Reports

Regulation of Interprotein Electron Transfer by Residue 82 of Yeast Cytochrome c

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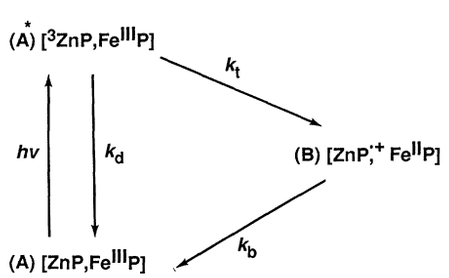
Yeast iso-1-cytochrome c (Cc) mutants have been constructed with Phe, Tyr, Gly, Ser, Leu, and Ile at position 82, each with Thr substituted for Cys at position 102. Their long-range electron transfer with zinc-substituted cytochrome c peroxidase (ZnCcP) has been studied by two kinetic techniques. The charge-separated complex, $[(ZnCcP)^+,Fe^{II}Cc]$ converts to $[ZnCcP,Fe^{III}Cc]$ by a single, intracomplex electron transfer step that is not governed by "gating" through possible rapid dissociation of the complex or isomerization (for example, heme-ligand) by $Fe^{II}Cc$ subsequent to its formation from $Fe^{III}Cc$. In every variant with an aliphatic residue at position 82 of Cc, the rate of this electron transfer process is $\sim 10^4$ slower at ~0°C than for the two variants with aromatic residues.

AMONG THE FACTORS THAT CONTROL the rate of electron transfer between metalloproteins (*1*), the role of the protein matrix that intervenes between the donor and acceptor sites is perhaps most intriguing and difficult to assess. Our recent studies of the complex between zinc-substituted yeast cytochrome c peroxidase and yeast iso-1-cytochrome c (*2*) addressed this issue through the use of site-directed mutagenesis to modify Phe^{82} (*3*) of the cytochrome, a phylogenetically conserved residue proposed to be involved in electron transfer between these proteins (*4*). The rate constant k_b for thermal electron transfer from $Fe^{II}Cc$ to $(ZnCcP)^+$ (*3*) within the protein-protein complex is $\sim 10^4$ greater for the wild-type (WT) protein and the Tyr^{82} variant than for the Ser^{82} and Gly^{82} variants. However, we noted that the smaller size of Ser (89 Å^3) and Gly (60 Å^3) residues relative to that of Phe (190 Å^3) and Tyr (194 Å^3) (*5*) might perturb the structure of Cc or the [CcP,Cc] complex, and crystallographic studies (*6*) have established that replacement of Ser for Phe at position 82 creates a discrete solvent channel adjacent to the heme prosthetic group. To interpret the sharp division between Cc variants with large and small values of k_b, we have measured k_b by two alternate techniques for a suite of cytochromes with Phe(WT), Gly, Ser, Tyr, Leu, and Ile at position 82, each with a $Cys^{102} \rightarrow$ Thr mutation to enhance its stability (*7*). These experiments unambiguously determine the kinetic mechanism, identify the intermediate state involved in electron transfer within the [ZnCcP,Cc] complex, and address the most likely major consequences of possible Cc structural perturbations. The importance of the size and aromaticity of residue 82 is tested by the variants in which position 82 is occupied by the aliphatic residues, Leu and Ile, whose side chain volumes (167 Å^3) are comparable to those of the aromatics, Phe and Tyr.

One strategy we use to study long-range electron transfer between CcP and Cc involves substituting zinc protoporphyrin (ZnP) for heme in CcP (*8*). Electron transfer from ^{3}ZnCcP to $Fe^{III}Cc$ can be initiated within the preformed complex by flash photolysis, as embodied in the kinetic scheme (scheme 1), and thus this technique is free from diffusion processes and protein-protein binding steps. The resulting electron transfer intermediate, $[(ZnCcP)^+,Fe^{II}Cc]$ (**B**), returns to the ground state by electron transfer from $Fe^{II}P$ to the ZnP^+ π-cation radical in a thermal reaction that is analogous to the physiological oxidation of $Fe^{II}Cc$ by H_2O_2-oxidized CcP. However, the process studied here is less complicated in that the native CcP is oxidized by two equivalents, one of which is at an unknown site (*9*), whereas we find that the only form of $(ZnCcP)^+$ relevant to these studies contains the ZnP^+ radical.



For each Cc variant, an electron transfer titration of ZnCcP with $Fe^{III}Cc$ (*8*) was performed and indicated the formation of a stable 1:1 complex. The photoinitiated rate constant k_t for each position 82 mutant is within a factor of ~3 of k_t for the Phe(WT) protein, except for the Gly variant, which we earlier inferred to form a modified complex

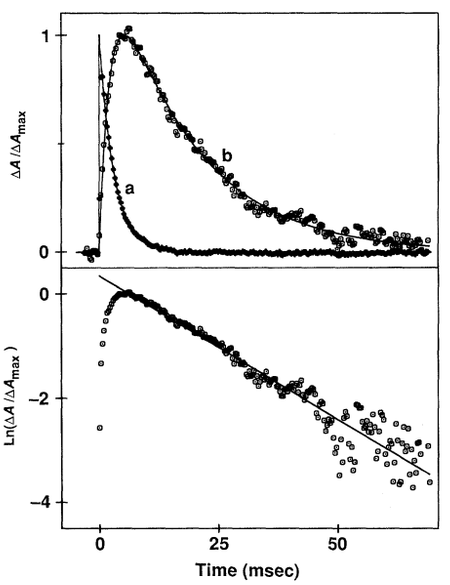


Fig. 1. Transient absorbance (**top**) and its logarithm (**bottom**) after photolysis of the $[ZnCcP,Fe^{III}Cc(Ser^{82})]$ complex. The kinetic scheme defining the rate constants of interest is presented in scheme 1. Trace a represents ^{3}ZnP monitored at 475 nm, which decays as a single exponential with rate constant $k_p = k_d + k_t$; trace b represent the electron transfer intermediate $[(ZnCcP)^+,Fe^{II}Cc]$, **B**, monitored at 551 nm, a ^{3}ZnCcP-ZnCcP isosbestic point. The absorbance rises and then falls as $\Delta A = A(\exp(-k_b t) - \exp(-k_p t))$ (*8*); the solid lines represent the theoretical fit with parameters $k_p = 317 \pm 3$ sec^{-1} and $k_b = 58 \pm 1$ sec^{-1}. In this experiment, the temperature was set to 25°C to test the kinetic order and to permit the rise and fall to be obtained in a single trace. At 0°C, k_b is lower for this system (Table 1) and the two phases are acquired separately (*2*). Conditions: 5 μM ZnCcP with 15 μM Cc in 1.0 mM potassium phosphate buffer, pH 7. The logarithm of the transient absorbance of the intermediate **B**, with data presented out to ~6 half-lives, indicates that decay of **B** is a single exponential. The solid line is the linear least-squares fit of the decay of **B**, with $k_b = 55 \pm 1$ sec^{-1}.

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(Table 1) (*2*). As a change of distance between redox sites by as little as 1 Å changes k_t by a factor of $\gamma > 2$ (*10*), the strong binding and similarity of rates lead us to conclude that the [ZnCcP,Fe^{III}Cc] complex is essentially the same for all position 82 mutants examined except Gly, and that the intracomplex, ^{3}ZnP → Fe^{III}P transfer process is largely unaffected by changes in the size and electronic properties of the residue at position 82.

The rate constant k_b of the thermal return reaction ($Fe^{II}P \rightarrow ZnP^+$, scheme 1), can be determined by measuring the time course of the absorbance difference associated with **B** (*8*). When $k_b > k_p = (k_d + k_t)$, the intermediate rises with rate constant k_b and decays with k_p, and it is only at the ^{3}ZnCcP-ZnCcP isosbestic points (551 and 444 nm) that its presence can be detected. However, as illustrated in Fig. 1 for the [ZnCcP,Cc(Ser)] complex, when $k_b < k_p$, the intermediate decays slowly, with rate constant k_b, after its formation with rate constant k_p.

Although the Phe(WT), Tyr, Ser, Leu, and Ile cytochrome variants behave similarly in the photoinitiated reaction, the thermal electron transfer process responds sharply to the identity of residue 82. For the complexes of ZnCcP and Cc with Phe or Tyr at position 82, this process is extremely rapid, $k_b \sim 2 \times 10^4$ sec^{-1} at 0°C (*2*). However, we now report that the three variants, Ser, Leu, and Ile, behave quite differently. For each of these three, the kinetic transients observed at 551 nm, where the kinetic difference spectrum of **B** is dominated by Fe^{II}Cc, and at 444 nm, where $(ZnCcP)^+$ dominates, rise and fall together, and follow precisely the kinetic formula (*2*) based upon the mechanism (scheme 1) for $k_b < k_p$. The decay of **B** is first order for at least six half-lives even up to 25°C (Fig. 1), and thus the thermal electron transfer process is not governed by dissociation of the intermediate and a slow, second-order return to the initial state (*11*). In each of the variants with non-aromatic residues at position 82 of Cc, Ser, Leu, and Ile, the rate of thermal electron transfer k_b is $\sim 10^4$ slower at ~0°C than for the two variants with aromatic residues (Table 1). Thus, it does not appear that the size of residue 82 controls k_b, and, in the absence of structural data on Leu or Ile proteins to the contrary, it would seem unlikely that k_b is reduced in all three variants primarily because their presence at position 82 permits solvent to be trapped at the molecular interface.

Table 1. Rate constants for electron transfer within the complexes of yeast iso-1-cytochrome c and zinc-substituted cytochrome c peroxidase. See scheme 1. The measured value for k_d was 92 ± 2 sec^{-1}. Conditions: 1.0 m*M* potassium phosphate buffer, *p*H 7.0, 0°C. Error limits were obtained from the nonlinear least-squares fits as averaged over multiple observations.

Residue at position 82*	k_t (sec^{-1})† (^{3}ZnP → Fe^{III}P)	k_b (sec^{-1})‡ ($ZnP^+ \leftarrow Fe^{II}P$)
Phe	166 ± 4	1.9 ± 0.6 × 10^4
Tyr	173 ± 1	1.5 ± 0.6 × 10^4
Ser	151 ± 5	2.3 ± 0.5
Leu	93 ± 5	2.0 ± 0.5
Ile	56 ± 3	3.0 ± 0.5
Gly	13 ± 2	1.4 ± 0.3

*Each cytochrome also has the Cys^{102} → Thr modification. †Measured in absorption at 475 nm, where contributions of **B** are negligible (Fig. 2) and from phosphorescence decays. Earlier work (*2*) monitored 432 nm and contributions from multiple kinetic components gave minor (<30%) errors in analysis. ‡Measured as described in text.

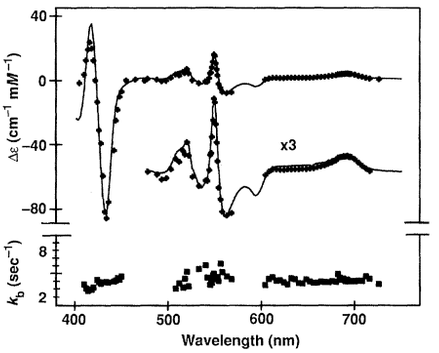
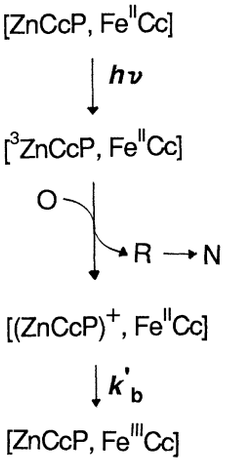


Fig. 2. Transient absorbance of the electron transfer intermediate [$(ZnCcP)^+$,Fe^{II}Cc], **B**, and the corresponding thermal transfer rate constants. Solid lines represent static difference absorptivity [$\epsilon(ZnCcP)^+ + \epsilon(Fe^{II}Cc)$] − [$\epsilon(ZnCcP) + \epsilon(Fe^{III}Cc)$]; the data points on those curves represent transient absorbance of **B**; spectral overlap with the actinic source precluded data collection from 580 to 600 nm. The spectra from 470 to 770 nm also have been offset and enlarged three times for clarity. The bottom set of data points represents k_b at corresponding wavelengths. Conditions: 4.5 μ*M* ZnCcP with 9 μ*M* Cc in 1.0 m*M* potassium phosphate buffer, *p*H 7, at 10°C.

Through study of a complex for which $k_b < k_p$, the intermediate **B** can be identified unambiguously because it persists after the ^{3}ZnP has decayed, and its spectrum can be examined at all wavelengths for times $\tau > 1/k_p$. Among the mutants, the Ser^{82} variant is particularly suitable for careful study because it has the highest ratio, $[k_t/(k_p - k_b)]$, and thus (*8*) exhibits the greatest buildup of **B**. The full, time-resolved kinetic difference spectrum of the [$(ZnCcP)^+$,$Fe^{II}Cc(Ser^{82})$] intermediate has been obtained by fitting the data for the slow kinetic phase (Fig. 2). The spectrum prominently displays the α and β bands of Fe^{II}Cc at 520 and 550 nm as well as the 688-nm band of $(ZnCcP)^+$, and it quantitatively fits a synthetic spectrum of [$(ZnCcP)^+$,Fe^{II}Cc] prepared by adding equal proportions of the two components. The rate constant k_b is independent of wavelength across the kinetic difference spectrum of **B**; the same value is obtained when monitoring features characteristic of the loss of ZnP^+ and Fe^{II}P, as well as the recovery of the ($ZnP,Fe^{III}P$) state.

Thus **B** is indeed the proposed [$(ZnCcP)^+$,Fe^{II}Cc] complex, and it behaves as a single kinetic entity with a rigorously first-order return to the initial redox state. However, the first-order rate constant k_b need not be associated with the $Fe^{II}P \rightarrow ZnP^+$ electron transfer process. Instead, it could be that structural perturbations in the mutant Cc cause the first-order decay of **B** to be governed by an isomerization of Fe^{II}Cc subsequent to its formation, such as the heme-ligand interchange seen upon reduction of Cc at alkaline *p*H (*12*). Thus we first examined the *p*H dependence of electron transfer. Contrary to expectations based on a *p*H-dependent ligand isomerization, k_b and k_t for the Ser and Leu variants are the same at *p*H 6 and 7, and the absorbance change associated with **B** is the same to within a factor of 2.

This possibility has been tested directly by measuring k_b in a [$(ZnCcP)^+$,Fe^{II}Cc] complex prepared initially with Fe^{II}Cc, which exhibits only one form (*11*). The $(ZnCcP)^+$ is generated within the preformed complex by rapid oxidative quenching of ^{3}ZnCcP according to scheme 2 through the use of an



oxidant O that accepts an electron to form a species R that decomposes to N before back electron transfer can occur (*13*). As illustrated in Fig. 3, photolysis of the [ZnCcP,$Fe^{II}Cc(Ser^{82})$] complex in the presence of $[Co^{III}(NH_3)_5Cl]^{2+}$ produces $(ZnCcP)^+$ through the oxidation of ^{3}ZnCcP by the cobalt complex on a time scale that is rapid compared with subsequent reactions. The rate constant for the $Fe^{II}Cc \rightarrow (ZnCcP)^+$ electron transfer step within [$(ZnCcP)^+$,Fe^{II}Cc] so prepared at ~10°C retains a similar $\sim 10^4$ discrimination be-

tween Cc variants with large and small values of k_b to that observed in the framework of the kinetic scheme 1. This result indicates that k_b is not controlled by a possible rate-limiting isomerization of a nonequilibrium $Fe^{II}Cc$ conformation formed by the photoinitiated electron transfer reaction $^3ZnP \rightarrow Fe^{III}P$. In the control for this measurement, the same procedure applied to [ZnCcP,Fe^{III}Cc(Ser^{82})] leaves a stable [$(ZnCcP)^+$,Fe^{III}Cc(Ser^{82})] complex (Fig. 3). This control further rules out a more complicated kinetic scheme in which an additional long-lived redox intermediate is generated through oxidation of a protein residue by ZnP^+.

The results presented here demonstrate that the intermediate **B** is indeed the charge-separated complex [$(ZnCcP)^+$,Fe^{II}Cc] and that the thermal, $Fe^{II}P \rightarrow ZnP^+$ process is a single, intracomplex redox step that is not governed by "gating" through rapid dissociation of the complex or through large-scale isomerization by Fe^{II}Cc subsequent to its formation. What, then, is the basis for the dramatic control of electron transfer by the residue at position 82 of Cc, and in particular for the apparent rate enhancement by an aromatic residue as compared to a comparably large aliphatic residue? The obvious interpretation is that "hole" transfer from ZnP^+ to $Fe^{II}P$ is rapid because the heme π-electron systems of the partner proteins are coupled by superexchange interactions through the intervening aromatic rings of Cc residue 82 and His^{181} of CcP (*1, 4, 14*).

However, the sharply lower rate observed with aliphatic residues at position 82 might well involve conformational "gating" (*15*), although the most obvious forms of this mechanism have been eliminated. An individual redox state of the [CcP,Cc] complex is not conformationally static, but rather dynamically samples an ensemble of protein conformations and binding geometries. This is suggested by nuclear magnetic resonance (NMR) studies of Cc (*16*), by the x-ray diffraction study of crystals of the [yeast-CcP,tuna-Cc] complex, in which it was found that the Cc is orientationally disordered (*17*), by molecular dynamics simulations (*18*), and by parallel studies of electron transfer (*19*). Thus a restricted subset of conformations that exhibit rapid electron transfer might be rendered relatively inaccessible by some of the mutations at position 82 of Cc. It is important to recognize, however, that the electronic and conformational mechanisms are not mutually exclusive. Both imply that certain structural states of the system exhibit exceptionally efficient electron transfer. Such conformations might well be sampled dynamically, whether or not they involve the placement of residue 82 in a position favorable for superexchange.

The unresolved issues regarding control of intracomplex electron transfer that are raised by the results to date will be addressed by examining the temperature dependence of the processes discussed here, as well as by the use of complementary mutations at His^{181} of CcP, all in conjunction with additional kinetic measurements as recently discussed (*15*). Their resolution will require parallel x-ray structural studies on the mutants as well as NMR studies and computer simulations of protein dynamics.

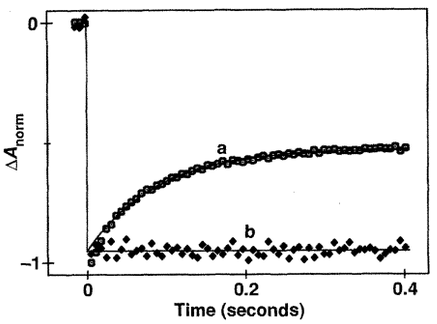


Fig. 3. Transient absorbance after flash photolysis of [ZnCcP,Cc(Ser^{82})] complex in the presence of $[Co(NH_3)_5Cl]^{2+}$ as an oxidative quencher. For trace a, the initial complex was [ZnCcP,Fe^{II}Cc]. The nearly instantaneous fall represents the generation of $(ZnCcP)^+$ by oxidation of ^{3}ZnCcP with the Co^{III} complex, which represents O in scheme 2; the $[Co(NH_3)_5Cl]^+$ (R in scheme 2) thus formed decomposes to $[Co(H_2O)_6]^{2+}$ and cannot react further. The subsequent rise reflects electron transfer from Fe^{II}Cc to $(ZnCcP)^+$. The corresponding solid line fits the data to $\Delta A = A(1 + Be^{-k_b t})$ with $k_b = 11 \pm 1$ sec^{-1}. The first few data points have a contribution from ^{3}ZnCcP; they are not included in the fit. For trace b, the initial complex was [ZnCcP,Fe^{III}Cc]. In this case, $(ZnCcP)^+$ is seen to be stable subsequent to its formation. Conditions: 4.5 μM ZnCcP with 9 μM Cc in 1 mM potassium phosphate buffer, pH 7, at 10°C and 0.1 mM $[Co(NH_3)_5Cl]Cl_2$. Monitoring wavelength is 432 nm (ZnCcP Soret band).

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7. Measurements with yeast iso-1-cytochrome c and its mutants are difficult because a surface sulfhydryl group, Cys^{102}, near the COOH-terminus of the yeast iso-1-cytochrome c, renders the protein susceptible to disulfide dimerization and autoreduction. Thus we have prepared a suite of mutants at position 82 further modified by the Cys → Thr mutation at position 102, which eliminates both problems [R. L. Cutler, G. J. Pielak, A. G. Mauk, M. Smith, *Protein Eng.* **1**, 95 (1987)]. We find that the kinetic properties of the Cys^{102} and Thr^{102} variants are indistinguishable under all conditions where they can be compared.
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