} oxidase with the same mutation (14), but the effect of the same mutation in the holoenzymes from \textit{P. denitrificans} is surprisingly less dramatic (60–80% of wild-type) (16).

Asp^{214} is one of the most conserved residues in CcO, and it is even conserved in another Cuₐ-containing enzyme, nitrous-oxide reductase (56), which physiologically accepts electrons from Cc (57). In the structure of CcO, Asp^{214} is located close to Trp^{143}, the residue likely to be the site of the closest approach of the Cc heme edge to Cuₐ, as identified in this study (19, 37) and a similar study in \textit{P. denitrificans} (30). Moreover, it is hydrogen-bonded to His^{217}, one of the Cuₐ ligands, and to Glu^{148}, making an unusual structural motif observed in other metal sites (37). This structural motif is probably important in maintaining the native conformation in the region of Cuₐ that is important for rapid electron transfer.

**Bell-shaped Ionic Strength Profile for Cc Oxidation by CcO**—

The effects of ionic strength on turnover number generally yielded bell-shaped profiles with maximal rates for the wild-type oxidase at 75 mM ionic strength. The formation of the bell-shaped profile is accounted for by the differential effects of ionic strength on the \( k_{in} \) and \( k_{off} \) rates as already discussed. The ionic strength at which the peak rate is achieved will depend on the balance of effects on \( k_{in} \) and \( k_{off} \), which will be influenced by the number and position of charges involved in the interaction and thus is inversely proportional to the binding constant (\( K_D \)). Direct binding studies in the accompanying paper (19) confirm this relationship and provide additional evidence that low activity at low ionic is not due to formation of an ineffective complex as sometimes suggested (16, 58, 59).

**The Importance of Trp^{143}—**

Trp^{143} is a highly conserved residue in all of the known CcO sequences and in quinol oxidases. In the crystal structure (11, 12), this residue is located on the surface of subunit II about 5 Å above the Cuₐ center, forming a hydrogen bond with Cys^{256}, one of the Cuₐ ligands, and in van der Waals contact with Met^{263}, another Cuₐ ligand (Fig. 1; see Fig. 9 in the accompanying paper (19)). It is centrally located with respect to the conserved carboxyl groups that appear to be involved in Cc binding (Fig. 8). In W143A and W143F, EPR analyses show that the Cuₐ center was essentially unaltered. In addition, the binding strength of W143F with Cc is the same as with wild-type oxidase (19), indicating that Cc binds in the normal configuration for electron transfer. However, the steady-state activity of W143F and W143A is decreased about 50–100-fold compared with wild-type, and more significantly, the intrinsic electron transfer rates from Cc to Cuₐ are decreased up to 1000-fold in the rapid kinetic assay (19). The significance of this finding with regard to current theories of the mechanism of electron transfer in protein is discussed in the accompanying paper (19).

Importantly, in these two strongly inhibited mutants, W143A and W143F, the Cc concentration dependence still exhibits the same biphasic character seen with the wild-type enzyme (Fig. 7) with maximal turnover rates similar to the intrinsic rates of electron transfer measured from Cc to Cuₐ (19). This provides compelling evidence that Cuₐ is the sole electron entry site for both phases of the kinetics, a long-standing issue (3, 17).

**Model of the Interaction Domain of CcO with Cc**—

In the space-filling model of the soluble domain of subunit II (Fig. 8), based on the coordinates of the \textit{P. denitrificans} CcO (33), Asp^{214}, Asp^{195}, Glu^{157}, and Glu^{148} are shown to be located in a region surrounding the highly conserved aromatic stretch, with Asp^{214} located next to Trp^{143} on the surface. The strong inhibitory effect of even conservative mutations of Trp^{143} in W143F indicate the likelihood that it is the point of closest approach to heme c and the electron entry site to Cuₐ.

The high degree of conservation and physical closeness of Asp^{214} to Trp^{143} are consistent with its location in the middle of the Cc binding domain, as suggested by the major changes in the kinetics associated with alteration of this residue. Glu^{157}, Glu^{148}, and Asp^{195}, with moderate effects on kinetics, are concluded to be located in the binding domain but not so central as to strongly affect the chemistry of the interaction and the electron transfer process itself. In this model, Asp^{151} and Glu^{152} are located at the edge of the binding domain, and Asp^{188} and Glu^{189} are well outside it, unlikely to be involved in Cc binding, as the data suggest.

In horse Cc, Lys^{6}, Lys^{131}, Lys^{72}, and Lys^{6987}, located on the upper front surface of the molecule, were implicated in its binding to electron transfer partners by chemical modification studies (2, 3) and confirmed as interfacial participants by the crystallographic analysis of the Cc-CcP complex (49, 50). Based on the arrangement of these lysine residues on Cc and the corresponding positions of the four designated carboxyl groups on CcO, an interface between Cc and CcO can be envisioned where Lys^{5}, Lys^{131}, and Lys^{6987} interact with Asp^{195}, Glu^{157}, and Glu^{148}, respectively. This arrangement would place Lys^{72} in Cc close to Asp^{214} in oxidase and the indole ring of Trp^{143} close to the heme edge of Cc. Such a complex (which may also include significant bound water at the interface, as in the case of the Cc-CcP complex) is consistent with all of the kinetic observations reported here on the oxidase mutants and on the natural variants of Cc but is only one of several possibilities. In the following papers, direct binding studies, time-resolved electron transfer measurements (19), and a systematic computational docking analysis (37) are reported that fully support and refine this model.

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