

Article

Accelerated Evolution of Cytochrome *c* in Higher Primates, and Regulation of the Reaction between Cytochrome *c* and Cytochrome Oxidase by Phosphorylation

Sue Ellen Brand ¹, Martha Scharlau ², Lois Geren ², Marissa Hendrix ³, Clayre Parson ² , Tyler Elmendorf ⁴, Earl Neel ⁵, Kaila Pianalto ², Jennifer Silva-Nash ⁶, Bill Durham ² and Francis Millett ^{2,*}

¹ North Murray High School, Chatsworth, GA 30705, USA

² Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701, USA

³ Independent Researcher, P.O. Box 603, Dardanelle, AR 72834, USA

⁴ School of Medicine, University of Kansas Medical Center, 2060 W 39th Ave, Kansas City, KS 66103, USA

⁵ Tulsa Bone and Joint Associates, Tulsa, OK 74146, USA

⁶ Independent Researcher, 5 Kingdom Court, Maumelle, AR 72113, USA

* Correspondence: millett@uark.edu



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Abstract: Cytochrome *c* (Cc) underwent accelerated evolution from the stem of the anthropoid primates to humans. Of the 11 amino acid changes that occurred from horse Cc to human Cc, five were at Cc residues near the binding site of the Cc:CcO complex. Single-point mutants of horse and human Cc were made at each of these positions. The Cc:CcO dissociation constant K_D of the horse mutants decreased in the order: T89E > native horse Cc > V11I Cc > Q12M > D50A > A83V > native human. The largest effect was observed for the mutants at residue 50, where the horse Cc D50A mutant decreased K_D from 28.4 to 11.8 μ M, and the human Cc A50D increased K_D from 4.7 to 15.7 μ M. To investigate the role of Cc phosphorylation in regulating the reaction with CcO, phosphomimetic human Cc mutants were prepared. The Cc T28E, S47E, and Y48E mutants increased the dissociation rate constant k_d , decreased the formation rate constant k_f , and increased the equilibrium dissociation constant K_D of the Cc:CcO complex. These studies indicate that phosphorylation of these residues plays an important role in regulating mitochondrial electron transport and membrane potential $\Delta\Psi$.

Keywords: Cytochrome *c*; Cytochrome *c* oxidase; electron transfer; phosphorylation

1. Introduction

Cytochrome *c* (Cc) is a very ancient protein occurring first in anaerobic photosynthetic bacteria over 3 billion years ago, possibly resembling cytochrome *c*₂ (Cc₂) in the current photosynthetic bacterium *Rhodobacter sphaeroides* [1]. Cc is a very highly conserved protein with the heme iron liganded by a His nitrogen and a Met sulfur (Figure 1) [2]. Extensive studies have identified a highly conserved ring of positively charged lysines surrounding the heme crevice of Cc that form electrostatic interactions with negatively charged Asp and Glu residues at the binding site of the Cc partners (Figure 1). Chemical modification, mutagenesis, and X-ray crystallography studies have shown that the *Rb. sphaeroides* Cc₂—reaction center complex is stabilized by electrostatic interactions between the ring of lysines surrounding the heme crevice of cyt *c*₂ and Asp and Glu residues on the reaction center [3–5]. The rate constant for electron transfer from cyt *c*₂ to the photooxidized chlorophyll dimer D⁺ was $k_{et} = 1.1 \times 10^6 \text{ s}^{-1}$ in both the crystal complex and the complex in solution, demonstrating that the structure of the complex was the same in the crystal and in solution [5]. The experimental rate constant of $k_{et} = 1.1 \times 10^6 \text{ s}^{-1}$ was in good agreement with calculations based on Marcus theory using the closest distance $R = 14.2 \text{ Å}$ between the conjugated ring atoms of the cyt *c*₂ heme and the chlorophyll dimer D⁺ [5].

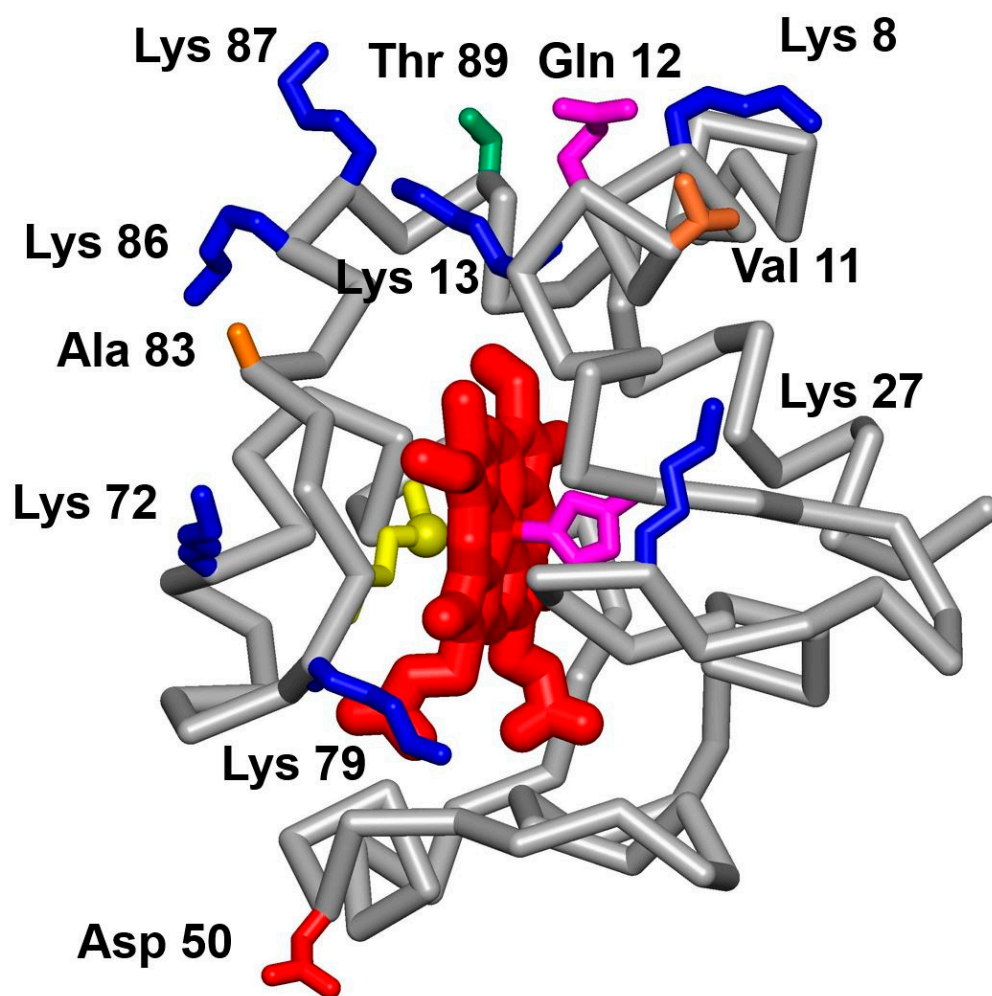


Figure 1. Horse Cc crystal structure (PDB 1HRC) with the heme shown in red, the histidine 18 and methionine 80 iron ligands shown in purple and yellow, and the lysines involved in binding electron transfer partners shown in blue. The amino acids Val 11, Gln 12, Asp 50, Ala 83, and Thr 89 that are mutated in human Cc are also shown.

Chemical modification studies demonstrated that lysines 8, 13, 27, 72, 79, and 87 surrounding the heme crevice of horse Cc are involved in binding negatively charged residues on cytochrome b_5 , in agreement with a computational model for the Cc–cyt b_5 complex [6,7]. A new laser-activated ruthenium technique was developed to measure the rate constant for intracomplex electron transfer between cyt b_5 labeled with a ruthenium complex at Cys-65 (Ru-65-Cyt b_5) and Cc to be $4 \times 10^5 \text{ s}^{-1}$, which is in good agreement with theoretical predictions of the rate constant [8,9].

Chemical modification studies demonstrated that the ring of lysines surrounding the heme crevice of Cc is involved in binding negatively charged residues on yeast cytochrome c peroxidase [10,11], in agreement with an X-ray crystal structure of the yeast Cc–cytochrome c peroxidase complex [12]. A new ruthenium-labeled yeast Cc derivative, Ru-39-Cc was designed to measure the actual rate of intracomplex electron transfer to the Trp-191 radical cation and the oxyferryl heme in cytochrome c peroxidase compound I [13]. Photo-reduced Ru-39-Cc initially transferred an electron to the radical cation in CMPI with rate constant $k_{\text{eta}} = 2 \times 10^6 \text{ s}^{-1}$, and then reduced the oxyferryl heme with rate constant $k_{\text{etb}} = 5000 \text{ s}^{-1}$. Calculations based on Marcus theory using the distance $R = 16 \text{ Å}$ between the Ru-39-Cc heme and the Trp-191 radical cation in the X-ray crystal structure gave a rate constant of $k_{\text{etb}} = 3 \times 10^5 \text{ s}^{-1}$, somewhat less than the experimental rate constant [13].

Extensive chemical modification studies have demonstrated that lysines 8, 13, 27, 72, 79, 86, and 87 surrounding the heme crevice of Cc are involved in binding negatively charged residues on both cytochrome *bc*₁ (cyt *bc*₁) and cytochrome *c* oxidase (CcO) in the mitochondrial respiratory chain, indicated that Cc must first bind to cyt *bc*₁ and accept an electron, then be released, bind to CcO and transfer an electron to CcO [14–25]. Lang and Hunte [26,27] have determined an X-ray crystal structure of the yeast Cc–cyt *bc*₁ complex that is in agreement with the chemical modification studies. Laser excitation of a 1:1 complex between reduced yeast Ru-39-Cc and yeast cyt *bc*₁ at low ionic strength leads to rapid reduction of heme *c*, followed by intracomplex electron transfer from cyt *c*₁ to heme *c* with a rate constant of 77,000 s^{−1} [28,29]. Mutation of Arg-13 and lysines, 27, 72, 79, 86, and 87 to Ala in yeast Ru-39-Cc resulted in significant decreases in the rate of complex formation with cyt *bc*₁, in agreement with the crystal structure [28]. There is a Π –cation interaction between Cc Arg-13 and cyt *c*₁ Phe-230 which has been shown to play an important role in the interaction between the two proteins [27]. Intracomplex electron transfer from laser excited horse Ru-72-Cc to either bovine or *Rb. Sphaeroides* cyt *bc*₁ heme *c*₁ had a rate constant of 60,000 s^{−1} [30]. Mutagenesis studies of the acidic residues Glu-74, Glu-101, Asp-102, Glu-104, Asp-109, Glu-162, Glu-163, and Glu-168 surrounding the heme crevice of *Rb. Sphaeroides* cyt *c*₁ demonstrated they are involved in binding Cc [30], in agreement with the X-ray crystal structure of the yeast Cc–cyt *bc*₁ complex [27]. Calculations based on Marcus theory using the distance $R = 9.4 \text{ \AA}$ between the Ru-39-Cc heme and the cyt *c*₁ heme in the X-ray crystal structure gave a rate constant of $k_{\text{etb}} = 1.8 \times 10^5 \text{ s}^{-1}$, somewhat larger than the experimental rate constant [30].

CcO is the terminal protein in the electron transport chain of mitochondria as well as many bacteria [31,32]. Cc binds to subunit II of CcO and transfers an electron to Cu_A [33–38]. Electrons are then transferred sequentially from Cu_A to heme *a*, heme *a*₃ and Cu_B in CcO subunit I, reducing oxygen to water and generating a proton gradient across the membrane. The highly conserved ring of seven lysines surrounding the Cc heme crevice have been shown by chemical modification studies to be involved in electrostatic interactions with CcO [14–18,22,39]. The X-ray crystal structures of bovine [40], *Paracoccus denitrificans* [41], and *Rb. sphaeroides* CcO [42,43] have shown a central hydrophobic domain surrounded by a prominent ring of acidic residues on subunit II involved in the binding of Cc [44–51]. Computational studies by Roberts and Pique [52] indicate that the interaction between horse Cc and bovine CcO involves a central hydrophobic domain surrounded by electrostatic and hydrogen bonding interactions (Figure 2). Laser excitation of the 1:1 complex between horse Ru-39-Cc and bovine CcO at low ionic strength led to electron transfer from heme *c* to Cu_A with rate constant $k_3 = 6 \times 10^4 \text{ s}^{-1}$, followed by electron transfer from Cu_A to heme *a* with rate constant $k_5 = 1.8 \times 10^4 \text{ s}^{-1}$ [34]. The ruthenium complex is on the backside of Ru-39-Cc, and does not affect any of the steady-state kinetic parameters of the reaction with CcO [34] or cyt *bc*₁ [29]. The K13A, K72A, K86A, and K87A Ru-39-Cc mutants greatly decreased the rate constant $k_{2\text{nd}}$ for binding Ru-Cc to CcO at 150 mM ionic strength, indicating that lysines 13, 72, 86, and 87 are involved in electrostatic binding to CcO (Figure 2) [53]. However, the K13A, K72A, K86A, and K87A Ru-39-Cc mutants had nearly the same intracomplex rate constant k_3 as wild-type Ru-39-Cc, demonstrating that these lysines are not involved in the electron transfer pathway (Figure 2) [53]. The *Rb. sphaeroides* (Rs) CcO mutants E148Q, E157Q, D195N, and D214 N significantly decreased the second-order rate constant $k_{2\text{nd}}$ for the reaction with horse Ru-55-Cc, indicating that the negative charges on E148, E157, D195, and D214 (homologous to bovine CcO E109, D119, D139, and D158) are involved in binding Cc [48]. The *Rb. sphaeroides* W143F mutant (bovine W104) decreased k_3 by 450-fold but did not affect the dissociation constant K_D of the Cc:CcO complex or the redox potential of Cu_A [48]. These results are consistent with the computational model for the Cc:CcO complex with the electron transfer pathway: heme *c* → CcO-W104 → CcO-M207 → Cu_A, which has a Van-der-Waals interaction between heme *c* CBC methyl group and the W104 C-H bond, a separation of 2.8 Å between the W104 CH and the sulfur atom of Met-207, and 9 covalent bonds (Figure 2) [52]. Calculations based on Marcus theory using the distance

$R = 14.1 \text{ \AA}$ between the Ru-39-Cc heme and the Cu_A center in the computational complex give a rate constant of $k_3 = 5 \times 10^4 \text{ s}^{-1}$, in good agreement with the experimental rate constant $k_3 = 6 \times 10^4 \text{ s}^{-1}$ [53]. The 450-fold decrease in k_3 caused by the *Rb. sphaeroides* CcO W143F mutation [48] is consistent with an additional $2.4 \text{ \AA}$ gap between the Cc heme CBC methyl group and CcO F143 [53].

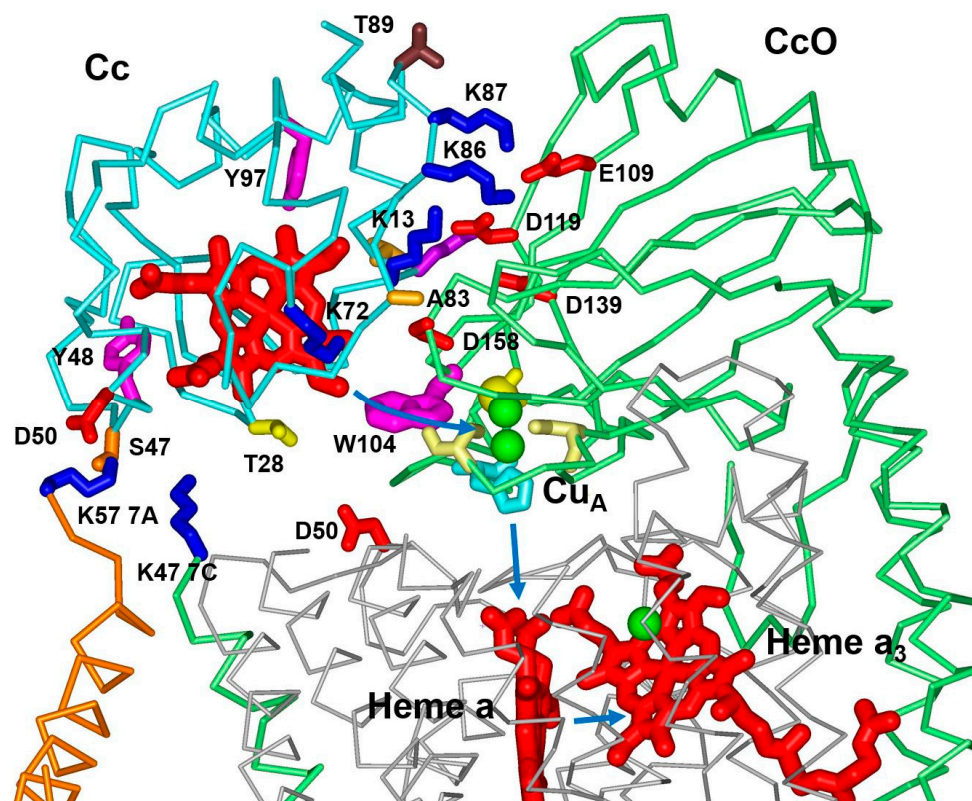
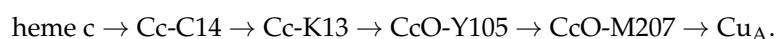


Figure 2. Computational model of the Cc:CcO complex based on the crystal structures of Cc (PDB 1HRC) and CcO (PDB 1OCC) [52]. Cc is colored blue, CcO subunit II is green, subunit I is grey, subunit 7A is orange, and subunit 7C is green. The positively charged Cc lysine residues are colored blue, and the negatively charged CcO glutamate and aspartate residues are colored red. The Cc amino acids mutated in this study are also shown. Blue arrows show the electron transfer pathway.

A crystal structure for the Cc–CcO complex [54] is very different from the computational model of the complex [52]. The binding domain of the Cc–CcO crystal structure is quite small, with several interactions of Cc residues K8, Q12, K13, and K87 with CcO residues. Calculations based on Marcus theory using the distance $R = 18.7 \text{ \AA}$ between the Ru-39-Cc heme and the Cu_A center in the crystal structure give a rate constant of $k_3 = 90 \text{ s}^{-1}$, much smaller than the experimental rate constant $k_3 = 6 \times 10^4 \text{ s}^{-1}$ [53]. A new pathway for electron transfer from Cc heme to Cu_A in the Cc:CcO crystal structure was proposed:



This is a long pathway with a running distance of $41.9 \text{ \AA}$ [54], with a calculated rate constant of $k_{\text{et}} = 3.3 \times 10^{-5} \text{ s}^{-1}$ using the Beratan tunneling pathway theory [53]. The Ru-39-Cc K13A mutant had very little effect on the experimental rate constant k_3 for electron transfer from Cc heme c to Cu_A , indicating that Cc Lys 13 is not on the electron transfer pathway [53]. The Rs CcO subunit II Y144 (bovine Y105) mutation caused only a 3.8-fold decrease in k_3 , indicating that Y105 is also not on the electron transfer pathway [53]. The Cc:CcO crystal structure [54] is therefore unlikely to be active in electron transfer. The

configuration of the Cc:CcO crystals could have been affected by crystal packing forces or by the conditions used in crystallization.

Cc is one of the most highly conserved proteins and is an excellent marker for evolutionary changes in species over the history of life on earth. Cc underwent three periods of accelerated evolution, the first early in vertebrate evolution, the second at the stem of the anthropoid primates, and the third in the catarrhine stem leading to the Old-World monkeys, apes, and humans [55]. Of the 11 amino acid changes that occurred from horse Cc to human Cc, eight occurred in the lineage from anthropoid primates to humans [55]. Five of these changes from horse to human Cc occurred at residues near the binding site of the Cc:CcO complex, V11I, M12Q, D50A, A83V, and T89E (Figures 1 and 3). The other six changes occurred farther from the binding site, and may also play a role in the function of Cc: A15S, F47Y, T58I, K70G, E62D, and E92A. There are only two amino acid changes from horse to bovine Cc, K60G and T89G [2], which should not affect the function of Cc. The binding site on CcO for Cc underwent a parallel accelerated evolution in the lineage from anthropoid primates to humans [56]. The CcO residues near the Cc binding site had over four times the number of changes as residues in other regions. It has been suggested that the rapid co-evolution of Cc and CcO has resulted in a very specific interaction between human Cc and human CcO [57]. One plausible reason for the accelerated evolution of Cc and CcO in primates is the greater need for energy production to support the expansion of the neocortical portion of the brain [58]. Another reason is the need to regulate the rate of mitochondrial electron transfer and membrane potential $\Delta\Psi$ to minimize formation of reactive oxygen species (ROS) and support the longer lifespan of humans [59]. In the present studies, the effect of single-point mutations at residues 11, 12, 50, 83 and 89 in horse and human Cc on electron transfer and binding Cc to CcO was determined. It was found that the dissociation constant K_D of the Cc:CcO complex was much smaller for human Cc than for horse Cc, indicating much tighter binding for human Cc. In the human mutants, K_D decreased in the order: native horse Cc > human A50D > V83A > I11V > N. Human Cc > M12Q > E89T. Corresponding results were found for horse Cc mutants.

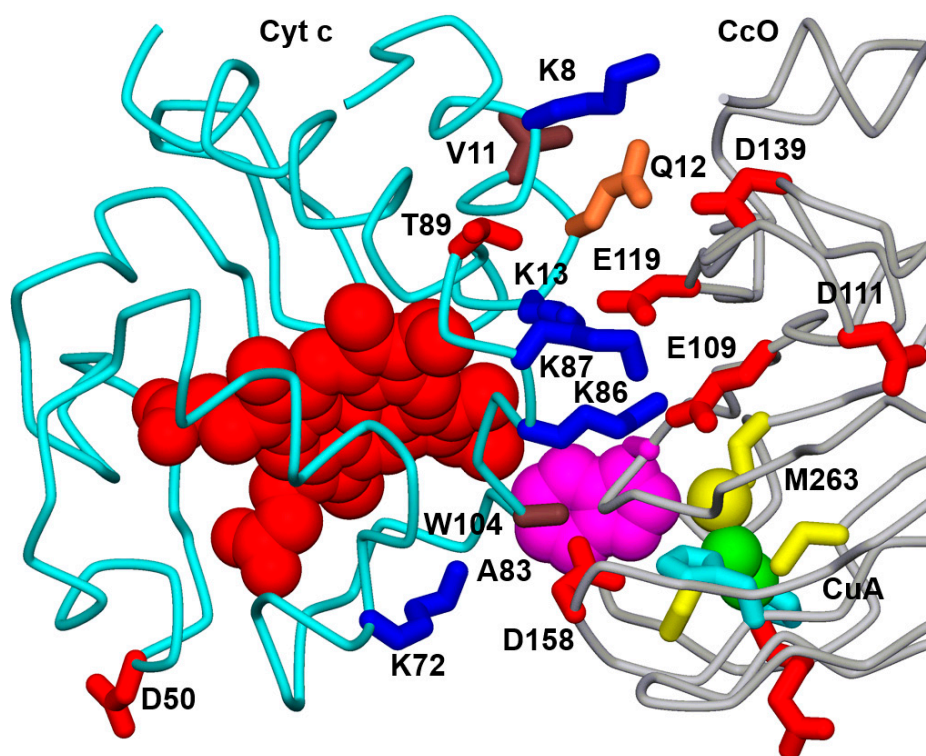


Figure 3. Structure of the computational horse Cc:CcO complex [52] showing the amino acid residues that are mutated from horse to human Cc. Cc lysines and CcO aspartates and glutamates are also shown.

The reaction between Cc and CcO plays a key role in regulating electron transfer and energy production in the entire mitochondrial respiratory chain [60]. Phosphorylation of key serine, threonine, and tyrosine amino acid residues on Cc and CcO has been found to regulate mitochondrial electron transfer and membrane potential $\Delta\Psi$, and minimize formation of ROS [59–63]. Defects in regulation of the reaction between Cc and CcO have been linked to many degenerative diseases and aging, and also to reperfusion injury following stroke and heart attack [60]. Cc is also the key activator of apoptosis, which involves release of dephosphorylated Cc from the mitochondria to the cytoplasm, and formation of the apoptosome, leading to cell death [59]. Phosphorylation of Cc minimizes release from the mitochondria and activation of apoptosis [59]. Cc can be phosphorylated at five different residues, Tyr97, Tyr48, Thr28, Ser47, and Thr58 in different tissues of the body [59,62,63]. In the present studies, the following phosphomimetic human Cc mutants have been prepared: Cc T28E, S47E, Y48E, Y97E, and the double mutant S47E-Y48E. The negatively charged carboxyl group on the glutamate mimics the phosphorylated serine, threonine or tyrosine. Laser kinetic studies indicated that the Cc T28E, S47E, and Y48E mutants increased the dissociation rate constant k_d of the Cc:CcO complex and decreased the formation rate constant k_f . Ultracentrifuge studies indicated that the equilibrium dissociation constant K_D of the Cc:CcO complex was increased by the Cc T28E, S47E, and Y48E mutants. These studies demonstrate that introduction of a negative charge at Cc T28, S47, or Y48 decreases the strength of the interaction between Cc and CcO, and indicates the phosphorylation of these residues plays an important role in regulating mitochondrial electron transport and membrane potential $\Delta\Psi$.

2. Materials and Methods

2.1. Materials

Horse heart cytochrome c (Type VI), lauryl maltoside, carboxyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy free radical (3CP), and aniline were obtained from Sigma-Aldrich, Inc., P.O. Box 14508, St. Louis, MO 68178, United States. The horse cytochrome c mutant, K39C Cc was prepared as described by Engstrom et al. [29]. Bovine CcO was prepared as described by Pan et al. [33].

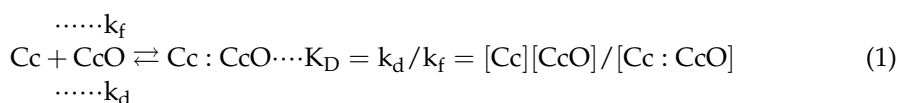
2.2. Site-Directed Mutagenesis

The native horse Cc and human Cc plasmids were a generous gift from Gary Pielak and are described in Olteanu et al. [64,65]. Single point mutations in native human or horse plasmid were made using the Stratagene QuikChange Site-Directed Mutagenesis Kit. The oligonucleotides for the horse mutations (D50A, V11I, A83V, T89E, and Q12M) and human mutations (A50D, I11V, V83A, E89T, M12Q, T28E, S47E, Y48E, and Y97E) were synthesized by Integrated DNA Technologies, Coralville, IA. Native and mutant Cc plasmids were sequenced by the University of Arkansas DNA resource center, Fayetteville, AR. Expression and purification procedures were followed as described by Olteanu et al. [64] except a waters 600 E HPLC and waters 991 photodiode array detector was used. To confirm the total mass and mass of digested protein fragments, MALDI TOF mass spectrometry was done on all mutants at the University of Arkansas Statewide Mass Spectrometry Facility, Fayetteville, AR.

2.3. Analytical Ultracentrifuge Experiments

Solutions for ultracentrifuge experiments contained 5 μM native or mutant Cc, 6 μM bovine CcO, 0.2% lauryl maltoside, and 5 mM phosphate buffer pH 7.0 and 0–350 mM NaCl. The sample and blank were injected into a double sector cell assembly and placed in an AN-60Ti rotor. The sample was spun at 48,000 r.p.m. and 22 °C using a Beckman XL-A analytical ultracentrifuge, and the 410 nm absorbance was measured every 10 min for a total time of 75 min. The sedimentation velocities are unbound Cc: 1.74 S, unbound CcO: 9.7 S and the 1:1 Cc:CcO complex: 11.4 S [48]. The ultracentrifuge experiments

were analyzed assuming that oxidized Cc forms a 1:1 complex with CcO, which has been previously verified under these conditions:

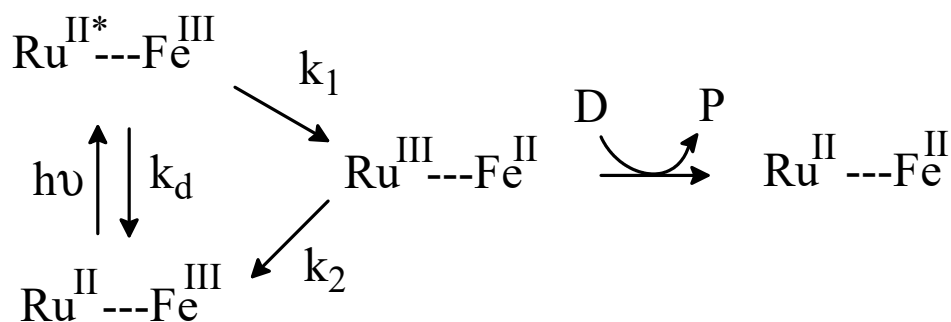


The concentration of free Cc, [Cc], was calculated from the absorbance in the free Cc plateau region using an extinction coefficient of $106 \text{ mM}^{-1} \text{ cm}^{-1}$ at 410 nm. The concentration of the 1:1 Cc:CcO complex was $[\text{Cc}:\text{CcO}] = [\text{Cc}_T] - [\text{Cc}]$, and $[\text{CcO}] = [\text{CcO}_T] - [\text{CcO}]$, where $[\text{Cc}_T]$ and $[\text{CcO}_T]$ are the total concentrations of Cc and CcO, respectively. Following each run, the concentration of salt within the sample cell was increased by adding 1 M NaCl. The salt concentrations for each ultracentrifuge run were 0, 70, 100, 120, 150, and 350 mM. The final salt concentration (350 mM) was used to completely inhibit Cc binding to CcO.

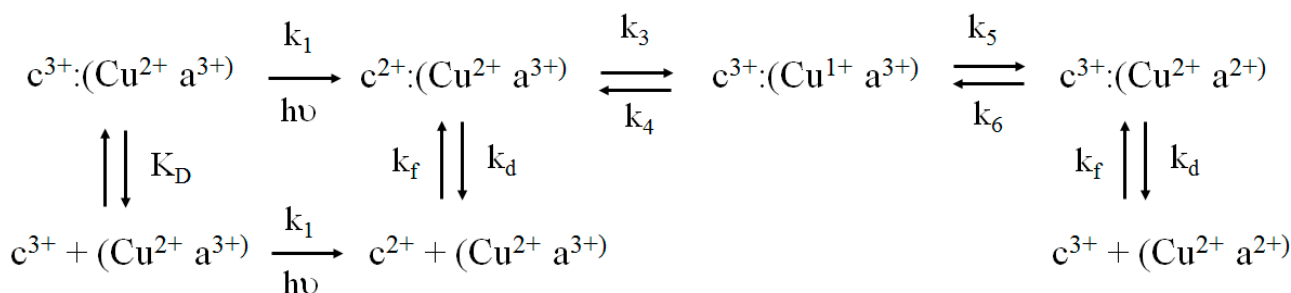
2.4. Flash Photolysis Experiments

Laser flash photolysis experiments involving Ru-39-Cc were carried out as previously described [34,48,53]. The reactions of Cc heme, CcO CuA, and CcO heme a were monitored at 550 nm, 830 nm, and 605 nm, respectively [34,48,53,66–68].

Reactions were carried out in 2 mM sodium phosphate, pH 7.0 or 5 mM TrisCl, pH 8.0 with 0.1 % dodecyl maltoside at 22 °C. 10 mM aniline and 1 mM 3CP were included as sacrificial electron donors D to reduce Ru^{III} to Ru^{II} (Scheme 1). Numerical integration was used to fit the time dependence of the absorbances to Scheme 2 [53].



Scheme 1. Mechanism of photoreduction of heme Fe^{III} in Ru-39-Cc.



Scheme 2. Mechanism of photoinduced electron transfer from Ru-39-Cc to CcO.

At low ionic strength when CcO was in excess of Ru-39-Cc, only a single phase of Ru-39-Cc oxidation was observed, which is due to intracomplex electron transfer from photo-reduced heme c to CcO CuA in the 1:1 Ru-39-Cc:CcO complex. As the ionic strength was increased, a second slow phase with rate constant k_{obs} is observed due the second-order reaction of free Ru-39-Cc^r with CcO. At intermediate ionic strength, k_f , k_d , and K_D could be measured from the fraction of the fast phase and the rate constant of the slow second-order

phase as shown below [34]. Previous studies have established that $k_3 \gg k_d$ [34]. Therefore, the rate equation below is valid for the slow second-order reaction k_{obs} :

$$\begin{aligned} d[Cc^r]/dt &= -k_f [CcO][Cc^r] = -(k_f[CcO])[Cc^r] = -k_{obs} [Cc^r] \\ k_{obs} &= k_f[CcO] \cdots k_f = k_{obs}/[CcO] \end{aligned} \quad (2)$$

The fraction of the fast phase is $f = \Delta A_{fast}/(\Delta A_{fast} + \Delta A_{slow})$

$[Cc_T]$ and $[CcO_T]$ are the total concentrations of Cc and CcO.

$$\begin{aligned} [Cc] &= (1 - f)[Cc_T] \cdots [Cc:CcO] = f [Cc_T] [CcO] = [CcO_T] - f [Cc_T] \\ K_D &= [Cc][CcO]/[Cc:CcO] = ((1 - f)([CcO_T] - f[Cc_T]))/f \end{aligned} \quad (3)$$

$$k_f = k_{obs}/([CcO_T] - f [Cc_T]) \quad (4)$$

$$k_d = k_f K_D = k_{obs} (1 - f)/f \quad (5)$$

At high ionic strength where $K_D \gg [CcO_T]$, and $f = 0$

$$k_f = k_{obs}/[CcO_T] \quad (6)$$

At low ionic strength where $K_D \ll [CcO_T]$, a slow second-order reaction can only be observed when $[Cc_T] > [CcO_T]$. Under these conditions, $f = [CcO_T]/[Cc_T]$ and

$$k_d = k_{obs}([Cc_T] - [CcO_T])/[CcO_T] \quad (7)$$

Experiments were also carried out to study the kinetics of the reaction between Cc and CcO using laser-excited lumiflavin to rapidly transfer an electron to unbound oxidized Cc to form Cc^r [34,69]. Excited lumiflavin cannot transfer an electron to Cc in the Cc:CcO complex because the heme crevice of Cc is not accessible. Excited lumiflavin also cannot reduce CcO Cu_A or heme a [69]. The lumiflavin flash photolysis technique is limited to the measurement of rate constants k_{obs} of less than 2000 s⁻¹. It was used to measure k_d at low ionic strength, and k_f at high ionic strength.

3. Results

3.1. Effect of Mutations from Horse Cc to Human Cc on the Reaction with CcO

The role of amino acid residues that are mutated from horse Cc to human Cc was studied by preparing horse Cc mutants in which one of the Cc residues V11, Q12, D50, A83, and T89 was changed to the amino acid in human Cc. A similar set of human Cc mutants was prepared.

The effect of the horse and human Cc mutants on the dissociation constant K_D of the 1:1 Cc:CcO complex was measured using analytical ultracentrifuge sedimentation velocity experiments of 5 μ M Cc and 6 μ M CcO in 5 mM sodium phosphate pH 7.0 and 0–300 mM NaCl, as previously described [48]. At ionic strengths below 50 mM, the K_D values of native horse and human Cc were <0.2 μ M, too small to measure, indicating very strong binding. At ionic strengths greater than 150 mM, the K_D values were >30 μ M, indicating very weak binding. The K_D values of horse and human Cc and all the mutants at an ionic strength of 110 mM are shown in Table 1. Native human Cc has a K_D value of 4.7 μ M, compared to 28.4 μ M for native horse Cc, indicating much stronger binding of human Cc to bovine CcO. The largest effect of the mutations was at residue 50, where the A50D human Cc mutant increased K_D to 15.7 μ M, and the D50A horse Cc mutant decreased K_D to 11.8 μ M. The E89T human Cc mutant decreased K_D to 2.1 μ M, while the T89E horse Cc mutant increased K_D to >35 μ M, indicating that the negative charge on E89 decreases binding strength.

K_d was measured by an analytical ultracentrifuge using 5 μ M native or mutant Cc, 6 μ M native Bovine CcO in 5 mM Pi pH 7.0, 100 mM NaCl, 0.2% Lauryl maltoside. The error limits are $\pm 20\%$.

Table 1. Dissociation constants (K_d) of native or mutant Cc complex with bovine CcO.

Cyt c	K_d (μM)	Cyt c	K_d (μM)
Horse	28.4	Horse	28.4
Human	4.7	Human	4.7
Horse V11I	23.4	Human I11V	2.9
Horse Q12M	14.3	Human M12Q	1.7
Horse D50A	11.8	Human A50D	15.7
Horse A83V	6.9	Human V83A	5.9
Horse T89E	>35	Human E89T	2.1

Horse, human, and human A50D Ru-39-Cc mutants were prepared to study the rapid kinetics of Ru-39-Cc with CcO. Laser excited $\text{Ru}^{\text{II}*}$ transfers an electron to heme c Fe^{III} in Ru-39-Cc to form $\text{Ru}^{\text{III}}-\text{Fe}^{\text{II}}$ with a rate constant of $k_1 = 600,000 \text{ s}^{-1}$ (Scheme 1) [34]. $\text{Ru}^{\text{III}}-\text{Fe}^{\text{II}}$ is rapidly reduced to $\text{Ru}^{\text{II}}-\text{Fe}^{\text{II}}$ by the sacrificial donors aniline and 3CP to prevent the back reaction k_2 [34]. Laser excitation of a 1:1 complex between human Ru-39-Cc and bovine CcO at low ionic strength led to electron transfer from photoreduced heme c to Cu_A with rate constants $k_3 = 62,000 \pm 10,000 \text{ s}^{-1}$ and $k_4 = 30,000 \pm 6000 \text{ s}^{-1}$, followed by electron transfer from Cu_A to heme a with rate constants $k_5 = 20,000 \pm 4000 \text{ s}^{-1}$ and $k_6 = 2700 \pm 600 \text{ s}^{-1}$ (Scheme 2) (Figure 4). The kinetics of heme c, Cu_A , and heme a were measured at 550 nm, 830 nm, and 605 nm and fitted to the top line of Scheme 2 as previously described [53]. The ratios k_3/k_4 and k_5/k_6 are consistent with the redox potentials of Cc, Cu_A and heme a [34,70,71]. There was no change in the rate constants from 0 to 60 mM NaCl. At ionic strengths above 60 mM, the amplitude of the fast intracomplex phase decreased, indicating that the Ru-39-Cc:CcO complex partially dissociated. The resulting uncomplexed Ru-39-Cc reacted with uncomplexed CcO in a new slow phase bimolecular reaction (Figure 5, Scheme 2) [34,53]. The fraction of the fast intracomplex phase was $f = 0.5 \pm 0.1$ at 115 mM ionic strength, and the equilibrium dissociation constant $K_D = 8.0 \pm 2 \mu\text{M}$ was calculated from Equation (3) in the Section 2. The bimolecular phase had a rate constant of $k_{\text{obs}} = 2400 \pm 300 \text{ s}^{-1}$, and $k_d = 2400 \pm 400 \text{ s}^{-1}$, and $k_f = 290 \pm 40 \text{ s}^{-1} \mu\text{M}^{-1}$ were calculated from Equations (4) and (5). At NaCl concentrations above 150 mM, no intracomplex phase was detected, $K_D > 50 \mu\text{M}$, and k_f could be determined from Equation (6). k_f decreased with increasing ionic strength as expected for a reaction involving electrostatic interactions between the partners. The value of k_d was determined at low ionic strength in experiments using 10 μM Ru-39-Cc and 5 μM CcO as described in the experimental procedures section. Under these conditions $K_D < 0.1$ and k_d could be determined from Equation (7). For the reaction between human Ru-39-Cc and CcO, $k_d = 16 \text{ s}^{-1}$ at 30 mM NaCl, and increased to 96 s^{-1} at 70 mM NaCl (Figure 5). Horse Ru-39-Cc dissociated from CcO at a lower ionic strength than human Ru-39-Cc, had a larger k_d at low ionic strength, and a smaller k_f at high ionic strength (Figure 5). These results are consistent with a stronger electrostatic interaction between Human Cc and CcO. Experiments were carried out in 5 mM sodium phosphate pH 7 buffer as well as in 5 mM TrisCl buffer to determine whether the kinetics were sensitive to pH. The ionic strength dependence of k_f for the reaction of human Ru-39-Cc with CcO was nearly the same at pH 8.0 (Figure 5) and pH 7.0 (Figure 6). The value of k_f was $290 \pm 40 \mu\text{M}^{-1} \text{ s}^{-1}$ at pH 8.0 and 115 mM ionic strength (Figure 5), compared to $k_f = 260 \pm 40 \mu\text{M}^{-1} \text{ s}^{-1}$ at pH 7.0 and 110 mM ionic strength (Figure 6). The ionic strength dependence of k_f for the reaction of horse Ru-39-Cc with CcO was also nearly the same at pH 8.0 (Figure 5) as at pH 7.0 [34]. The value of k_d was only determined at pH 7.0 (Figures 5 and 6).

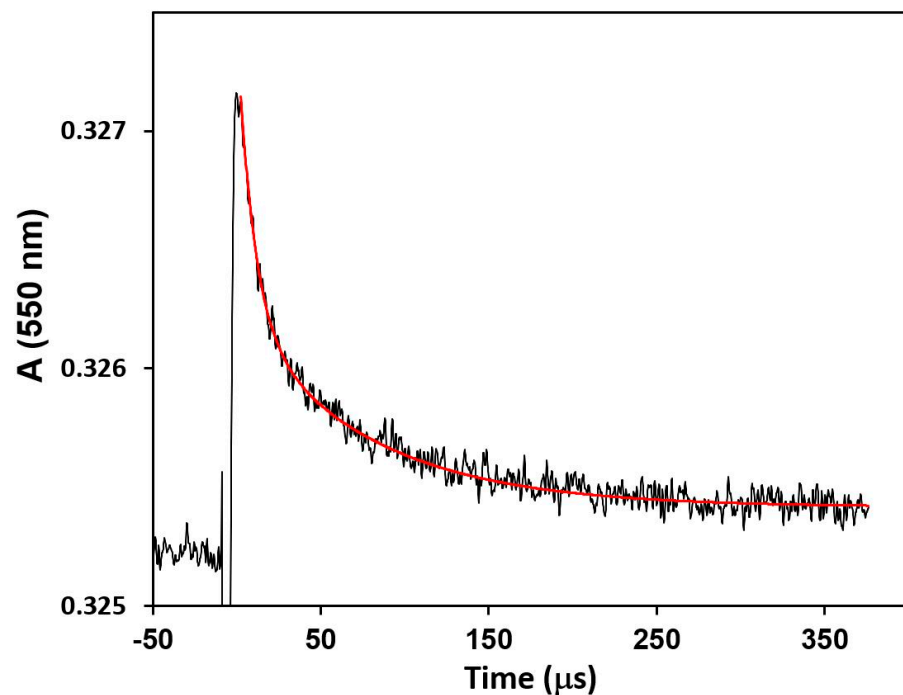


Figure 4. Photoinduced electron transfer from human Ru-39-Cc to bovine CcO showing the laser induced reduction of Cc heme c at 550 nm followed by electron transfer to CcO Cu_A. The solution contained 7.6 μM Ru-39-Cc and 12 μM CcO in 5 mM TrisCl, pH 8.0, 0.1% dodecyl maltoside, 10 mM aniline, and 1 mM 3CP. The solid line is the best fit to the transient using numerical integration of Scheme 2 as described in the text. $k_3 = 62,000 \text{ s}^{-1}$ and $k_4 = 30,000 \text{ s}^{-1}$.

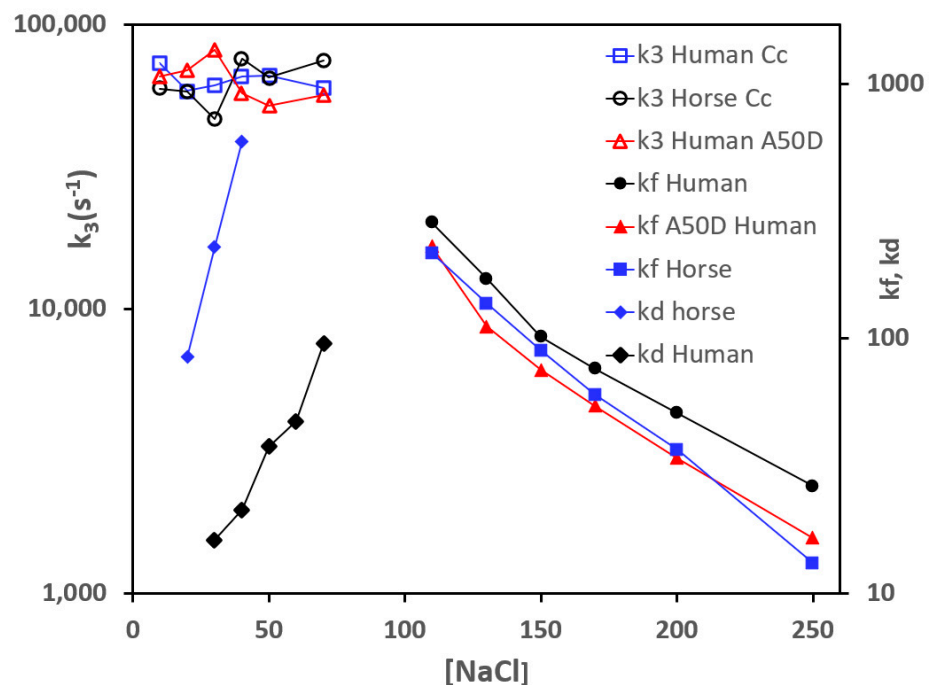


Figure 5. Laser-induced electron transfer from horse, human, and human A50D Ru-39-Cc to bovine CcO. The rate constants k_3 , k_d , and k_f were measured as described in the text. k_3 and k_d are in s^{-1} , while k_f is in $\mu\text{M}^{-1} \text{ s}^{-1}$. The solutions for measuring k_3 and k_f contained approximately 8 μM Ru-39-Cc and 15 μM CcO in 5 mM TrisCl, pH 8.0, 0.1% dodecyl maltoside, 10 mM aniline, and 1 mM 3CP. The solutions for measuring k_d contained 10 μM Ru-39-Cc and 5 μM CcO in 5 mM sodium phosphate, pH 7.0, 0.1% dodecyl maltoside, 10 mM aniline, and 1 mM 3CP.

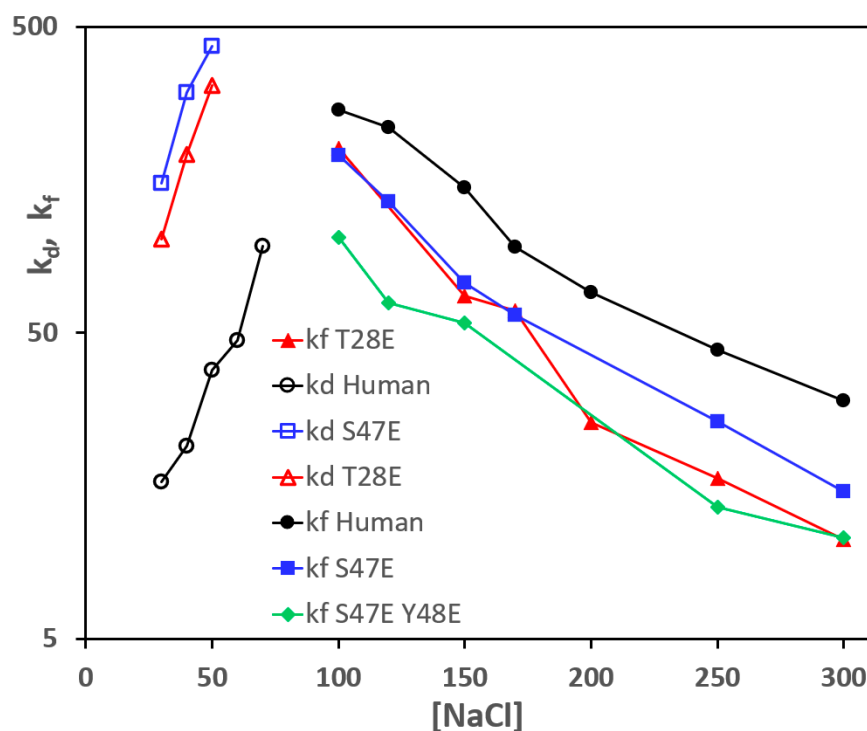


Figure 6. Laser-induced electron transfer from native human Cc wild type, T28E, and S47E and S47E-Y48E mutants to bovine CcO. The k_d and k_f values of the human Cc mutants were measured using the lumiflavin laser flash photolysis method as described in the text. k_d is in s^{-1} , while k_f is in $\mu M^{-1} s^{-1}$. The samples contained 10 μM human Cc, 5 μM bovine CcO in 5 mM sodium phosphate, pH 7.0, 0.1 % lauryl maltoside, 100 μM lumiflavin, 0.5 mM EDTA and the indicated concentrations of NaCl.

3.2. Effect of Phosphomimetic Mutations of Human Cc on the Reaction with CcO

The phosphomimetic human Cc mutants T28E, S47E, Y48E, and Y97E were prepared to determine the effect of phosphorylation of Cc T28, S47, Y48, and Y97 on the binding of Cc to CcO and the regulation of the reaction. The effect of the human Cc mutants on the dissociation constant K_D of the 1:1 Cc:CcO complex was measured using analytical ultracentrifuge sedimentation velocity experiments of 5 μM Cc and 6 μM CcO in 5 mM sodium phosphate pH 7.0 and 0–300 mM NaCl. At ionic strengths below 60 mM, the K_D values of native Cc and all the mutants were $<0.2 \mu M$, indicating very strong binding. At ionic strengths greater than 150 mM, the K_D values were $>30 \mu M$, indicating very weak binding. The K_D values of wild-type human Cc and all the phosphomimetic mutants could be measured at an ionic strength of 110 mM (Table 2). The K_D values of Cc T28E, S47E, Y48E were all significantly larger than that of wild-type human Cc, indicating that introducing a negative charge at that residue decreases the interaction strength. However, the Cc Y97E did not have a significant effect on K_D .

Table 2. Human Phosphomimetic Mutant Kinetics.

Cyt c	K_d (μM)	k_d (s^{-1})	k_f ($\mu M^{-1} s^{-1}$)
Human	4.7	1203	256
Human T28E	18.0	3456	192
Human S47E	24.0	4344	181
Human Y48E	10.5		
Human Y97E	5.1		

K_D , k_f , and k_d were measured in 5 mM sodium phosphate, pH 7.0 with 100 mM NaCl. K_D was measured by the ultracentrifuge method, and k_f and k_d were measured by the laser flash photolysis method. The error limits are $\pm 20\%$.

The kinetics of the reaction between human Cc phosphomimetic mutants and CcO was studied using laser-excited lumiflavin to rapidly transfer an electron to unbound oxidized Cc^o to form reduced Cc^r [34,69]. Excited lumiflavin cannot transfer an electron to Cc^o in the Cc^o:CcO complex because the heme crevice of Cc is not accessible. Excited lumiflavin also cannot reduce CcO Cu_A or heme a [69,72]. The lumiflavin flash photolysis technique is limited to the measurement of rate constants k_{obs} of less than 2000 s^{-1} . Flash photolysis of a sample containing 10 μM human Cc, 4.5 μM CcO, 100 μM lumiflavin, 0.5 mM EDTA, 30 mM NaCl in 5 mM sodium phosphate buffer resulted in reduction of unbound Cc, followed by electron transfer from Cc^r to CcO with a rate constant of $k_{obs} = 13\text{ s}^{-1}$. Since $K_D < 0.1\text{ }\mu\text{M}$ at low ionic strength, k_d can be determined from Equation (7) to be $k_d = 16\text{ s}^{-1}$ at 30 mM NaCl (Figure 6). As the ionic strength was increased to 100 mM NaCl, k_{obs} increased to 250 s^{-1} , and then decreased with further increases in NaCl. At ionic strengths above 150 mM, $K_D > 30\text{ }\mu\text{M}$, and k_f can be determined from Equation (6) (Figure 6). The T28E and S47E human Cc mutants significantly increased the value of k_d at low ionic strength, and decreased the value of k_f at high ionic strength (Figure 6, Table 2). The S47E Y48E double mutant decreased the value of k_f more than that of the S47E single mutant. These results indicate that phosphorylation of human Cc T28, S47, and Y48 decreases the strength of the interaction between Cc to CcO.

4. Discussion

The analytical ultracentrifuge experiments indicated that the dissociation constant $K_D = 4.7\text{ }\mu\text{M}$ for the 1:1 complex of human Cc with bovine CcO compared to $28\text{ }\mu\text{M}$ for the complex of horse Cc with bovine CcO complex at 110 mM ionic strength (Table 1). These results are in agreement with earlier studies showing that human Cc binds more strongly to bovine CcO than does horse Cc [72]. Five amino acid residues at the Cc binding domain which differ between horse and human Cc have been identified, 11, 12, 50, 83 and 89 (Figure 3). To explore the role of these amino acid residues in binding CcO, single-point mutations at residues 11, 12, 50, 83 and 89 in horse and human Cc were prepared. It was found that for the human mutants K_D decreased in the order: native horse Cc > human A50D > V83A > I11V > native human Cc > M12Q > E89T (Table 1). For the horse mutants K_D decreased in the order: T89E > native horse Cc > V11I Cc > Q12M > D50A > A83V > native human (Table 1). The largest effect was observed for the mutants at residue 50, where the horse Cc D50A mutant decreased K_D from 28.4 to $11.8\text{ }\mu\text{M}$, and the human Cc A50D increased K_D from 4.7 to $15.7\text{ }\mu\text{M}$. Cc residue 50 is located towards the back of Cc a distance from the main binding site on CcO subunit II (Figures 1–3). However, it is close to subunit 7A, and interactions with this subunit might account for the effect of the mutations (Figure 3). A large effect was also observed for mutations at Cc residue 89, where the horse Cc T89E mutant increased K_D to >35 , and the human Cc E89T decreased K_D to 2.1 . Residue 89 is near the CcO subunit II binding domain, and electrostatic repulsion by the negatively charged E89 could explain the effects of these mutants (Figure 3). Horse Cc residue A83 is very close to the interaction domain, and the horse A83V mutation could increase the hydrophobic interaction, accounting for the decrease in K_D . The horse Q12M and the human M12Q mutations both cause a decrease in K_D , which might be due to the location of Q12 within the interaction domain and subtle differences in the orientation of horse and human Cc.

Laser flash photolysis studies indicated that horse, human, and human A50D Ru-39-Cc had nearly the same rate constant $k_3 = 60,000\text{ s}^{-1}$ for electron transfer from heme c to Cu_A in the Ru-39-Cc:CcO complex at low ionic strength (Figure 5). The complex dissociation rate constant k_d for both horse and human Ru-39-Cc increased rapidly with increasing ionic strength, consistent with a strong electrostatic interaction between Cc and CcO (Figure 5). At $40\text{ }\mu\text{M}$ NaCl, $k_d = 592$ for horse Ru-39-Cc, compared to $k_d = 21$ for human Ru-39-Cc,

indicating much weaker binding for horse Cc at low ionic strength. As the ionic strength was raised above 120 mM the Cc:CcO complex completely dissociated, and the formation rate constant k_f was found to decrease with increasing ionic strength, as expected for an electrostatic interaction. The k_f values were smaller for horse and human A50D Ru-39-Cc than for human Ru-39-Cc, indicating weaker binding.

The phosphomimetic human Cc mutants T28E, S47E, Y48E, and Y97E were used to study the effect of phosphorylation of Cc T28, S47, Y48, and Y97 on the binding of Cc to CcO (Figure 6, Table 2). The human Cc mutants T28E, S47E, Y48E all increased the value of K_D significantly, indicating weaker binding, while the Y97E mutant had no significant effect on K_D . The T28E and S47E mutants significantly increased the complex dissociation rate constant k_d at low ionic strength, and decreased the complex formation rate constant k_f at high ionic strength, consistent with a decrease in the binding strength. Cc T28 is phosphorylated in kidney, causing a 50% decrease in oxygen consumption, and the V_{max} of the reaction of the phosphomimetic mutant Cc T28E with CcO was 78% reduced compared to wild-type Cc [63]. Cc S47 is phosphorylated in brain, causing a 48% decrease in oxygen consumption, and the reaction of the Cc S47E mutant with CcO was decreased by 54% [62]. Cc Y48 is phosphorylated in liver, and the K_m of the reaction of the phosphomimetic mutant Cc Y48E with CcO is 3.6-fold larger than the wild-type Cc, indicating weaker binding [73]. Cc Y97 is phosphorylated in heart, and the K_m for the reaction with CcO is increased 2-fold [74]. Tyr 97 phosphorylation was also characterized with the replacement of tyrosine with a phosphomimetic, p-carboxymethyl-L-phenylalanine (pCMF), which actually increased the CcO activity [75,76]. Cc is phosphorylated at T58 in rat kidney, which controls mitochondrial respiration and apoptosis [77]. However, isoleucine is present at Cc residue 58 in humans, which lack tissue-specific isoforms such as the testis isoform, and only express a single Cc. The present results are in agreement with previous studies and provide more detailed information on how the phosphomimetic Cc mutants affect the k_f and k_d for the reaction with CcO.

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Abbreviations

Cc	cytochrome c
CcO	cytochrome c oxidase
Ru-39-Cc	(dimethylbipyridine)(bisbipyridine)ruthenium-Cys-39-cytochrome c
Ru-55-Cc	(dimethylbipyridine)(bisbipyridine)ruthenium-Lys-55-cytochrome c
3CP	carboxyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy free radical
Ru ₂ Z	Ru(bipyrazine) ₂ -(quaterpyridine)
<i>Rb. sphaeroides</i>	<i>Rhodobacter sphaeroides</i>

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