Definition of the Interaction Domain for Cytochrome c on the Cytochrome bc₁ Complex

STEADY-STATE AND RAPID KINETIC ANALYSIS OF ELECTRON TRANSFER BETWEEN CYTOCHROME c AND RHODOBACTER SPHAEROIDES CYTOCHROME bc₁ SURFACE MUTANTS*

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The interaction domain for cytochrome c on the cytochrome bc₁ complex was studied using a series of Rhodobacter sphaeroides cytochrome bc₁ mutants in which acidic residues on the surface of cytochrome c₁ were substituted with neutral or basic residues. Intra-complex electron transfer was studied using a cytochrome c derivative labeled with ruthenium trisbipyridine at lysine 72 (Ru-72-Cc). Flash photolysis of a 1:1 complex between Ru-72-Cc and cytochrome bc₁ at low ionic strength resulted in electron transfer from photoreduced heme c to cytochrome c₁ with a rate constant of kₑ = 6 × 10⁴ s⁻¹. Compared with the wild-type enzyme, the mutants substituted at Glu-72, Asp-102, Glu-104, Asp-109, Glu-162, Glu-163, and Glu-168 have significantly lower kₑ values as well as significantly higher equilibrium dissociation constants and steady-state Kᵘ values. Mutations at acidic residues 56, 79, 82, 83, 97, 98, 213, 214, 217, 220, and 223 have no significant effect on either rapid kinetics or steady-state kinetics. These studies indicate that acidic residues on opposite sides of the heme crevice of cytochrome c₁ are involved in binding positively charged cytochrome c. These acidic residues on the intramembrane surface of cytochrome c₁ direct the diffusion and binding of cytochrome c from the intramembrane space.

The cytochrome bc₁ complex is an essential component of the energy-transducing electron transfer chains in mitochondria and many prokaryotes (1). The complex from all organisms contains two b cytochromes (bL and bH) located on a single polypeptide chain, cytochrome c₁, and the Rieske iron-sulfur protein. The total number of polypeptide subunits depends on the species, ranging from three in some prokaryotes to 11 in beef heart mitochondrial cytochrome bc₁. It is generally accepted that electron transfer through the complex involves a Q-cycle mechanism in which four protons are translocated from the negative to the positive side of the membrane per two electrons transferred from ubiquinol to cytochrome c (2). The electron transfer reaction between cytochrome c and the cytochrome bc₁ complex involves, at a minimum, the following steps: 1) formation of a 1:1 reactant complex between cytochrome c⁺⁺ and reduced cytochrome bc₁, 2) intracomplex electron transfer from cytochrome c₁²⁺ to cytochrome c³⁺, and 3) dissociation of the product complex to release cytochrome c²⁺. The binding interaction between cytochromes c and bc₁ must stabilize the optimal orientation of the reactant complex for rapid electron transfer and allow both rapid reactant complex formation and product complex dissociation. Both steady-state and stopped-flow kinetic studies have shown that the reaction rate is inhibited by high ionic strength, indicating that the interaction has an electrostatic component (3–5). Extensive chemical modification studies have revealed that six or seven lysine amino groups surrounding the heme crevice of cytochrome c are involved in binding cytochrome bc₁ (6–9). Studies utilizing a water-soluble carbodiimide have implicated the acidic residues 66, 67, 76, and 77 on bovine cytochrome c₁ in cytochrome c binding as well as acidic residues on the hinge protein (10, 11). Acidic residues in sequence 165–174 have also been implicated in cytochrome c binding by photoaffinity cross-linking studies (12). The x-ray crystal structures of beef and chicken cytochromes bc₁ have revealed that the exposed heme CD edge of cytochrome c₁ on the cytoplasmic surface of the membrane is surrounded by acidic residues that could form a docking site for cytochrome c (13–15). The hinge protein (subunit 8) is located adjacent to cytochrome c₁, and acidic residues on its cytoplasmic surface could help direct cytochrome c to the docking site.

In this paper, the interaction domain for cytochrome c on the cytochrome bc₁ complex was defined using a series of Rhodobacter sphaeroides cytochrome bc₁ mutants in which acidic residues on the surface of cytochrome c₁ were substituted with neutral or basic residues. Steady-state kinetic studies were carried out to determine how mutation affected V_max and Kₘ for cytochrome c. A new ruthenium photoexcitation technique was also used to resolve a key step in the mechanism, intracomplex electron transfer between cytochrome c and purified cytochrome c₁ (16, 17). In this technique, a 1:1 electrostatic complex between cytochrome bc₁ and a cytochrome c derivative labeled with ruthenium trisbipyridine is formed at low ionic strength. The Ru(II) group is photoexcited to a metal-to-ligand charge transfer state, Ru(II)*, which is a strong reducing agent and rapidly transfers an electron to heme c (18). Electron transfer between photoreduced heme c and cytochrome c₁ can be measured on a microsecond time scale (17).

Electron transfer between ruthenium trisbipyridine-labeled cytochrome c and the cytochrome bc₁ complex isolated from bovine mitochondria as well as wild-type and mutant R. sphaeroides was studied over a wide range of conditions.

EXPERIMENTAL PROCEDURES

Materials—Bovine cytochrome bc₁ was purified as described by Yu et al. (19). Horse heart cytochrome c was obtained from Sigma. Horse

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heart Ru-72-Cc, labeled with ruthenium trisbipyridine at lysine 72, was prepared as described by Durham et al. (18). Cytochrome oxidase was purified as described by Capaldi and Hayashi (20). Aniline was purchased from Fisher, and 3CP was purchased from Aldrich. Stock solutions of aniline were prepared fresh daily. A stock solution of 3CP was adjusted to pH 7 using NaOH and was stored at -70 °C.

Construction of Mutations and Expression of Mutant Complexes in R. sphaeroides—Mutants were constructed by site-directed mutagenesis using the Altered Sites system from Promega Corp., and oligonucleotides used were synthesized at the Oklahoma State University Recombinant DNA/Protein Core Facility. The oligonucleotides used were GCAGTT-CCACCGCGCAAGGCCATGAGCCGGACG (D95K), CTCCGCTCA-GGCCCAAGGCCATGAGCCGGACG (E103Q), GGCAAGCCCACCAACCACTTCCCCCATTC (E108N), GCC-GCGGCCCAAGGCCATGAGCCGGACG (E79Q), GGCGGCCCCGAGCTGCCCCAGAACCAGGTCCGGGCCTAT-GAG (E74Q), CCGGGCCGCCCACTCGCCCAAGGGTACCGG (E89Q), GGGCCGGCCGACTCCGGCAAGGGTGCT (E216K), CAGGTCTATACCCAGGTCTG (E222K), and GTGCTGACGGGCGGGGAAACGGGTAAG (D220K). Steady-state parameters were monitored over the wavelength range of 540–560 nm using samples recorded over the wavelength range of 540–560 nm yielded a kinetic difference spectrum with a maximum at 546 nm (Fig. 2), consistent with the absorbance spectrum for electron transfer between cytochromes c and bc1 (3).

Flash Photolysis Experiments—Transient absorbance measurements were carried out as described by Heacock et al. (17) by flash photolysis of 300-μl solutions contained in a 1-cm glass semimicrocuvette. The excitation light pulse was provided by a pulse modulator (Hollis-Hoffeet), the initial velocities as described (22).

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RESULTS

Intracomplex Electron Transfer between Ru-72-Cc and Bovine Cytochrome bc1—The electron transfer reaction between cytochrome c and bovine cytochrome bc1 was studied using the ruthenium photoreduction technique previously described (17). Horse Ru-72-Cc was chosen because it has a high efficiency of photoreduction in a single flash (35%) and because it interacts favorably with purified bovine cytochrome c1 (17). Flash photolysis of a solution containing 9 μM Ru-72-Cc and 10 μM bovine cytochrome bc1, in 5 mM Tris-Cl (pH 7.4), 10 mM aniline, and 1 mM 3CP was excited with a 480-nm laser flash of 200-ns duration. Cytochrome c reduction and reoxidation were monitored at 548 nm, and cytochrome c1 reduction was monitored at 556 nm. The rate constant for the exponential transients at both wavelengths was (6.0 ± 1.5) × 104 s−1.

![Image](http://www.jbc.org/)
Electron Transfer between Cytochromes c and bc₁

was taking place in a complex between Ru-72-Cc and cytochrome bc₁, with a dissociation constant of <4 μM, according to the top line of Scheme 2. The rate constant remained the same (6.0 × 10⁴ s⁻¹) as the ionic strength was increased from 5 to 45 mM (Fig. 3). A second slow phase first appeared at 35 mM ionic strength with a relative amplitude of 36% and increased to 60% relative amplitude at 45 mM ionic strength with a rate constant of 6100 s⁻¹ (Fig. 3). The fast phase was completely eliminated above 55 mM ionic strength, indicating dissociation of the Ru-72-Cc-cytochrome bc₁ complex. The slow phase obeyed second-order kinetics, indicating that it was due to a reaction between solution Ru-72-Cc and cytochrome bc₁, according to Scheme 2. The rate constant of the slow phase decreased with further increases in ionic strength above 55 mM, consistent with a reaction between oppositely charged proteins.

Electron Transfer between Ru-72-Cc and Wild-type R. sphaeroides Cytochrome bc₁—Flash photolysis of a solution containing 10 μM Ru-72-Cc and 10 μM R. sphaeroides cytochrome bc₁ at low ionic strength led to a rapid increase in 548 nm absorbance, followed by an exponential decrease with a rate constant of 6.0 × 10⁴ s⁻¹ due to electron transfer from the cytochrome c heme Fe(II) to cytochrome c₁. The 556 nm transient also had a rate constant of 6 × 10⁴ s⁻¹, consistent with reduction of cytochrome c₁. The rate constant was independent of protein concentration, indicating that the reaction was due to intracomplex electron transfer. The kinetic difference spectrum was nearly the same as for the complex with beef cytochrome bc₁ (Fig. 2). The rate constant remained nearly the same as the ionic strength was increased to 45 mM; then the amplitude of the fast intracomplex phase decreased, and a new slow phase appeared (Fig. 4). The fast and slow phases were of equal amplitude at 65 mM ionic strength, with a rate constant of 5200 s⁻¹ for the slow phase. The fast phase was completely eliminated above 80 mM ionic strength, indicating dissociation of the complex. The rate constant of the slow phase was linearly dependent on the concentration of cytochrome bc₁ (data not shown), indicating bimolecular kinetics according to Scheme 2 with a second-order rate constant of k₂ = 3.1 × 10⁸ M⁻¹ s⁻¹ at 85 mM ionic strength. The rate constant of the slow phase decreased with increasing ionic strength above 65 mM (Fig. 4).

To compare the interaction of Ru-72-Cc and native horse cytochrome c with R. sphaeroides cytochrome bc₁, steady-state kinetics were carried out under high ionic strength conditions, where previous studies have shown that the Michaelis constant Kₘ is a good measure of the binding interaction (22). Native horse cytochrome c was found to have a Vₘₐₓ of 3.8 ± 0.5 μM cytochrome c reduced per min/μM cytochrome b and a Kₘ of 10 ± 1 μM in buffer containing 25 mM sodium phosphate (pH 7.0), 150 mM NaCl, and 0.3 mM EDTA. Under the same conditions, Ru-72-Cc has a Vₘₐₓ of 1.3 ± 0.2 μM cytochrome c reduced per min/μM cytochrome b and a Kₘ of 14.6 μM.

Characterization of R. sphaeroides Cytochrome bc₁ Surface Mutants by Steady-state Kinetics—Acidic residues distributed along the surface of the cytochrome c₁ subunit were mutated to neutral or basic residues to define the docking interface for cytochrome c₁. All of the mutants grew photosynthetically at rates comparable to that of the complement strain. The steady-state reactions of horse cytochrome c with the cytochrome bc₁ mutants were carried out at high ionic strength as described above. Wild-type cytochrome bc₁ and each of the mutants displayed monophasic kinetics with well defined Vₘₐₓ and Kₘ values (Fig. 5 and Table I). The largest effect on the steady-state kinetics was observed for the double mutant E100K/E101K, which has a Kₘ value of 73 μM, ~7-fold larger than that of wild-type cytochrome bc₁ (Fig. 5 and Table I). Mutants E74Q, E104Q, D109N, and E162Q/E163Q each have a Kₘ value that is ~5-fold larger than that of wild-type cytochrome bc₁ (Table I). The E169Q mutation led to a 3-fold increase in Kₘ. The increase in Kₘ for these mutants indicates that the binding affinity for cytochrome c has decreased. However, the steady-state Vₘₐₓ values for these mutants are comparable to that for

![Figure 2. Kinetic difference spectrum for photoinduced electron transfer between Ru-72-Cc and cytochrome bc₁. Absorbance transients were recorded for bovine (■) and R. sphaeroides (R. sp.; ●) cytochromes bc₁, under the conditions given in the legend to Fig. 1. The absorbance change at the indicated wavelength is the ratio of the transient absorbance change at the indicated wavelength to the initial absorbance increase at 550 nm.](http://www-jbc.org/doi/fig)
the wild-type enzyme. This observation partially explains why the growth rate is not impaired by these mutations since the steady-state enzymatic activity at high cytochrome c concentration is comparable to that of wild-type cytochrome bc₁. Mutants E56Q, E79Q, E82Q/D83N, D95K, E97K/E98K, D213K/D214N, D214K, E217K, D220K, and D223N have Km and Vmax values comparable to those of the wild-type strain (Table I).

Reaction between Ru-72-Cc and R. sphaeroides Cytochrome bc₁ Surface Mutants—The binding interaction was also defined by studying the reactions of Ru-72-Cc with the R. sphaeroides cytochrome bc₁ surface mutants. All of the mutants displayed intracomplex electron transfer with Ru-72-Cc at 5 mM ionic strength. The intracomplex rate constants for electron transfer were measured at low ionic strength in 5 mM Tris-Cl (pH 7.4) as described in the legend to Fig. 1. The error limits are ±20%.

The percent fast phase of electron transfer was measured at 45 mM ionic strength in 5 mM Tris-Cl (pH 7.4) as described in the legend to Fig. 8. The error limits are ±15%.

![Figure 3](http://www.jbc.org/)

**Figure 3.** Ionic strength dependence of the fast intracomplex (○) and slow bimolecular (●) phases of photoinduced electron transfer between Ru-72-Cc and bovine cytochrome bc₁. Transients at 548 and 556 nm were recorded under the conditions given in the legend to Fig. 1 with NaCl added to bring the ionic strength up to the indicated values.

![Figure 4](http://www.jbc.org/)

**Figure 4.** Ionic strength dependence of the fast intracomplex (open symbols) and slow bimolecular (closed symbols) phases of photoinduced electron transfer between Ru-72-Cc and wild-type and mutant R. sphaeroides cytochromes bc₁. Transients were recorded at 548 and 556 nm under the conditions given in the legend to Fig. 3. ○, wild-type R. sphaeroides cytochrome bc₁; ▲, E101K/D102K; ■, E104Q.

![Figure 5](http://www.jbc.org/)

**Figure 5.** Steady-state reactions of horse cytochrome c with R. sphaeroides cytochrome bc₁ mutants. The steady-state velocity was measured in buffer containing 25 mM sodium phosphate (pH 7.0), 0.3 mM EDTA, 150 mM NaCl, 25 μM Q₁, and 2–120 μM horse cytochrome c. The velocity is given in micromoles of cytochrome c reduced per min/nM cytochrome c. Vmax and Km for horse cytochrome c were measured in buffer containing 150 mM NaCl and 25 mM sodium phosphate (pH 7.0). Vmax is expressed as micromoles of cytochrome c reduced per min/nmol of cytochrome b. The error limits in Vmax and Km are ±15%.

![TABLE I](http://www.jbc.org/)

**Table I.** Characterization of R. sphaeroides cytochrome c₁ mutants

| Mutants | Surface region | Photosynthetic growth | Vmax [μM] | Km [μM] | ket [10⁵ s⁻¹] | Fast phase % |
|---------|----------------|-----------------------|----------|---------|----------------|-------------|
| Wild-type | +              | 3.8 10 60 80          |
| E74Q    | +              | 3.8 49 27 26          |
| E101K/D102K | 1      | 3.6 73 20 0          |
| E104Q   | +              | 3.3 48 31 9          |
| D109N   | ++             | 2.3 51 26 20         |
| E162Q/E163Q | 3     | 3.9 51 12 30         |
| E169Q   | +              | 3.6 29 14 30         |
| E56Q    | ++             | 3.1 12 70 75         |
| E79Q    | +              | 3.3 12 65 65         |
| E82Q/D83N | 1       | 2.5 16 50 65         |
| D95K    | +              | 3.2 16 32 30         |
| E97K/E98K | 1      | 2.8 44 80            |
| D213K/D214N | 2     | 2.8 14 48 70         |
| D214K   | +              | 2.5 7 55 65          |
| E217K   | 2              | 3.2 15 33 85         |
| D220K   | 2              | 2.7 8 57 70          |
| D223N   | 2              | 3.1 15 60 70         |

**a** Mutants with a significant effect on the kinetics are in the upper part of the table.

**b** Region on surface of cytochrome c₁ as defined in the legends to Figs. 9 and 10 and under “Discussion.”

**c** Vmax and Km for horse cytochrome c were measured in buffer containing 150 mM NaCl and 25 mM sodium phosphate (pH 7.0). Vmax is expressed as micromoles of cytochrome c reduced per min/nmol of cytochrome b. The error limits in Vmax and Km are ±15%.

**d** ket is the rate constant for intracomplex electron transfer with Ru-72-Cc, measured at low ionic strength in 5 mM Tris-Cl (pH 7.4) as described in the legend to Fig. 1. The error limits are ±20%.

**e** The percent fast phase of electron transfer was measured at 45 mM ionic strength in 5 mM Tris-Cl (pH 7.4) as described in the legend to Fig. 8. The error limits are ±15%.
from heme c to cytochrome c1 ranged from $1.2 \times 10^4$ s$^{-1}$ for E162K/E163Q to 7.0 $\times 10^4$ s$^{-1}$ for E56K (Table I). The mutants substituted at Glu-74, Asp-95, Glu-104, Asp-109, Glu-101, Asp-102, Glu-162, Glu-163, and Glu-169 had the largest effect on intracomplex electron transfer, with $k_{et}$ values 20–53% that of wild-type cytochrome bc$_1$ (Table I). The fast intracomplex phase for electron transfer was eliminated at a much lower ionic strength for these mutants than for wild-type cytochrome bc$_1$ (Figs. 4 and 6–8 and Table I). For example, the fast intracomplex phase of electron transfer was completely eliminated at 25 mM ionic strength for the E101K/D102K mutant and at 45 mM ionic strength for the E104Q mutant (Figs. 4 and 8). The percent of the fast intracomplex phase of electron transfer at 45 mM ionic strength was 30% or less for the E74Q, D95K, E100K/D101K, E104Q, D109N, E162Q/E163Q, and E169Q, mutants, compared with 80% for wild-type cytochrome bc$_1$ (Fig. 8 and Table I). The rate constant of the slow phase due to the bimolecular reaction was less than that of wild-type cytochrome bc$_1$ for these mutants and decreased with increasing ionic strength (Figs. 4, 6, and 7). Mutations at Glu-56, Glu-79, Glu-82, Asp-83, Glu-97, Glu-98, Asp-213, Asp-214, Asp-220, and Asp-223 had relatively minor effects on the rate constant for intracomplex electron transfer and the percent of the fast phase at 45 mM ionic strength, indicating that these residues are not involved in binding cytochrome c (Table I).

**DISCUSSION**

**Intracomplex Electron Transfer between Ru-72-Cc and Cytochrome bc$_1$**—The reaction between cytochrome c and the cytochrome bc$_1$ complex involves the following series of steps: 1) diffusion of ferricytochrome c to cytochrome bc$_1$ to form a transient reactant complex, 2) electron transfer within the reactant complex to form a product complex, and 3) dissociation of the product complex to release ferrocytochrome c. Extensive steady-state and stopped-flow spectroscopic studies have revealed many important features of this reaction (3–7, 9). However, it has not been possible to measure the rate constants of individual steps in the overall mechanism using these techniques. The ruthenium photoexcitation technique has made it possible to measure the rate constant for a key step in this mechanism, intracomplex electron transfer between cytochrome c and purified cytochrome c$_1$ (17). In this investigation, the reactions between cytochrome c and bovine and R. sphaeroides cytochromes bc$_1$ were studied using horse Ru-72-Cc. This derivative is the best currently available for this application. The rate constant for electron transfer from Ru(II)$^*$ to heme c in Ru-72-Cc is $k_{et} = 3 \times 10^7$ s$^{-1}$, which is very fast compared with intracomplex electron transfer between heme c and cytochrome c$_1$ (18). The yield of heme c reduced with a single laser flash is 35%, which is among the largest for the available ruthenium trisbipyridine-labeled cytochrome c deriv-
Electron Transfer between Cytochromes c and bc1

atives. This ensures that electron transfer between cytochromes c and c1 can be detected with good signal-to-noise ratio. This reaction is difficult to detect because the spectra of cytochromes c and c1 are very similar, leading to small absorbance changes. Ru-72-Cc has a 3-fold smaller \( V_{\text{max}} \) and a 1.5-fold higher \( K_a \), than native horse cytochrome c in the steady-state reaction with \( R. \) sphaeroides cytochrome bc1, at high ionic strength. This indicates that the ruthenium complex does have an effect on the interaction with cytochrome c1, consistent with the location of Lys-72 to the left side of the heme crevice. However, this effect is not too large, and Ru-72-Cc forms a strong 1:1 complex with cytochrome bc1, at low ionic strength. Previous studies have shown that among the ruthenium trisbipyridine-labeled cytochrome c derivatives that could be used for this application, Ru-72-Cc has the largest intracomplex electron transfer rate constant and binding constant for the reaction with purified beef cytochrome c1 (17). Unfortunately, the photoreduction yield is too small to detect intracomplex electron transfer for those ruthenium trisbipyridine-labeled cytochrome c derivatives that are labeled on the back of cytochrome c and have the same binding constant as native cytochrome c.

Flash photolysis of a 1:1 complex between Ru-72-Cc and bovine cytochrome bc1, at low ionic strength results in electron transfer from photoreduced heme c to cytochrome c1, with a rate constant of \( 6.0 \times 10^5 \text{ s}^{-1} \). The kinetic difference spectrum has the same wavelength dependence as the difference spectrum for electron transfer between native cytochrome c1 and cytochrome c obtained by equilibrium mixing techniques (3). The kinetic difference spectrum reflects the extent of the reaction, controlled by the equilibrium constant \( K_{eq} \), as well as the spectra of the two components. The equilibrium constant for the electron transfer reaction between purified ferrocytochrome c1 and ferrierythrocyanide is \( K_{eq} = 3.3 \) at high ionic strength (3), consistent with redox potentials of 228 and 261 mV for the two cytochromes, respectively. The reduction potential of cytochrome c decreases to 220 mV upon complex formation with cytochrome oxidase (24). If a similar change occurs upon complex formation with cytochrome bc1, this would lead to a decrease in \( K_{eq} \) and a larger kinetic difference spectrum for the reverse electron transfer reaction measured in the present experiments. Complex formation may also lead to slight changes in the \( \alpha \)-bands of the two cytochromes (17, 24). Since the spectra of cytochromes c and c1 are so similar to each other, very slight spectral changes could lead to larger changes in the kinetic difference spectrum.

Ionic Strength Dependence of the Reaction between Ru-72-Cc and Cytochrome bc1.—The reactions of cytochrome c with its redox partners in mammalian mitochondria are thought to occur by a three-dimensional diffusion process in the intermembrane space under physiological conditions of 100–150 mM ionic strength (25). We therefore studied the reaction of Ru-72-Cc with cytochrome bc1 as the conditions were changed continuously from low ionic strength, where intracomplex electron transfer was observed, to high ionic strength, where bimolecular kinetics occurred. The rate constant for intracomplex electron transfer between Ru-72-Cc and bovine cytochrome bc1 remains constant at \( k_{ct} = 6.0 \times 10^5 \text{ s}^{-1} \) as the ionic strength is increased from 5 to 45 mM. This indicates that the orientation of the 1:1 complex is optimal for electron transfer at low ionic strength and does not change as the ionic strength is increased. The rate constant for intracomplex electron transfer with \( R. \) sphaeroides cytochrome bc1 is also nearly independent of ionic strength. Similar behavior has also been observed in the reactions of cytochrome c with purified cytochrome c1 (17), cytochrome oxidase (16, 27), cytochrome c peroxidase (28–31), and cytochrome bc1 (32, 33).

As the ionic strength is increased above 35 mM, the amplitude of the fast intracomplex phase decreases, and a new slow phase appears due to bimolecular electron transfer between solution Ru-72-Cc and cytochrome bc1. The relative amplitudes of the fast and slow phases can be used to estimate the equilibrium dissociation constant \( K_{d} = k_{et}/k_{ct} \) for the 1:1 complex to be 9.5 \( \mu \text{M} \) at 45 mM ionic strength for bovine cytochrome bc1, and 5.5 \( \mu \text{M} \) at 65 mM ionic strength for \( R. \) sphaeroides cytochrome bc1. The presence of both the fast intracomplex phase and the slow bimolecular phase at intermediate ionic strength provides strong evidence that the complete bimolecular reaction involves formation of a 1:1 complex, followed by intracomplex electron transfer according to Scheme 2. The dissociation rate constant \( k_d \) must be much smaller than \( k_{et} \), since if \( k_d \) were larger than \( k_{et} \), then rapid equilibrium conditions would apply, and separate slow and fast phases would not be observed (31). Assuming that the bimolecular reaction obeys Scheme 2 and \( k_d < k_{et} \), the observed rate constant is given by Equation 1 (31),

\[
k_{obs} = k_{et}(E_0 - \frac{1}{2}(k_{et} + k_0 + k_d) - \frac{1}{2}((k_{et} + k_0)^2 - 4k_0k_d)^{1/2})
\]

where \( E_0 \) is the concentration of cytochrome bc1, and \( C_0 \) is the concentration of Ru-72-Cc. From this equation, the formation and dissociation rate constants can be estimated to be \( k_{et} = 9.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \) and \( k_d = 9.1 \times 10^3 \text{ s}^{-1} \) at 45 mM ionic strength for bovine cytochrome bc1, and \( k_{et} = 9.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \) and \( k_d = 5.2 \times 10^3 \text{ s}^{-1} \) at 65 mM ionic strength for \( R. \) sphaeroides cytochrome bc1. The complex formation rate constant of \( 10^9 \text{ M}^{-1} \text{ s}^{-1} \) is consistent with an electrostatically assisted, diffusion-controlled reaction between cytochrome c and the negatively charged surface of cytochrome c1. Similar complex formation rate constants have been measured for the reactions of cytochrome c with cytochrome c peroxidase (31), cytochrome c oxidase (27), and cytochrome bc1 (32, 33). The overall reaction between Ru-72-Cc and bovine cytochrome bc1 at 45 mM ionic strength thus involves formation of a 1:1 cytochrome c–cytochrome bc1 complex with rate constant \( k_{et} = 9.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \), intracomplex electron transfer with rate constant \( k_{ct} = 6.0 \times 10^5 \text{ s}^{-1} \), and product complex dissociation with rate constant \( k_d = 9.1 \times 10^3 \text{ s}^{-1} \).

The second-order rate constant for the reaction between Ru-72-Cc and both bovine and \( R. \) sphaeroides cytochromes bc1 decreases rapidly with increasing ionic strength. At ionic strengths above 100 mM, it is likely that \( k_d \) will become comparable to \( k_{et} \) and that \( k_{et} \) will be a function of all three rate constants in Scheme 2, as given in Equation 2.

\[
k_{et} = k_{et}(k_{et} + k_0)
\]

The decrease in \( k_{et} \) with increasing ionic strength is most likely due to a decrease in \( k_{et} \) as well as an increase in \( k_d \). \( k_d \) is unlikely to change very much at high ionic strength since it is unchanged from 5 to 65 mM ionic strength. The large ionic strength dependence of \( k_d \) is an indication of the strong electrostatic interaction between cytochromes c and bc1.

Definition of the Cytochrome c-binding Domain by Kinetic Studies of \( R. \) sphaeroides Cytochrome bc1 Surface Mutants—Among the three redox-active subunits of cytochrome bc1, from different species, the cytochrome c1 subunit is the least conserved. Except for the heme ligands (His-41 and Met-160 in the bovine sequence), heme anchors (Cys-37 and Cys-40), Arg-120 (which forms a salt bridge with heme c propionate), and the heme-embracing sequence –PDL– (ADL in \( R. \) sphaeroides), there is no conserved sequence with known function. Both bovine and \( R. \) sphaeroides cytochrome c1 subunits belong to
Ambler’s class I cytochrome c family. Three conserved helices, namely α1, α2, and α5 in horse cytochrome c and other types of class I cytochromes, are also present in chicken and bovine cytochromes, with the same orientation relative to each other and essentially occupy the same space. These three helices form the functional domains of cytochrome c, and essentially align the conserved secondary structure in all type I cytochromes with the same orientation relative to each other.

The x-ray crystal structure of bovine cytochrome bc1 shows that the surface acidic residues on cytochrome c1, which are conserved in all Ambler’s class I cytochromes, form the basis of this alignment. The α-helix is underlined, and the three acidic surface regions are shaded. Conserved amino acid residues are in boldface.

Acidic residues in each of the three acidic regions on the surface of R. sphaeroides cytochrome c1 were mutated to neutral or basic residues to define the interaction domain for cytochrome c. Mutations near bovine residue 79 in region 1 and residue 136 in region 3 (residues 100–109 and 162–169 in the R. sphaeroides sequence) had significant effects on the intracomplex electron transfer rate $k_{et}$ as well as on the steady-state Michaelis constant $K_m$ for cytochrome c (Fig. 10B and Table I). The amino terminus of subunit 8, containing eight consecutive glutamate residues, was also found to be important in binding cytochrome c in the carbodiimide modification studies (10). However, when the same region was mutated in R. sphaeroides, the five extra acidic residues in the loop connecting α3 and heme ligand Met-207 may perform the function of cytochrome c1.

The 16-residue insertion (residues 132–147) before the α3 helix in R. sphaeroides cytochrome c1 does not contain any charged residues, indicating that this part is most likely buried in the protein. This is consistent with the relative location of the N terminus of the α3 helix, which is buried deep in the protein (Fig. 10). Compared with bovine cytochrome c1, the loop connecting the α3 helix and heme ligand Met-207 in R. sphaeroides cytochrome c1 is 21 amino acids longer in length. Only two acidic residues are located in this loop in bovine cytochrome bc1 (Fig. 10), which interacts with residues 50–60 of subunit 8 (containing six acidic residues). Since there is no subunit 8 in R. sphaeroides, the five extra acidic residues in the loop connecting α3 and heme ligand Met-207 may perform the function of cytochrome c1.

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compared with 80% for wild-type cytochrome \( bc_1 \) (Fig. 8 and Table I). These mutations therefore increase the equilibrium dissociation constant \( K_D \), consistent with the increase in steady-state \( K_m \). The mutations listed above also led to a decrease in the second-order rate constant at high ionic strength, paralleling the effects on \( k_{et} \), \( K_D \), and \( K_m \) (Figs. 4, 6, and 7). Not all of the acidic residues in region 1 are involved in binding cytochrome \( c \), however, since mutations E79Q, E82Q/D83N, and E97K/E98K in this region had no effect on the steady-state or rapid kinetic parameters. The D95K mutation had a significant effect on the rapid kinetic parameters, but only a small effect on the steady-state \( K_m \), suggesting a marginal role in binding. None of the mutations in acidic region 2 (residues 212–223 in the \( R. sphaeroides \) sequence) had a significant effect on the steady-state or rapid kinetics (Table I), indicating that acidic residues in region 2 are not involved in binding cytochrome \( c \). However, Broger et al. (12) reported that the photoaffinity analogue arylazidolysine 13-cytochrome \( c \) was cross-linked to bovine cytochrome \( c_1 \) somewhere in region 2, suggesting that this region may be close to the cytochrome \( c \)-binding site. The ruthenium complex may cause Ru-72-Cc to bind to a somewhat different domain on the surface of cytochrome \( c_1 \) than native horse cytochrome \( c \). However, the charge mutations have nearly the same pattern of effects on the rapid kinetics with Ru-72-Cc as on the steady-state kinetics with native horse cytochrome \( c \), indicating that any change in the binding domain is small.

The mutational studies indicate that acidic residues in regions 1 and 3 of \( R. sphaeroides \) cytochrome \( c_1 \) are involved in binding positively charged cytochrome \( c \). Regions 1 and 3 are located on the intramembrane surface of bovine cytochrome \( c_1 \) near the exposed CD edge of the heme. These two regions on opposite sides of the heme crevice appear to direct the diffusion and binding of cytochrome \( c \) from the intramembrane space. The region immediately surrounding the heme crevice is relatively hydrophobic. It thus appears that the interaction between cytochromes \( c \) and \( bc_1 \) consists of a central hydrophobic domain surrounded by complementary electrostatic interactions at the periphery. A similar type of interaction domain is observed in the complex between yeast cytochrome \( c \) and cytochrome \( c \)-peroxidase, which has been characterized by x-ray crystallography and mutagenesis studies (26, 30, 31).

Fig. 10. A, structure of cytochrome \( c_1 \) and subunit 8 of bovine heart mitochondria, viewed parallel to the membrane. Acidic residues exposed to the intramembrane space region are shown in red, and the three acidic regions identified in the legend to Fig. 9 are boxed. The heme, heme ligands (His-41 and Met-160), and the conserved heme-embracing tripeptide -PDL- are displayed in a stick model. The heme A-D edge, where electron transfer between the iron-sulfur protein and cytochrome \( c_1 \) is thought to occur, is identified. B, structure of cytochrome \( c_1 \) and subunit 8 viewed from the intramembrane space down the membrane. Acidic residues exposed to the surface of cytochrome \( c_1 \) are colored red and labeled. The conserved \( R. sphaeroides \) acidic residues are indicated in parentheses. The heme group is shown as a space-filling model colored red. The coordinates for bovine cytochrome \( c_1 \) were provided by Dr. Di Xia (personal communication).
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