Published in final edited form as:

Biochemistry. 2015 February 10; 54(5): 1188–1197. doi:10.1021/bi500888y.

Control of Cyclic Photoinitiated Electron Transfer between Cytochrome c Peroxidase (W191F) and Cytochrome c by Formation of Dynamic Binary and Ternary Complexes

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Abstract

Extensive studies of the physiological protein—protein electron-transfer (ET) complex between yeast cytochrome c peroxidase (CcP) and cytochrome c (Cc) have left unresolved questions about how formation/dissociation of binary and ternary complexes influence ET. We probe this issue through study of the photocycle of ET between Zn-ProtoporphyrinIX-substituted CcP(W191F) (ZnPCcP) and Cc. Photoexcitation of ZnPCcP in complex with Fe^{3+}Cc , initiates the photocycle: charge-separation ET $[\text{ZnPCcP}, \text{Fe}^{3+}\text{Cc}] \rightarrow [\text{ZnP}^+\text{CcP}, \text{Fe}^{2+}\text{Cc}]$ followed by charge recombination, $[\text{ZnP}^+\text{CcP}, \text{Fe}^{2+}\text{Cc}] \rightarrow [\text{ZnPCcP}, \text{Fe}^{3+}\text{Cc}]$. The W191F mutation eliminates fast hole hopping through W191, enhancing accumulation of charge-separated intermediate and extending the timescale for binding/dissociation of the charge-separated complex. Both triplet quenching and the charge-separated intermediate were monitored during titrations of ZnPCcP with Fe^{3+}Cc , Fe^{2+}Cc , and redox-inert CuCc. The results require a photocycle that includes dissociation/recombination of the charge-separated binary complex and a charge-separated ternary complex, $[\text{ZnP}^+\text{CcP}, \text{Fe}^{2+}\text{Cc}, \text{Fe}^{3+}\text{Cc}]$. The expanded kinetic scheme formalizes earlier proposals of “substrate-assisted product dissociation” within the photocycle. The measurements yield the thermodynamic affinity constants for binding the first and second Cc: $K_I = 10^{-7} \text{ M}^{-1}$, $K_{II} = 10^{-4} \text{ M}^{-1}$. However, two-site analysis of the thermodynamics of formation of the ternary reveals that Cc binds at the weaker-binding site with much greater affinity than previously recognized, and places upper bounds on the contributions of repulsion between the two Cc of the ternary complex. In conjunction with recent NMR studies, the analysis further suggests a dynamic view of the ternary complex, wherein neither Cc necessarily faithfully adopts the crystal-structure configuration because of Cc-Cc repulsion.

The complex between the physiological protein—protein electron-transfer (ET) partners, yeast cytochrome c peroxidase (CcP) and cytochrome c (Cc) was the first ET-active complex to be crystallized, and remains a paradigm for ET within a well-defined complex.¹

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Supporting Information Available

Triplet decay curves with fits for the Fe^{3+}Cc titration, fit parameters for Fe^{2+}Cc and CuCc intermediate progress curves to Scheme 1, extended and modified kinetic schemes used to analyze Fe^{2+}Cc and CuCc titration intermediate curves, comparisons of triplet decay rate constants for the Fe^{2+}Cc titration, and details of equations for the global triple exponential equation and the system of differential equations used in Schemes 1 are provided in the supplementary information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Despite extensive studies of this pair by NMR,²⁻⁴ crystallography,^{5, 6} photo-induced kinetic measurements,⁷⁻⁹ and other spectroscopic techniques,¹⁰⁻¹² there nonetheless remain fundamental questions about how ET between them is modulated by the formation and dissociation of the complex on the ET timescale, and about the possible role of a ternary complex in reactivity.⁴ The pair has been particularly amenable to study because the heme-iron of either partner can be substituted by Zn (or Mg) to form a complex that exhibits an ET photocycle.⁸ When the Fe of CcP is substituted to form ZnPCcP and is in complex with the iron form of the partner protein, Fe³⁺Cc, the complex can undergo a laser-initiated ET photocycle comprised of 'forward' charge-separation (CS) ET (³ZnPCcP;Fe³⁺Cc→ZnP⁺CcP;Fe²⁺Cc), rate constant k_f , to produce the charge-separated intermediate protein pair, ZnP⁺CcP and Fe²⁺Cc—denoted **I** representing all states involving ZnP⁺CcP—followed by 'back' charge-recombination (CR) ET to regenerate the initial state (ZnPCcP;Fe³⁺Cc←ZnP⁺CcP;Fe²⁺Cc) with rate constant k_b ,¹³ a process analogous to physiological ET wherein Fe²⁺Cc reduces CcP Compound ES.

The CR process in the complex formed with ZnP-substituted, wild-type CcP is extremely rapid, $k_b \sim 3500\text{s}^{-1} \gg k_f$, because CcP residue tryptophan 191 on the proximal side of the heme 'short-circuits' direct heme-heme 'back' ET via 'hole hopping' in which the ZnP⁺ oxidizes W191 and the W191⁺ cation radical oxidizes Fe²⁺Cc. This process makes it difficult to examine the behavior of the CS intermediate, **I**, and altogether precludes examination of the dynamic processes within **I** on longer timescales.^{8, 14-17} To fully explore the role of complex formation/dissociation dynamics in the ET photocycle, we employ CcP W191F; this mutation negligibly perturbs the overall structure of CcP¹⁸ and does not impact the ability of Fe³⁺Cc to quench ³ZnPCcP, but prevents the short-circuit and slows CR.⁹ The [ZnPCcP(W191F), Fe³⁺Cc] complex thus is well-suited for studying direct interprotein heme-heme ET, and its use enables us to monitor **I** over a wide range of timescales.⁹

In the present study, the charge separated intermediate was monitored through titrations of ZnPCcP by Fe³⁺Cc, Fe²⁺Cc, and redox-inert CuCc.¹⁹ These studies allow a detailed examination of the dynamics of formation/dissociation of the binary [CcP, Cc] complex, revealing the importance of the charge-separated ternary complex, **I**₃(2+/3+) $\equiv$ [ZnP⁺CcP, Fe²⁺Cc, Fe³⁺Cc].²⁰ These findings are incorporated into an extended kinetic mechanism for the ET photocycle between ZnPCcP(W191F) and yeast Iso-1 Fe³⁺Cc that formalizes and enhances earlier proposals of "substrate-assisted product dissociation",²¹ by incorporation of a dynamic ternary complex.²²

The measurements of CS further yield the consensus values of the thermodynamic affinity constants for binding the first and second Cc. However, a two-site analysis of the thermodynamics of formation of the ternary that explicitly incorporates repulsion between the two Cc, reveals that Cc binds at the weaker-binding site with much greater affinity than previously recognized, and places upper bounds on the free energy of repulsion between the two Cc of the ternary complex. In conjunction with recent descriptions of the equilibrium between encounter and docked complexes,^{23, 24} the analysis further suggests a modified view of the ternary complex's structure, as a dynamic association of CcP and two Cc wherein neither Cc necessarily adopts the crystal-structure configuration because of Cc-Cc repulsion.

Materials and Methods

Protein preparation

Recombinant yeast cytochrome c peroxidase (CcP) W191F mutant based on the MKT construct and recombinant yeast Iso-1 cytochrome c (Cc) were isolated and purified using previously published protocols.^{25, 26} Holoenzyme CcP purity was assayed using a 408nm/282nm ratio of 1.3 and SDS-PAGE. ApoCcP was prepared and subsequently reconstituted with Zn-protoporphyrinIX in the dark as previously described²⁷ and purity was assayed using a 432nm/282nm ratio greater than 3 and SDS-PAGE. To prepare metal-free Cc, in a chemical hood, 3–10mL pyridine-HF were added to ~100mg lyophilized FeCc in a polyethylene beaker and gently stirred in the dark with a magnetic stirrer for 10 minutes with a stream of nitrogen blowing over the surface. The reaction was quenched with 10mL of 50mM Acetate buffer, pH 5 and allowed to gently stir for 30 minutes with nitrogen blowing over the surface. Appropriate HF respirators, gloves, laboratory coats, and aprons were used at all times when HF was in use. Excess Fe and pyridine were removed using a short Sephadex G-50 column. Metal-free Cc was diluted by half with water and loaded onto a short Whatman CM52 column equilibrated with 25mM Acetate buffer, pH 5. The column was washed extensively with buffer to remove pyridine and eluted with 25mM Acetate 500mM NaCl buffer, pH 5. CuCc was prepared from metal-free Cc as previously described.²⁸

Kinetic Measurements—Transient absorption kinetics measurements were performed on a laser flash-photolysis apparatus described elsewhere. Wild type and mutant FeCc were freshly oxidized with at least 10-fold excess potassium ferricyanide the day of experiments. Excess potassium ferricyanide was removed by passing the Fe^{3+}Cc and potassium ferricyanide solution through a 2mL CM-52 column preequilibrated with water. Fe^{3+}Cc was eluted using 500mM NaCl, 25mM KPi buffer, pH 7. Proteins were exchanged into potassium phosphate buffers and concentrated using ultracentrifugation. Protein stocks were flushed with argon using a Schlenk line prior to addition and transferred to sample cuvettes using Hamilton gastight syringes 30 minutes before experiments to allow for further oxygen scrubbing. For all samples, the day before experiments, 2.00mL buffer in an airtight quartz cuvette was flushed with argon for 1 hour using a Schlenk line after which a catalase oxygen-scavenging system was added to ensure complete deoxygenation (final concentrations: 10 μM catalase, 10 μM glucose oxidase, 10 μM glucose). Concentrations were determined using $\epsilon_{432\text{nm}} = 196\text{mM}^{-1}\text{cm}^{-1}$ for ZnPCcP, $\epsilon_{409\text{nm}} = 106\text{mM}^{-1}\text{cm}^{-1}$ $\epsilon_{416\text{nm}} = 129\text{mM}^{-1}\text{cm}^{-1}$ for Fe^{3+}Cc and Fe^{2+}Cc respectively, and $\epsilon_{422\text{nm}} = 138\text{mM}^{-1}\text{cm}^{-1}$ for CuCc.

Model Simulation and Fitting—Global fits of titration kinetic traces to analytical expressions and numerical fits to the set of kinetic differential equations were calculated with MATLAB using routines written in-house. Numerical integration of the set of differential equations for Scheme 1 employed the MATLAB ode45 solver (which uses the explicit Runge-Kutta (4,5) formula, Dormand-Prince pair). The 95% confidence intervals for the kinetic parameters were calculated with the built-in *nlparci* function in MATLAB. Details of the differential equations and global fitting equations are presented in SI.

Results

Low salt (10mM KPi) Fe³⁺Cc titration

ET Quenching—Fe³⁺Cc was titrated into a sample of ~5μM ZnPCcP(W191F) in 10mM KPi buffer, pH 7 at 20 C. In the absence of Fe³⁺Cc, ³ZnPCcP decays exponentially with $k_1 \approx 90\text{s}^{-1}$. With further additions of Fe³⁺Cc, the triplet state decay traces can be described with a double exponential function with decay constants, $k_1 \approx 90\text{s}^{-1}$ and $k_2 \approx 320\text{s}^{-1}$ and respective fractions, f_1 and f_2 with initial amplitude A_0 .²⁹ As long as $0 < [\text{Fe}^{3+}\text{Cc}] < [\text{ZnPCcP}]_0$, k_1 and k_2 are nearly unchanging while f_2 increases nearly linearly, as appropriate for a tightly-bound 1:1 complex in slow exchange with its dissociated components;³⁰ at the titration endpoint, when $[\text{Fe}^{3+}\text{Cc}] \approx [\text{ZnPCcP}]_0$, and the formation of a binary is roughly complete ($f_2 \approx 1$), the triplet decay is approximately exponential with $k_2 \sim 320\text{s}^{-1}$. However, past this point, when $[\text{Fe}^{3+}\text{Cc}] > [\text{ZnPCcP}]_0$, the triplet decay again becomes biexponential with the appearance of a faster phase, decay constant k_3 , associated with the formation of a 2:1 complex in slow exchange with the 1:1 complex, with enhanced quenching in the ternary: ternary quenching rate constant, $k_3 > k_2$. A correlation between the yield of charge-separated intermediate (see below) and the rate constant for quenching suggests that much of the quenching in the ternary is by energy transfer.

To incorporate both binary and ternary complexes, the triplet decay traces were fit with a triple exponential function, Fig S1, which corresponds to a model in which equilibrium populations of free, 1:1, and 2:1 complexes are in slow exchange and decay independently. To test this protocol and to further reduce the total number of fit parameters, the entire set of triplet decay traces was also fit globally to rate constants, k_1 , k_2 , and k_3 , and affinity constants, K_I and K_{II} . Although the two fitting approaches give comparable values, the confidence intervals for the global approach are better (smaller). The values of the rate constants and affinity constants obtained in this fashion are given in Table 1, and are consistent with previous reports.^{8, 9}

Charge-Separated Intermediate—The progress curves of the charge-separated intermediate state (**I**) were followed through an Fe³⁺Cc titration by monitoring the transient absorbance upon photolysis at a ³ZnPCcP - ground isosbestic, 445 nm, a wavelength where both the (ZnP⁺CcP - ZnPCcP) and (Fe²⁺Cc - Fe³⁺Cc) absorbance differences contribute to the transient absorbance. As with the quenching measurement, the behavior of **I** is not consistent with a simple 1:1 stoichiometry in slow exchange. If charge separation and recombination occurred as a simple, intracomplex photocycle, one would expect a simple exponential rise and fall for the absorbance of **I**, with *unchanging* rate constants throughout the titration, one corresponding to the intracomplex triplet decay constant, the other to intracomplex CR associated with the return of **I** to ground; only the signal amplitude would change during a titration.

Instead, although we observe an increase in the maximum accumulation of **I**³¹ the shapes of the progress curves for **I** are complex, with an apparently exponential rise and biphasic fall, one exponential phase decaying within ~50 ms, the other, a second order phase, decaying only over 1 s or longer, with the proportions of the slower phase increasing during the course of the titration, Fig 1. Throughout the titration, plots of the reciprocals of the

absorbance-difference are linear at times where it is dominated by the slow component, Fig 2, which shows that this phase of CR is second order. Indeed, any description of the slow phase in the recovery of the ground state must incorporate dissociation and recombination of **I**: the complex could not remain bound for the duration of the intermediate signal, ~1–2 seconds. For **I** to remain just 90% bound for at least 1 second, the dissociation rate constant would need to be $< 0.1\text{s}^{-1}$, which is two orders of magnitude smaller than that calculated using the measured binding constant, $K_I = 2 \times 10^7\text{M}^{-1}$, and the reported association rate constant of at least $k_{\text{on}} \sim 10^8\text{M}^{-1}\text{s}^{-1}$.³²

The results to this point indicate that two extensions of the simple photocycle are required. The results of the Fe^{3+}Cc quenching titration clearly indicate the formation of a ternary complex in the triplet state, and thus presumably in **I** as well; the behavior of the CR shows that the description must furthermore incorporate dissociation and reassociation of the charge-separated intermediate complex, $[\text{ZnP}^+\text{CcP}, \text{Fe}^{2+}\text{Cc}] \equiv \text{I2}(2+)$. However, these ‘simple’ extensions profoundly complicate the kinetic scheme for a photocycle. A full kinetic scheme that incorporates binary and ternary complexes of the triplet state and **I**, as well as dissociation/reassociation of each species in all possible combinations, is presented in Scheme S1. This scheme includes 18 species, 36 equilibrium processes, and at least six ET processes. Clearly, it is not practicable to determine all the parameters in such a scheme. However, it is possible to assess the roles of dissociation and of the ternary charge-separated complexes on the CR process, and determine key kinetic parameters by considering successively more complex *sub-schemes* extracted from Scheme S1, beginning with a *minimal* kinetic sub-scheme described previously,⁹ Scheme 1, and then including additional kinetic routes as needed to arrive at a *core* model, Scheme 2 below, that captures the key features needed to describe the progress curve for **I**.

Minimal kinetic scheme—To begin, we recognize that although a full fit to the triplet decay traces requires a 2:1 binding model, the majority of bound $^3\text{ZnPCCP}$ exists as the binary complex even at the highest $[\text{Fe}^{3+}\text{Cc}]$. Therefore, Scheme 1 includes only the dominant channel for intracomplex photo-initiated CS, $[^3\text{ZnPCCP}, \text{Fe}^{3+}\text{Cc}] \rightarrow [\text{ZnP}^+\text{CcP}, \text{Fe}^{2+}\text{Cc}]$. The present measurements cannot yield meaningful parameters values for minor channels, as the small contributions from such channels are overwhelmed by the smallest deviations from the triplet/ground isosbestic, where the measurements must be made, and where the absorbance difference for **I** is not maximal. Once formed, **I2**(2+) can undergo intracomplex CR, rate constant $^{\text{app}}k_{\text{b}}$, but also is free to dissociate Fe^{2+}Cc with the apparent dissociation rate constant, $^{\text{app}}k_{\text{off}(2+)}$, and recombine with the association rate constant, $^{\text{app}}k_{\text{on}(2+)}$.

Each *individual* progress curve for **I** collected during an Fe^{3+}Cc titration can be well fit to Scheme 1 using numerical integration of the corresponding equations (**SI**), as shown in Fig 1. Moreover, as required, in the fit of each trace, the rate constant for the appearance of **I**, $k_{\text{rise}} = 310 \pm 12\text{s}^{-1}$ corresponds to the rate constant, k_2 , for the decay of the triplet state in the bound complex. However, during a titration up to the addition of five equivalents of Cc per ZnPCCP , the fit parameter $^{\text{app}}k_{\text{off}(2+)}$ increases linearly, by a factor of six, while $^{\text{app}}k_{\text{b}}$ decreases by 1/3, from $\sim 60\text{s}^{-1}$ to $\sim 40\text{s}^{-1}$, Fig 3, with the main changes occurring well

beyond the point where ZnPCcP is fully bound. The fits to Scheme 1 further yield a systematic decrease in $^{app}k_{on(2+)}$ from $\sim 1.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ to $\sim 8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. However, a large uncertainty introduced by the limitation of data collection to 1 s, before all the intermediate has recombined, precludes detailed analysis. If Scheme 1 were adequate to describe the behavior of **I**, all three rate constants, $^{app}k_b$, $^{app}k_{off(2+)}$, and $^{app}k_{on(2+)}$, would remain invariant during the titration—only the amplitude of the signal would change, rising with the fraction of bound complex, and this would reach an unchanging maximum when all ZnPCcP is complexed. The progress curves for **I** show none of these characteristics.

The changes in CR rate constants beyond complete formation of the 1:1 complex instead reflect the presence of a ternary charge-separated complex, and thus complement the finding that a description of triplet quenching requires the presence of a ternary photoexcited complex. To understand how to incorporate a ternary charge-separated complex into Scheme 1, it is important to consider all the processes that the binary charge-separated complex, **I2(2+)**, can undergo following its formation through the dominant CS process. If this complex dissociates before CR can occur, the free ZnP^+CcP thus formed must rebind Fe^{2+}Cc in order to undergo CR, but instead it can bind Fe^{3+}Cc , which acts as an ‘inhibitor’ of charge recombination by taking the place of the Fe^{2+}Cc (the equilibrium binding constants for the association of Fe^{3+}Cc and Fe^{2+}Cc with WT CcP are similar³³). Such an inhibited complex can further bind a second Fe^{3+}Cc to form a ‘doubly-inhibited’ ternary inhibitor $[\text{ZnP}^+\text{CcP}, (\text{Fe}^{3+}\text{Cc})_2] \equiv \text{I3}(3+/3+)$; it is the progressive accumulation of these species with increasing $[\text{Fe}^{3+}\text{Cc}]$ that acts to slow CR.

In addition to undergoing CR or dissociating, **I2(2+)** also can bind an Fe^{3+}Cc to form the **I3(2+/3+)** ternary complex. There are multiple possible alternative structures of such a ternary complex. Depending on the CR pathways within such a ternary, and on the probabilities of releasing Fe^{3+}Cc to reform **I2(2+)**, or of releasing Fe^{2+}Cc to form **I2(3+)**, formation of **I3(2+/3+)** could either enhance or suppress CR as revealed by changes in $^{app}k_{off(2+)}$ and $^{app}k_b$ beyond one equivalent of $[\text{Fe}^{3+}\text{Cc}]$. To address these issues, two types of ‘competition’ titrations were performed.

Fe²⁺Cc Titration: Assignment of the slower decay phase of **I** to accumulation of the ‘inhibited’ complex **I2(3+)**, which cannot undergo CR, implies that addition of excess Fe^{2+}Cc should enhance the regeneration of the ET-active **I2(2+)**, thereby speeding CR and decreasing the lifetime of **I**. To examine this prediction, Fe^{2+}Cc was titrated into a 1:1 mixture of $\sim 5 \mu\text{M}$ ZnPCcP, $\sim 5 \mu\text{M}$ Fe^{3+}Cc (pH 7 KPi buffer, 20C), to a final concentration of $22 \mu\text{M}$ added Fe^{2+}Cc . The fraction of photoexcited $^3\text{ZnPCcP}$ bound in complex with Fe^{3+}Cc , Fig S2, as determined from biexponential fits of the triplet decay traces with rate constants k_1 and k_2 , Fig S3, decreased throughout the titration, establishing that Fe^{3+}Cc and Fe^{2+}Cc bind to ZnPCcP/ $^3\text{ZnPCcP}$ with similar affinities.

The excess Fe^{2+}Cc also alters the time course for the **I**, Fig 4. The trace for **I** generated without addition of Fe^{2+}Cc , which reproduces the corresponding trace of Fig 1 above, exhibits a slow phase of roughly 50% of the signal. As expected, progressive additions of Fe^{2+}Cc decrease the intensity of the signal for **I**, but have little effect on its rate of appearance, $k_{\text{rise}} = 370 \pm 16 \text{ s}^{-1}$ which corresponds to the unvarying rate constant for triplet

decay, k_2 . However, added Fe^{2+}Cc obviously suppresses the slow component of CR, as anticipated.³⁴

In fits of these curves to Scheme 1 the ‘intracomplex’ CR rate constant $^{\text{app}}k_b$, associated with the more rapidly decaying phase decreases by $\sim 1/3$ (from $\sim 60\text{s}^{-1}$ to $<40\text{s}^{-1}$, Fig S4), while the suppression of the slow component is manifest as a speeding up of decay with the result being that the more slowly decaying component coalesces with the other, Fig 4. The observation that increasing $[\text{Fe}^{2+}\text{Cc}]$ speeds up CR confirms the basic tenet of Scheme 1, that $\text{I2}(2+)$ dissociates, and that the slow CR phase is rate-limited by recombination of Fe^{2+}Cc with ZnP^+CcP . Likewise, the apparent *decrease* in the intracomplex CR rate constant $^{\text{app}}k_b$ with increasing Fe^{2+}Cc suggests that $\text{I2}(2+)$ can bind a second Fe^{2+}Cc , forming a ternary $\text{I3}(2+/2+) \equiv [\text{ZnP}^+\text{CcP}, (\text{Fe}^{2+}\text{Cc})_2]$ complex. Apparently as a paradox, the binding of the second Fe^{2+}Cc electron donor acts to decrease the net rate constant associated with $\text{Fe}^{2+}\text{Cc} \rightarrow \text{ZnP}^+\text{CcP}$ charge-recombination ET. This, however, simply implies that neither Fe^{2+}Cc in $\text{I3}(2+/2+)$ adopts the favorable bound conformation of the ET-active $\text{I2}(2+)$ binary complex, while the observation that the fast phase that decays exponentially indicates that $\text{I2}(2+)$ and $\text{I3}(2+/2+)$ are in rapid exchange. As discussed in more detail below, a parallel decrease of $^{\text{app}}k_{\text{off}(2+)}$ with $[\text{Fe}^{2+}\text{Cc}]$, Fig S5, in contrast to an increase with $[\text{Fe}^{3+}\text{Cc}]$ (see above) and $[\text{CuCc}]$ (below), is further evidence of the inhibiting effect of the latter two Cc. As with the Fe^{3+}Cc titration, the $^{\text{app}}k_{\text{on}(2+)}$ values could not be further analyzed.

CuCc Titration: To test whether the increases in $^{\text{app}}k_{\text{off}(2+)}$ during the titration with Fe^{3+}Cc and Fe^{2+}Cc are caused exclusively by formation of the ternary complex through binding of an inert Cc, and not in some part through a self-exchange between bound Fe^{3+}Cc and Fe^{2+}Cc , we performed an analogous titration with redox-inert CuCc.¹⁹ CuCc was titrated into a sample $\sim 5\mu\text{M}$ ZnPCCP , $\sim 7\mu\text{M}$ Fe^{3+}Cc (pH 7, KPi buffer, 20C), to a final concentration of $\sim 16\mu\text{M}$ CuCc. Inspection of the triplet decay traces, Fig S6, shows a small increase in the lifetime of $^3\text{ZnPCCP}$, an indication that the inert CuCc was replacing Fe^{3+}Cc in the photoexcited complex, but the effect is too small to precisely quantify. The overall behavior of **I** during a titration with CuCc, Fig 5, is similar to that during the Fe^{3+}Cc titration, Fig 1, with $k_{\text{rise}} = 300 \pm 20\text{s}^{-1}$. However, fits of the **I** time courses with Scheme 1 reveal that as with the Fe^{3+}Cc , $^{\text{app}}k_{\text{off}(2+)}$ increases linearly with progressive additions of CuCc, Fig S7, establishing the formation of the ternary complex, $\text{I3}(2+/\text{Cu}) \equiv [\text{ZnP}^+\text{CcP}, \text{Fe}^{2+}\text{Cc}, \text{CuCc}]$; again $^{\text{app}}k_{\text{on}(2+)}$ values were not further analyzed. We observe a modest, but significant decrease in $^{\text{app}}k_b$ with increasing CuCc, Fig S8, directly confirming that in the ternary complex the presence of the second, nonreactive Cc makes the Fe^{2+}Cc less reactive. We return to this finding in Discussion.

Core Kinetic Scheme: The experiments described above yield mechanistic constraints to guide expanding Scheme 1 to develop a core kinetic model of the ET photocycle for Fe^{3+}Cc and ZnPCCP(W191F) as these proteins form dynamic binary and ternary complexes whose dissociation and combination occurs on the long timescale of direct heme-heme ET in the CcP(W191F) , FeCc complexes. These constraints and the modification that follow may be summarized:

- a. Dissociation/reassociation competes with ET during charge recombination; Scheme 1 includes dissociation of the binary CS intermediate, $I2(2+)$.
- b. The ZnP^+CcP liberated upon dissociation of $I2(2+)$, can bind $Fe^{3+}Cc$ to form the 'inhibited' complex, $I2(3+)$, slowing the second order recombination process of the slow CR component;. Scheme 1 must be extended to include formation of $I2(3+) \equiv [ZnP^+CcP, Fe^{3+}Cc]$.
- c. An MCc ($M=Fe^{2+}, Fe^{3+}, Cu$) can interact with $I2(2+)$ to form a ternary complex whose structure is less suited for ET than is that of the binary complex; to describe the 'normal' titration of $ZnPCcP$ by $Fe^{3+}Cc$, Scheme 1 must be modified to include the formation of the ternary complex, $I3(2+/3+)$, which is allowed to dissociate either one of the Cc to form one of two binary complexes, $I2(2+)$ or $I2(3+)$.

Scheme 2 presents a *core kinetic model* for the $ZnPCcP(W191F)$ and $Fe^{3+}Cc$ ET photocycle that incorporates these features, and in fact represents a subset of Scheme S1: k_{on} and $k_{off(2+/3+)}$ are the association and dissociation rate constants, respectively, for the binary complexes; $^{ter}k_{on(2+/3+)}$ and $^{ter}k_{off(2+/3+)}$ are rate constants for formation and dissociation of the ternary complex; all are provisionally taken to be identical for the photoexcited and charge-separated complexes but unique for each $Fe^{2+}Cc$ and $Fe^{3+}Cc$. Again, the CR rate constant for the binary is k_b ; in principle the ternary can also undergo CR, rate constant $^{ter}k_b$. The scheme does not explicitly include the $I3(3+/3+)$ complex because it is not kinetically differentiable from the 'inhibitor' complex, $I2(3+)$. Analogous schemes for the $Fe^{2+}Cc$ and $CuCc$ titration ET photocycles are given by Schemes S2 and S3.

In Scheme 2, $I2(2+)$ can dissociate after photoinitiated CS ET, with rate constant, $k_{off(2+)}$, and the liberated ZnP^+CcP is free to bind nonreactive $Fe^{3+}Cc$ molecules, thereby forming the 'inhibitor' complex, $I2(3+)$,³⁵ and given that $[Fe^{3+}Cc] \gg [ZnP^+CcP]$, we infer that ZnP^+CcP spends the majority of the time in complex. Alternatively, $I2(2+)$ can bind a second $Fe^{3+}Cc$ molecule to generate the ternary complex, $I3(2+/3+)$, which can dissociate either Cc , reforming $I2(2+)$ or the inhibited binary complex, $I2(3+)$. This process effectively enhances the apparent rate of dissociation of $Fe^{2+}Cc$ from $I2(2+)$ by providing an additional route to form the inhibited complex, $I2(3+)$, and it causes the concentration-dependent increase of the apparent rate constant, $^{app}k_{off(2+)}$, for $CuCc$ and $Fe^{3+}Cc$, but decrease for $Fe^{2+}Cc$, as noted above, amplifying the slow phase of CR.

Even an attempt to fit titration data with Scheme 2, a sub-scheme of the full kinetic Scheme S1, presents formidable difficulties because of the large number of parameters and the impossibility of independently monitoring the individual ZnP^+CcP species within Scheme 2: transient absorbance spectroscopy monitors only the sum of the charge-separated species. However, we can obtain parameters partially describing Scheme 2 by identifying them with the parameters obtained by fitting the progress curves to Scheme 1. The two critical parameters in Schemes 2, S2, and S3 are $k_{off(2+)}$ and $^{ter}k_{on(M)}$, ($M = Fe^{2+}, Fe^{3+}, Cu$), the rate constants for the processes that form the species, ZnP^+CcP and $[ZnP^+CcP, Fe^{2+}Cc, MCc] \equiv I3(2+/M)$ respectively, which represent the slower decay phase. Perhaps surprisingly both a dissociation and an association constant can be obtained from the two parameters that describe the linear dependence of the *apparent* rate constant for the dissociation of $I2(2+)$ of

Scheme 1 (denoted $^{app}k_{off(2+)}$) on the concentration of added titrant, $[MCc]_0$, as found in the fits to Scheme 1. The correlation can be written as follows.

$$^{app}k_{off(2+)} = k_{off(2+)} + {}^{ter}k_{on(M)} [MCc]_0 \quad (1)$$

Firstly, the concentration of titrant MCc is much greater than the concentration of $I2(2+)$ at all times during a photocycle, $[MCc] \gg I2(2+)$, so one may equate the instantaneous value of free $[MCc]$ with the total concentration, $[MCc] \approx [MCc]_0$. As a result, within Scheme 2 the formation of the ternary complex, $I2(2+) + MCc \rightarrow I3(2+/M)$, is pseudo-first order. Secondly, this formation of $I3(2+/M)$ manifests itself through the description of the titrations with Scheme 1 as a contribution to the apparent rate constant for $I2(2+)$ dissociation that increases with $[MCc]_0$ during a titration, $^{app}k_{off(2+)} = {}^{ter}k_{on(M)} [MCc]_0$. Addition of this contribution to the true rate constant for dissociation, $k_{off(2+)}$, leads to eq 1.³⁶

The variations of $^{app}k_{off(2+)}$ with $[MCc]_0$ obtained through analysis of the $Fe^{3+}Cc$ and $CuCc$ titrations with Scheme 1 and their description with eq 1 yields the values of ${}^{ter}k_{on(M)}$ shown in Table 2. These dissociation rate constants for the ternary complexes are approximately the same, confirming that the formation of a ternary complex is the cause of the concentration dependence of the apparent dissociation of the binary complex, $^{app}k_{off(2+)}$.

Discussion

Introduction of the W191F mutation greatly slows the CR ‘half’ of the ET photocycle associated with the $[ZnPCcP, Fe^{3+}Cc]$ complex by eliminating fast hole-hopping from $Fe^{2+}Cc$ to the ZnP cation radical in ZnP^+CcP through W191.⁹ This mutation both enhances the accumulation of the CS intermediate, **I**, and greatly extends the timescales over which binding/dissociation of this paradigmatic protein-protein ET pair can be probed through ET photocycle measurements. This study reports progress curves for the triplet decay and charge-separated intermediate curves of the titration of $ZnPCcP(W191F)$ with $Fe^{3+}Cc$, as well as titrations of the $[ZnPCcP, Fe^{3+}Cc]$ complex at fixed concentration with $Fe^{2+}Cc$ and with $CuCc$. The results of these experiments require an expansion of the CR branch of the simple photocycle for intracomplex ET between $ZnPCcP(W191F)$ and $Fe^{3+}Cc$ (Scheme 1) to incorporate (i) dissociation/recombination of the charge-separated intermediate state; (ii) two types of binary intermediate complex, the reactive intermediate, $I2(2+)$, and the ‘inhibited’ complex, $I2(3+)$; (iii) the ternary intermediate complex in which ZnP^+CcP binds both $Fe^{2+}Cc$ and $Fe^{3+}Cc$, $I3(2+/3+)$, Scheme 2. Incorporation of this expanded CR scheme within the ET photocycle formalizes and expands earlier proposals to explain apparently anomalous kinetic and binding measurements,^{21, 37} and further allows us to introduce a distinctive perspective on the structure of the ternary CS complex.

An early proposal suggested that “substrate-assisted product dissociation” added a concentration-dependent term to the dissociation of Cc from CcP.²¹ Such a term offered an explanation for observed steady-state turnover rates at high protein concentrations that were greater than the dissociation rate constants reported at low concentrations. Those results

yielded a [Cc]-dependent, second order dissociation rate constant in agreement with an earlier proton NMR study³⁷ that observed concentration-dependent exchange rates between free Cc and Cc bound to CcP: an increase in absolute protein concentration at constant CcP:Cc ratio shifted the exchange from slow-exchange on the NMR timescale, to fast-exchange.³⁷ The present measurements of the CS recombination kinetics show a corresponding concentration-dependent increase of the apparent rate of dissociation of the binary CS complex, **I2(2+)**

Stopped-flow measurements^{22, 38} also were interpreted in terms of a concentration-dependence of the apparent dissociation of the binary complex, caused by transient formation of the ternary complex in which binding at the reactive site is decreased by repulsion between bound Cc. The detailed kinetic Scheme 2 correspondingly incorporates the reaction between **I2(2+)** and an additional Fe^{3+}Cc to form the weakly bound ternary complex, **I3(2+/3+)**, which is free to release *either* Fe^{2+}Cc or Fe^{3+}Cc . When the kinetics of charge recombination are interpreted within the contracted kinetic Scheme 1, which does not explicitly include the **I3(2+/3+)**, the influence of the additional channel for liberating Fe^{2+}Cc by dissociation from the ternary complex is manifest as an enhancement of the *apparent* dissociation constant of **I2(2+)**, and leads to the appearance of “substrate-assisted product dissociation”.²¹ However, to understand these results and most especially the the strength and effects of Cc repulsion within the ternary complex, it is necessary to incorporate the contributions of repulsion into the thermodynamics of binding within a two site binding model.

Thermodynamics of Two-Site Binding

It is well-known that binding measurements for a system that includes binary and ternary complexes provide only two thermodynamic affinity constants.³⁹ These can be formulated as one constant for the binding to CcP of one Cc to form a binary, K_I , the second constant for the binding of a second Cc to form the ternary, K_{II} with corresponding free energies G_I and G_{II} , respectively.

Alternatively, the second constant can be defined as that for the direct binding of two Cc by CcP, affinity constant $K_T = K_I K_{II}$, a formulation that is seen below to be particularly useful for the present discussion.

It is also well known that the two constants K_I and K_{II} of eqs 2 and 3 are *not* site binding constants,³⁹ nor do they imply *any* specific binding model. Nonetheless, it is also well known that the thermodynamic formulation *formally* corresponds to *independent* binding at

two pre-existing sites, 1 and 2, with binding constants, K_1 and K_2 , where $K_I = K_1 + K_2$ and $K_{II} = K_1 K_2 / (K_1 + K_2)$ ⁴⁰ *whether or not* the system is physically represented by this model. In fact, calculations and experiments have confirmed that the two Cc cannot bind independently, and that the stability of the ternary is diminished by electrostatic repulsions between the two positively charged Cc (anti-cooperative binding).^{41, 42}

To describe the thermodynamics of two-site binding of two Cc by CcP we consider two CcP binding sites, 1 and 2, where Cc can bind to form alternative binary complexes with true site constants, K_1 and K_2 respectively, and with binding free energies G_1 and G_2 ,

$$-RT \ln K_i = \Delta G_i \quad i=1, 2 \quad (5)$$

as illustrated in Fig 6A. Regarding the formation of the ternary. (i) Electrostatic repulsion between the two bound Cc molecules of the ternary complex necessarily causes the binding to be 'anti-cooperative' and can be captured by explicitly introducing a free energy of repulsion ($G_\Phi > 0$) into the free energy for formation of the ternary complex from its unbound components (Eq 6, G_T).

$$-RT \ln K_T = \Delta G_T = {}^{ter} \Delta G_1 + {}^{ter} \Delta G_2 + \Delta G_\Phi \quad (6)$$

(ii) The free energies of binding a Cc to the CcP surface at each site *within the ternary complex* need not have the same values as for the two binary complexes, *namely*, in general $G_i \neq {}^{ter} G_i$. This is so because upon binding of a second Cc to CcP, the binding geometries at both sites and even the protein charges can adjust so as to create the most stable ternary complex: in minimizing the total free energy of the ternary, G_T , a diminution in binding free energies to the CcP surface can be more than compensated by mitigation of the repulsion, as shown in Fig 6B. Stated differently, the ternary can lose free energy of interfacial binding interactions, but benefit overall by a more than compensating decrease in repulsion.

To understand the role of repulsions in the ternary complex between CcP and Cc quantitatively, it is nonetheless useful to first consider anti-cooperative binding in the *rigid limit* of the two-site model, where the site binding constants, K_1 and K_2 , are *assigned* as being unchanged upon binding of a second Cc, and thus the site-binding free energies within the ternary complex (${}^{ter} G_i$, $i = 1, 2$) are assigned to equal those within the binary complexes, $G_i = {}^{ter} G_i$, Fig 6B. Anticooperativity is introduced by repulsion between the two Cc, decreasing K_{II} , through the incorporation of a repulsion factor $\Phi = e^{-G_\Phi/RT}$. In this limit, the relationship between the measured affinity constants, K_I and K_T , and the site-affinity constants, K_1 and K_2 , and the free energy for forming the ternary complex from its components, then become

$$K_I = K_1 + K_2 \quad (7)$$

$$K_T = K_I K_{II} \Phi \quad (8)$$

$$-RT \ln K_T = \Delta G_T = \Delta G_1 + \Delta G_2 + \Delta G_\Phi \quad (9)$$

Kinetic measurements and mutagenesis, both in our laboratory^{8, 43–45} and others^{12, 46–48} generally arrived at measured affinity constants for the ‘first’ and ‘second’ Cc, which correspond to thermodynamic constants of $K_I \sim 10^7 \text{ M}^{-1}$, $K_{II} \sim 10^4 \text{ M}^{-1}$ at 10mM potassium phosphate buffer, pH 7 20C, values corroborated in the present study. Use of these consensus values permits us to discuss the actual site constants and to set limits on the repulsion free energy.

That the crystal and NMR^{49–51} structures reveal a 1:1 CcP/Cc complex with similar conformations would *seem* to suggest that one binding site (site 1) is overwhelmingly more favorable than any second site. However, in the extreme case of this rigid limit, if the site constants were equal to the measured thermodynamic affinity constants ($K_I \approx K_I$ and $K_2 \approx K_{II}$), then repulsion would be zero, and this is ruled out by the measurements of Erman and coworkers,⁴² as noted above. This requires that the actual site-binding constant K_2 must be larger than the measured value of $K_{II} = 10^4$, although still enough smaller than K_I that the binary is predominantly in the tightly-binding form in accordance with the NMR studies.⁵⁰ We propose that if K_2 were merely 10-fold less than K_I , rather than the 10^3 -fold that would apply if one inappropriately assigned $K_2 = K_{II}$, then it is likely that the binary complex with binding at site 1, the ‘tight’ site, would dominate both the crystallization process that led to the X-ray structure, as well as the solution structures. If we then use this assumption within eqs 7 and 8 for concreteness, yielding the plausible value, $K_2 \sim 10^6$, then one arrives at a calculated repulsion constant of $\Phi \approx 10^{-2}$, corresponding to $G_\Phi = 2.7 \text{ kCal/mol}$. If one further relaxes the *rigid limit* two-site model, allowing the Cc at each site within the ternary complex to adjust its binding conformation relative to that of the binary, this further lowers the possible influence of repulsions. Such relaxation leads to the inequalities, ${}^{\text{ter}}K_I < K_I$ and/or ${}^{\text{ter}}K_2 < K_2$; through eq 7 this in turn would correspondingly reduce the free energy of repulsion, $G_\Phi < 2.7 \text{ kCal/mol}$.

We may conclude this discussion by noting that eq 7 places an upper limit to the affinity at the weaker-binding site, $K_2 = K_I = K_I/2 = 5 \times 10^6$. This limiting value in turn leads to an absolute upper bound to the repulsion free energy in *any* two site model, through use of eq 8. Use of the consensus measured thermodynamic affinity constants and this upper bound to K_2 within the *rigid-limit* two-site model gives an upper bound to the repulsion free energy at 10mM KPi buffer, pH 7, 20C: $G_\Phi = 3.3 \text{ kCal/mol}$; this is an absolute upper bound, as relaxation of the rigid limit would only lower this value. The experiments designed to probe the repulsion free energy, which employed a ternary that constrains the movement of one Cc, yielded a free energy for the repulsion between the two Cc of $G_\Phi = 6 \text{ kCal/M}$, roughly twice the actual upper limit for the non-covalent ternary revealed by the present thermodynamic analysis.⁴² However those experiments were conducted at lower ionic strength, and higher pH and temperature (10mM *ionic strength* KPi buffer, pH 7.5 and 25C),

all of which differences would enhance repulsion. Thus, we suggest that the two measurements are satisfactorily supportive.

The Nature of Binary and Ternary Complexes

Despite the beautiful visualization of complementary docking between CcP and Cc provided by the crystal structure, there is good reason to believe that this structure is actually a member of an ensemble of solution binding configurations, as suggested long ago by Brownian dynamics simulations.⁴⁶ Such a situation was supported by the “Velcro” model in which a large surface region, or charged patch, on CcP participates in complex formation.⁵² and most persuasively, has recently been underscored by the NMR studies of Ubbink and co-workers, who found that within the binary complex, Cc spends up to 30% of the time in an encounter complex with CcP as a collection of non-specific binding configurations around the ‘tight’ site.²³ Consistent with this view, small and subtle changes in the binding surfaces of Cc and CcP introduced by point mutations significantly change the structure of the docked complex.⁶ Collectively, these measurements lead to an expanded, more dynamic view of the association between CcP and a single Cc in solution. The present thermodynamic analysis of binding extends such ideas by showing that the second binding site has a much greater affinity than previously recognized. Stronger second-site binding would in turn contribute to a broader ensemble of binary structures.

Combining the analysis of affinities with the kinetic measurements of the charge-recombination process, further contributes to our understanding of the structure of the ternary, and to the view that it is even more dynamic than the binary, and that the most-probable binding sites/geometries for the two Cc not necessarily identical to those most characteristic of the alternative binaries. This picture is supported by the present experimental observation that increasing concentrations of MCc not only increase $^{app}k_{off(2+)}$, but also decrease $^{app}k_b$. If the two Cc within the ternary complex, I3(2+/3+) or I3(2+/Cu), were situated as in the corresponding binary complexes, the value $^{app}k_b$ would remain constant, contrary to observation; only $^{app}k_{off(2+)}$ (and possibly $^{app}k_{on}$) would change. Instead, the presence of both binary and ternary complexes (I2(2+) and I3(2+/3+)) in rapid exchange generates an observed $^{app}k_b$ that is a weighted average of k_b and $^{ter}k_b$. The finding that $^{app}k_b$ decreases with increasing concentration of the ternary indicates that the presence of the second Cc forces the $Fe^{2+}Cc$ to bind in a less-reactive conformation than in the binary complex, and the inequality, $0 < ^{ter}k_b < k_b$.⁵³

In conclusion, we have developed a generalized kinetic scheme for the ET photocycle of the complex between ZnPCcP(W191F) and $Fe^{3+}Cc$ that formalizes, expands, and revises the picture of the kinetic behavior and structure of the charge-separated intermediate. The resulting kinetic Scheme 2 incorporates binary complexes in two different redox states, I2(2+), I2(3+), along with a ternary complex, I3(2+/3+), and accounts for the dynamics of complex interconversion over timescales from μ sec to sec. In formalizing and enhancing earlier proposals of “substrate-assisted product dissociation”, we have developed a thermodynamic description of two-site binding which reveals that Cc binds at the weaker-binding site with much greater affinity than previously recognized, and places upper bounds on the contributions of repulsion between the two Cc of the ternary complex. In conjunction

with recent NMR studies, the analysis further suggests a dynamic view of the ternary complex, wherein neither Cc necessarily faithfully adopts the crystal-structure configuration because of Cc-Cc repulsion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge the support of the National Institute of Health (HL 63203)

We thank Dr. Judith Nocek and Dr. Diana Mayweather for many useful discussions and Dr. John Magyar for providing protocols used in the preparation of CuCc. We further acknowledge two anonymous reviewers, whose incisive comments and suggestions directly led to major improvements in this report.

Abbreviations

ET	Electron Transfer
CcP	Cytochrome c Peroxidase
Cc	Cytochrome c
ZnPCcP	Zn-ProtoporphyrinIX-substituted CcP
CS	Charge-Separation
CR	Charge-Recombination

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Figure 1.

Titration of a sample $\sim 5\mu\text{M}$ ZnPCcP with Fe^{3+}Cc ($5\text{--}26\mu\text{M}$). Progress curves for the charge-separated intermediate (black) normalized to A_0 for the corresponding triplet decay curves with fits to Scheme 1 (red) *Conditions:* 10mM, pH 7 KPi buffer, 20C.

Figure 2.

Titration of a sample $\sim 5\mu\text{M}$ ZnPCcP with Fe^{3+}Cc ($5\text{--}26\mu\text{M}$). Reciprocal-absorbance plots of the progress curves for charge separation measured at 445nm .

Figure 3.

Titration of a sample ~5 μM ZnPCcP with Fe³⁺Cc (5–26 μM). (Top) $appK_b$ through the titration. Dotted line to guide the eye. (Bottom) $appK_{off(2+)}$ through the titration with fit of to eq 2 (black line). *Conditions:* 10mM, pH 7 KPi buffer, 20C.

Figure 4.

Titration of a sample $\sim 5\mu\text{M}$ ZnPCcP, $\sim 5\mu\text{M}$ Fe^{3+}Cc with $0\text{--}17\mu\text{M}$ Fe^{2+}Cc . Progress curves for the charge-separated intermediate, I, normalized to A_0 of the corresponding decay at 475nm (black) with fits to Scheme 1 (red). *Conditions:* pH 7.0, 10mM KPi, 20C.

Figure 5.

Titration of a sample $\sim 5\mu\text{M}$ ZnPCcP, $\sim 7\mu\text{M}$ Fe^{3+}Cc with 0– $16\mu\text{M}$ CuCc. Progress curves for the charge-separated intermediate, **I**, normalized to A_0 of the corresponding decay at 475nm (black) with fits to Scheme 1 (red). *Conditions:* pH 7.0, 10mM KPi, 20C.

Figure 6. Representations of Cc bound to the surface of CcP within the binary and ternary complexes. **A)** Two unique binary complexes with equilibrium affinity constants, K_1 and K_2 . **B)** Rigid and dynamic models of the ternary complex with incorporated free energy of repulsion, G_ϕ .

Scheme 1.

Scheme 2.

Table 1

Kinetic parameters for global triple exponential fits of the triplet decay traces during a Fe^{3+}Cc titration shown with 95% confidence intervals.

k_1	$89.7 \pm 0.2 \text{ s}^{-1}$
k_2	$297 \pm 0.7 \text{ s}^{-1}$
k_3	$1190 \pm 63 \text{ s}^{-1}$
K_{I}	$2.3 \pm 0.2 \times 10^7 \text{ M}^{-1}$
K_{II}	$9.2 \pm 0.3 \times 10^3 \text{ M}^{-1}$

Table 2

Kinetic parameters for linear fits of $^{app}k_{off(2+)}$ values from fits using Scheme 1 with 95% confidence intervals

Protein titrant	$^{ter}k_{on(M)} (M^{-1}s^{-1})$
Fe ³⁺ Cc	$6.6 \pm 0.7 \times 10^6$
CuCc	$6.9 \pm 0.2 \times 10^6$