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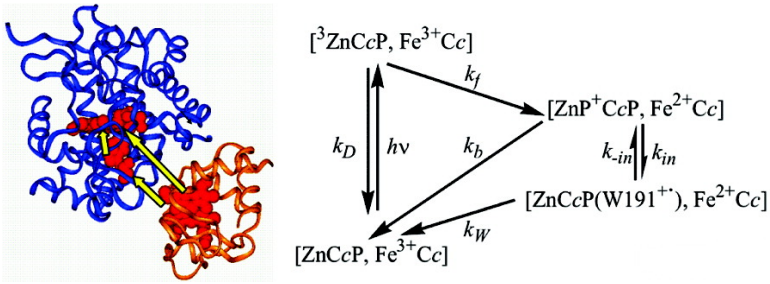

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Hopping in the Electron-Transfer Photocycle of the 1:1 Complex of Zn−Cytochrome *c* Peroxidase with Cytochrome *c*

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The physiological electron-transfer (ET) partners, cytochrome *c* peroxidase (C*c*P) and cytochrome *c* (C*c*)[1], often are viewed as a paradigmatic protein−protein ET pair,[2] but in fact form a quite complex system because C*c*P contains two redox centers, the heme and an adjacent tryptophan, W191.[3−6] These protein partners nonetheless occupy a central role in the study of interprotein ET, in considerable part because the heme of either partner can be modified to exhibit photoinitiated ET through substitution of Zn (or Mg) for Fe.[4] Laser excitation of the Zn−porphyrin (ZnP) to its triplet excited state (^{3}ZnP) initiates direct "heme−heme" ET to the ferriheme center across the protein−protein interface, with ET rate constant, k_f (eq 1)

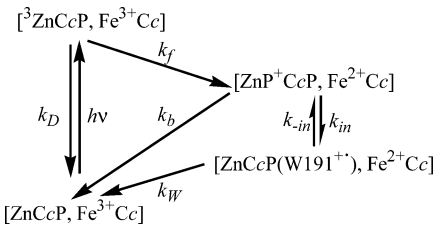
$$^3\mathrm{ZnC}c\mathrm{P} + \mathrm{Fe}^{3+}\mathrm{C}c \xrightarrow{k_f} \mathrm{ZnP}^{+}\mathrm{C}c\mathrm{P} + \mathrm{Fe}^{2+}\mathrm{C}c \qquad (1)$$

This photoinitiated ET produces the charge-separated intermediate, **I** = [ZnP$^+$C*c*P, Fe^{2+}C*c*], with a metalloporphyrin π-cation radical (ZnP$^+$) in the donor protein and a ferroheme in the acceptor protein. **I** returns to the ground state by a thermal ET process that has been viewed as involving direct heme−heme back-ET, with rate constant, k_b (eq 2), to complete a simple photocycle.

$$\mathrm{ZnP}^{+}\mathrm{C}c\mathrm{P} + \mathrm{Fe}^{2+}\mathrm{C}c \xrightarrow{k_b} \mathrm{ZnC}c\mathrm{P} + \mathrm{Fe}^{3+}\mathrm{C}c \qquad (2)$$

However, the occurrence of *direct* back-ET can be questioned when the metal substitution occurs in C*c*P. While the ^{3}ZnC*c*P can only be quenched by Fe^{3+}C*c* through direct heme−heme electron or energy transfer, the ZnP$^+$ formed through eq 1 *is* potentially able to oxidize W191, and this opens the possibility of the two-step, "hopping"[7] return of **I** to ground shown in Scheme 1. We here establish this hopping mechanism by contrasting intracomplex ET between yeast iso-1 C*c* and ZnC*c*P(WT) (wild-type) with that for two ZnC*c*P(X) variants: X = W191F, with redox-active W191 replaced by Phe;[8,9] WYM4,[10] a W191F mutant with further replacement of four other potentially redox-active sites[11,12] (W51F, Y187F, Y229F, and Y236F).

Scheme 1



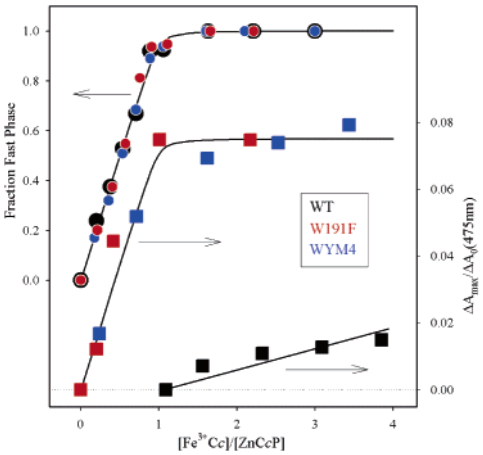


Figure 1. Titrations of ZnC*c*P(X) by Fe^{3+}C*c*. ZnC*c*P(X), X = WT (black), W191F (red), and WYM4 (blue). *Binding profile (left axis; circles)*: Fraction of ^{3}ZnC*c*P bound in 1:1 complex (decay constant, k_{obs}) plus collective fit to 1:1 isotherm, $K_A = 5 \times 10^7$ M^{-1}. *Maximum absorbance of* ***I*** *(right; squares)*: ΔA_{max}(547 nm) for **I** normalized to the $t = 0$ triplet absorbance difference [ΔA_0(475 nm)], with the same isotherm overlaid. *Conditions*: 5 μM ZnC*c*P, 10 mM KPi, pH 7.0, $T = 20$ °C.

For all three ZnC*c*P(X), the decay of photoexcited ^{3}ZnC*c*P is exponential, with decay constant $k_D \sim 100$ s^{-1}. When substoichiometric yeast iso-1 Fe^{3+}C*c* is added, the partners form the 1:1 complex visualized by X-ray diffraction.[13,14] This complex exchanges slowly on the time scale of the triplet lifetime, and the ^{3}ZnC*c*P decay becomes biexponential; the unbound fraction decays with k_D; the fraction of C*c*P involved in the [ZnC*c*P, Fe^{3+}C*c*] complex decays with a rate constant increased by the quenching constant (k_q), $k_{obs} = k_D + k_q$.[15] Figure 1 plots the fraction of bound ZnC*c*P-(X) formed for all three proteins during titrations with Fe^{3+}C*c*. A joint fit of the three titrations to a one-site binding isotherm gives the association constant, $K \approx 5 \times 10^7$ M^{-1}, as previously found for ZnC*c*P(WT).[15,16] The quenching constant also is unchanged by the mutations, showing that W191 is not involved in eq 1, as can be seen in the excellent overlay of ^{3}ZnC*c*P(X) decay traces (Figure 2) for the three variants in 1:1 complex with Fe^{3+}C*c*: k_{obs}(X) ≈ 300 s^{-1}, giving k_8(X) ≈ 220 s^{-1}. The same result is obtained with crystals of the 1:1 complex of [ZnC*c*P, Fe^{3+}C*c*].[14]

When the solution titration proceeds beyond stoichiometric C*c*, the ^{3}ZnC*c*P decay again becomes exponential, and the decay constant increases linearly with [Fe^{3+}C*c*], $k = k_{obs} + k_2[\mathrm{C}c]_{free}$, where $[\mathrm{C}c]_{free}$ is the concentration of unbound C*c* and k_2 is a second-order quenching constant, due to reaction at a second, weakly-binding domain on the C*c*P surface.[4,16−18]

Figure 2 presents progress curves for **I** collected at the 547 nm ^{3}ZnC*c*P/ZnC*c*P isosbestic point for the three ZnC*c*P(X) in ∼1:1 complexes with Fe^{3+}C*c*; the maximum absorbances during the progress curves for the titrations of the three ZnC*c*P are plotted in

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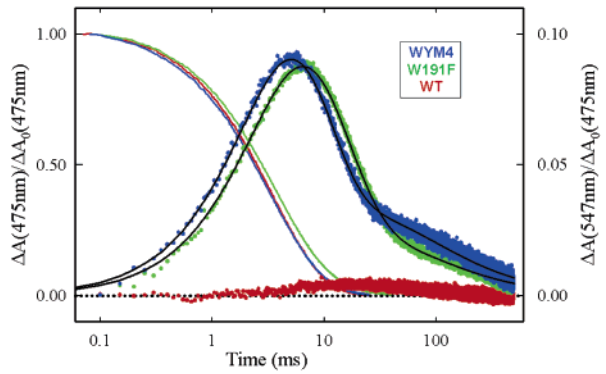

Figure 2. Kinetic progress curves for [ZnC*c*P(X), Fe^{3+}C*c*], X = WT, W191F, and WYM4. Triplet decay traces (475 nm, 20 shots, *left axis*) plus corresponding traces for **I** (547 nm, 100 shots, *right*), normalized to triplet [ΔA_0(475 nm)]. *Conditions*: 5 μM ZnC*c*P with 1 equiv of C*c*(y) in 10 mM KPi, pH 7.0, T = 20 °C. Multiple repeated traces overlay showing that the photocycle is reversible.

Figure 1. As reported previously,[16] little or no intermediate can be seen with ZnC*c*P(WT) up to the 1:1 point in the titration (Figures 1 and 2). Likewise, **I** is not seen upon photolysis of the crystalline complex.[14] In the solution titration of ZnC*c*P(WT), a signal from **I** appears as the concentration of Fe^{3+}C*c* increases beyond a 1:1 ratio and increases linearly with excess Fe^{3+}C*c* (Figure 1) due to second-site ET quenching (k_2), eq 1.

In sharp contrast to the results for ZnC*c*P(WT), quenching of the two variant ^{3}ZnC*c*P(X) (X = W191F, WYM4) by Fe^{3+}C*c* gives signals from **I** (Figure 2), and the amount of intermediate increases synchronously with the fraction of ZnC*c*P(X) bound in a tight-site complex up to the 1:1 ratio, rather than lagging until 1:1 (Figure 1).[19] Intermediate **I** for the mutants appears exponentially, with a rate constant that corresponds to the triplet decay of the complex, and it returns to the ground state through thermal back-ET with a smaller rate constant, $k_b < k_{obs}$. A "second phase" in the decay of **I** at long time (Figure 2) reflects partial dissociation of **I** (rate constant k_{off}) into the separated ZnP^+C*c*P and Fe^{2+}C*c* components, which subsequently undergo second-order back-ET to the ground state. Fits of the progress curves for **I** formed with the ZnC*c*P(X) variants give comparable rate constants: X = W191F; $k_b = 74\ s^{-1}$ $k_{off} = 16\ s^{-1}$; X = WYM4; $k_b = 140\ s^{-1}$, $k_{off} = 37\ s^{-1}$; second-order "charge recombination" of the dissociated partners, $k_r \sim 10^7$ $M^{-1}\ s^{-1}$ for both. On the basis of the measured extinction coefficient difference for **I**,[16] ca. $^1/_4$ of the quenching of ^{3}ZnC*c*P by Fe^{3+}C*c* leads to detectable accumulation of **I**; whether the remainder of the quenching is by energy transfer or through an additional channel for prompt return to ground[9] remains to be determined. The small differences between the two variants likely reflect subtle differences in structure/conformation;[20] contributions from hopping to one of the additional W/Y residues removed in WYM4 would increase k_b, contrary to observation.

The absence of accumulated **I** during triplet quenching in the [ZnC*c*P(WT), Fe^{3+}C*c*] 1:1 complex, both in solution and in single crystal, is compatible with the simple photocycle of eqs 1 and 2 only if the quenching is by energy, not electron transfer, or if k_b is much faster than k_f, and thus the ET intermediate does not build up to detectable levels. However, neither is the case. Consider the first alternative. The quenching process is not altered by the mutations, and it involves direct heme−heme ET in the two mutants. Thus, for ZnC*c*P(WT), the quenching must likewise involve heme−heme ET (eq 1). Now consider the second alternative. It cannot be that **I** fails to accumulate because direct heme−heme thermal back-ET (eq 2) is too fast, because the results with the mutants show that it is *not* fast: $k_b < k_{obs}$. Thus, the simple photocycle cannot apply. Instead, we conclude that W191 acts as an ET mediator and "short-circuits" the direct heme−heme back-ET through the two-step, hopping process of Scheme 1; the ZnP^+ cation radical formed by eq 1 rapidly oxidizes W191, and the resultant $W191^+$, in turn, rapidly oxidizes Fe^{2+}C*c*.[21] The absence of a significant signal from **I** requires that k_W, $k_{in} \gg k_{obs}$, which is compatible with W191−heme ET rates measured in studies of *intra*protein ET within C*c*P[6] and with studies of ET from ^{3}ZnC*c* to high-valence states of FeC*c*P.[22,23] ET hopping through W191 is abolished by mutating it to Phe, which is not oxidized by ZnP^+, thus slowing the return of **I** to ground and allowing it to accumulate. The detection of **I** in reaction of Zn(C*c*P) with horse C*c*, both in crystal[14] and in solution,[24] implies that k_W is decreased with the heterologous C*c*.

Elimination of W191 in C*c*P indeed allows us to treat the ZnC*c*P-(X)/Fe^{3+}C*c* partners (X = W191F, WYM4) as forming a paradigmatic interprotein ET complex with an heme-to-heme photocycle described by eqs 1 and 2, rather than the more complex one of Scheme 1. This finding opens the way to measuring both eqs 1 and 2 in solution and in single crystal with the mutant C*c*P's down to cryogenic temperatures, as we have done for ET within mixed-metal Hb hybrids.[25] For our solution studies, the modified [ZnC*c*P-(W191F), Fe^{3+}C*c*] complexes further offer new opportunities for studying the role of intracomplex dynamics in controlling ET, opportunities which we are actively pursuing.[9]

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