Cytochrome c Peroxidase Simultaneously Binds Cytochrome c at Two Different Sites with Strikingly Different Reactivities: Titrating a "Substrate" with an Enzyme

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The study of protein–protein complexes represents a central theme of current electron-transfer research.[1] Knowledge of the binding stoichiometries and affinities for such complexes provides a foundation for determining interfacial dynamics and recognition,[2-5] as well as electron-transfer (ET) pathways,[6] yet the binding stoichiometry with which CcP[7] binds Cc has long been in contention. Numerous measurements found only a 1:1 Cc:CcP complex,[8-14] despite early results from size-exclusion chromatography[15] that indicated a 2:1 binding stoichiometry. Recently, our studies[2c] of photoinduced electron transfer between ZnCcP and Cc confirmed the 2:1 binding stoichiometry and disclosed that the surface of CcP presents high- and low-affinity sites with widely differing reactivities. The present report describes photoinduced electron transfer in the "inverse" complex between ZnCc and Fe^{3+}CcP. However, this experiment does *not* simply repeat the prior study because it monitors the "substrate" (S), here ZnCc, during a titration performed by varying the concentration of the "enzyme" (E), here Fe^{3+}CcP. It results in a titration curve unlike any before seen, where the quenching of the ^{3}ZnCc shows a maximum value and then *decreases* with increasing Fe^{3+}CcP (enzyme) concentration. These measurements prove that CcP can bind Cc simultaneously at two distinct sites with dramatically different ($\sim 10^3$) affinities and reactivities, and they yield well-defined values for the *site* binding and ET quenching rate constants.

In the absence of Fe^{3+}CcP, the decay of ^{3}(ZnCc)[16] is exponential[18a] and the intrinsic rate constant of $k_D = 67\ s^{-1}$ is independent of buffer concentration. When Fe^{3+}CcP is added, ^{3}(ZnCc) is quenched by electron transfer.[18] The observed decay remains exponential[18a] (rate constant, k_{obs}), which indicates that exchange between bound and free ^{3}ZnCc is rapid. As shown in Figure 1, the rate constant for the quenching of ^{3}ZnCc in 2.5 mM KP_i buffer, $k_q = k_{obs} - k_D$, increases upon addition of the Fe^{3+}CcP quencher up to a ratio of CcP:Cc $\sim$ 1:2. *The quenching constant, k_q, then decreases with subsequent additions of Fe^{3+}CcP quencher.* The resulting maximum in k_q requires that a 1:2, [CcP,$(ZnCc)_2$], complex is involved and further implies that the electron-transfer quenching is much more efficient in a 1:2 complex than in a 1:1 complex. The gentle linear increase of k_q toward very high [Fe^{3+}CcP] (Figure 1A, inset) is attributable to bimolecular electron transfer between complex and free Fe^{3+}CcP.

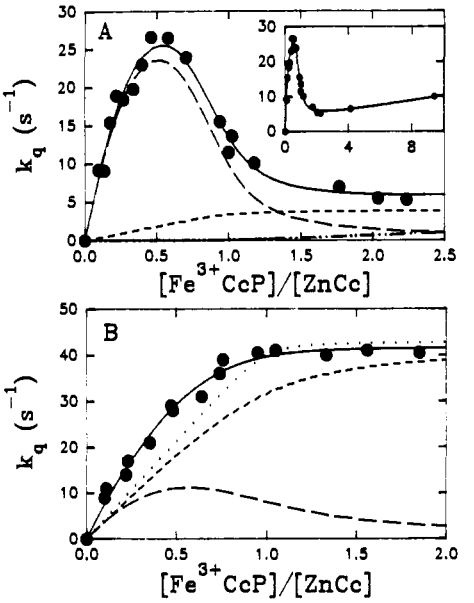
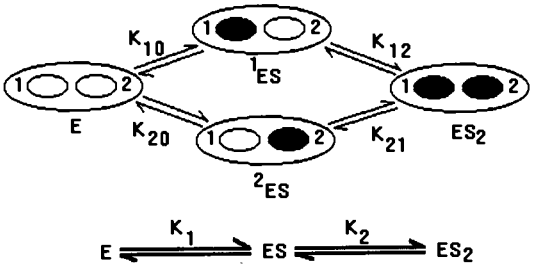


Figure 1. Quenching titration of ^{3}(ZnCc) by Fe^{3+}CcP. The solid lines (—) are calculated with eq 2 using the parameters given in Table I. The (i) (– – –), (ii) (- - -), and (iii) (–··–) lines are respectively the contributions (individual terms in eq 2) to the quenching of ^{3}(ZnCc) by Fe^{3+}CcP from (i) 1:2 and (ii) 1:1 complexes and (iii) from the reaction between 1:1 complex and free Fe^{3+}CcP. (A) Quenching constant (k_q) vs Fe^{3+}CcP/ZnCc for [KP_i] = 2.5 mM. Inset: k_q at very high [Fe^{3+}CcP]. Conditions: [ZnCc] = 8.5 μM, in 2.5 mM KP_i buffer (pH 7.0) at 20 °C. (B) Quenching constant (k_q) vs Fe^{3+}CcP/ZnCc for [KP_i] = 10 mM. The (···) line is the fit to a 1:1 isotherm with $k = 43\ s^{-1}$ and $K = 5 \times 10^7\ M^{-1}$. Conditions: [ZnCc] = 10 μM, in 10 mM KP_i buffer (pH 7.0) at 20 °C. Uncertainties in k_q, $\pm$ 2 s^{-1}.

Scheme I



$$^3(ZnCc):CcP + Fe^{3+}CcP \rightarrow ZnCc^+:CcP + Fe^{2+}CcP \quad (1)$$

The dependence of k_q on [Fe^{3+}CcP] can be quantitatively described by a mechanism involving different electron-transfer rate constants for complexes of 1:1 and 1:2 stoichiometry, augmented by eq 1. Scheme I describes the binding of two substrate ZnCc to CcP in terms of site binding constants K_{ij}, i,

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(7) Abbreviations: cytochrome *c* peroxidase, CcP; zinc cytochrome *c* peroxidase, ZnCcP; cytochrome *c*, Cc; zinc cytochrome *c*, ZnCc; potassium phosphate buffer, KP_i.
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(18) (a) Supplementary Figure S1 presents semilog plots of the triplet decay traces. (b) Studies of the electron-transfer intermediate as observed by transient absorption are in progress.

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Table I. Stoichiometric Constants for the 1:2 Binding by Fe^{3+}*Cc*P and ZnC*c*[a]

	$[KP_i]$ = 2.5 mM	$[KP_i]$ = 10 mM
K_1 (M^{-1})	7.7×10^6	4.9×10^5
K_2 (M^{-1})	7.5×10^3	4×10^3
k_1 (s^{-1})	4	40
k_2 (s^{-1})	1630	1620
k_3 (s^{-1} M^{-1})	8.2×10^4	*b*

[a] Stoichiometric affinity constants are defined in Scheme I. Stoichiometric rate constants are defined by eq 2. Titrations performed with KP_i buffer, pH 7. [b] Undetermined.

j = 0–2, as well as in terms of stoichiometric binding constants K_i, i = 1, 2. In the rapid-exchange limit of this scheme that applies here, the quenching constant k_q can be written

$$k_q = k_1[ES]/[S]_0 + k_2[ES_2]/[S]_0 + k_3[E]_f \qquad (2)$$

Here $[S]_0$ is the total concentration of ZnC*c* and $[ES] = [^1ES] + [^2ES]$ is the total concentration of 1:1 complex independent of site occupancy: it can be calculated from the stoichiometric equilibria[16b] (the bottom of Scheme I). The parameters k_1 and k_2 are *stoichiometric* electron-transfer rate constants that respectively correspond to the net quenching by complex with 1:1 stoichiometry and by complex with 1:2 stoichiometry. Finally, k_3 is the bimolecular rate constant between complex and free Fe^{3+}C*c*P, denoted $[E]_f$ (eq 1). The quenching curve for 2.5 mM KP_i buffer is fit extremely well with eq 2 (Figure 1A); the resulting stoichiometric constants are listed in Table I. The decomposition of the titration curve into contributions from the three terms in eq 2 clearly demonstrates that although the concentration of 1:2 complex is only ~0.1% that of the 1:1 complex, yet the 1:2 stoichiometry dominates the quenching. As confirmation of this model, we find that a titration by Fe^{3+}C*c*P where site 1 is blocked gives a simple binding isotherm with k_q approaching k_2.[19]

The relationships between stoichiometric and site binding constants in the two-site case are given by eq 3.[20] Likewise, if

$$K_1 = K_{10} + K_{20} \qquad K_1K_2 = K_{10}K_{12} = K_{20}K_{21} \qquad (3)$$

we assume that each of the two sites on C*c*P has a fixed quenching rate constant, (^1k, ^2k), then these are related to the stoichiometric rate constants that we define by eq 4. We set aside for the present

$$k_1 = {}^1k(K_{10}/K_1) + {}^2k(K_{20}/K_1) \qquad k_2 = {}^1k + {}^2k \qquad (4)$$

such alternate possibilities[16b] as "reactivity cooperativity", where binding a second ZnC*c* at site 2 increases the quenching constant for site 1. Treatment of the stoichiometric constants (Table I) with eqs 3 and 4 gives well-defined values for two of the site constants, $K_{10} \sim K_1 = 7.7 \times 10^6$ M^{-1}, $^2k \approx k_2 = 1630$ s^{-1}, and sets limits on the other two independent site constants, $0 \le {}^1k \le k_1 \approx 4$ s^{-1}, $0 \le K_{20} \le 1.85 \times 10^4$ M^{-1}. However, these latter ranges can be further restricted. Given that two C*c* bound to C*c*P will repel each other electrostatically, it is reasonable to require that $K_{21} < K_{10}$, namely, that binding at site 1 is weaker when site 2 is filled. Then the site constants, K_{20} and ^1k, are limited to the ranges $^1k \approx 0$–2.4 s^{-1} and $K_{20} \approx 0.7$–1.9×10^4 s^{-1}.[21] Thus, $^2k \sim 10^{3.1}k$, whereas $K_{20} \sim 10^{-3}K_{10}$.

At the higher buffer concentration (10 mM KP_i), the rate constant for quenching of ^{3}(ZnC*c*) increases with $[Fe^{3+}CcP]$ until a 1:1 ratio is reached, and it then remains constant at a plateau value of $k_q \sim 41$ s^{-1} (Figure 1B). The measurements are consistent with those reported by Conklin and McLendon.[22] This behavior may *appear* consistent with a 1:1 stoichiometry of the ZnC*c*:C*c*P complex, but it is not possible to fit the titration curve with a simple 1:1 binding expression (Figure 1B). Instead, the curve can be fit with eq 2, yielding stoichiometric constants listed in Table I. The decompositions of Figure 1 show that the qualitative change between 1A and 1B arises from a reversal in relative contributions from the singly and doubly bound enzyme forms, ES_1 and ES_2. The same arguments used above give the site constants $K_{10} = 4.9 \times 10^5$ M^{-1}, $K_{20} \sim 1.2 \times 10^4$ M^{-1}, $^1k \sim 0$–2.4, and $^2k \sim 1620$. Thus, the titration curve changes shape because of the 10-fold reduction in the affinity at the strongly binding but poorly quenching site. At 10 mM buffer it is still true that $^2k \sim 10^{3.1}k$, but now $K_{20} \sim 10^{-2.4}K_{10}$.

This report completes the demonstration[2c] that C*c*P can bind two C*c* simultaneously and that the individual binding sites differ by *ca.* 10^3 in their affinities. The fixed-affinity model indicates that the weakly binding site is *ca.* 10^3 times more reactive. The alternative model of reactivity cooperativity would imply that binding the second ZnC*c* increases the reactivity toward the first by *ca.* 10^3, an equally remarkable result. The present procedure of titrating a substrate with a multisite enzyme offers a unique opportunity to characterize a weakly binding site in the presence of one with high affinity. Characterization of the full ET cycle and of the location of the high-reactivity, low-affinity site on the C*c*P surface should permit us to determine whether the reactivity differences reflect the importance of specific ET pathways within C*c*P.

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Supplementary Material Available: One figure of triplet decay traces (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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