fluenced by paramagnetic oxygen gas, and the hydrogen atom was stable with respect to the oxygen molecules near the D4R cage. The fluctuation of the local magnetic field at the encapsulated atomic hydrogen is induced by triplet oxygen and affects spin-lattice relaxation. The saturation power depended on the O_2 pressure in a manner quite similar to the way the apparent intensity does. These observations show clearly that the hydrogen atom is encapsulated in the D4R cage and can experience the magnetic field induced by O_2 molecules outside the cage without chemical attack by the latter. The D4R cage in the crystalline state has free space around itself. In that space, oxygen molecules can reach near the cage. The free space is maintained by bulky trimethylsilyl groups.

Relaxation times were determined by electron spin echo (ESE) for the main lines that are split only by the hydrogen nucleus. Echo signals were not obtained at room temperature because of their fairly small relaxation times, so the measurement was performed for the degassed sample at 77 K. From two-pulse and three-pulse ESE decays of the atomic hydrogen sample (Fig. 4), the phase memory time, t_M, of 0.87 μs and the spin-lattice relaxation time, t_1, of 13 μs were obtained. Thus, the phase memory time is considered to be constant because the line shape of the ESR signal was invariable with respect to temperature, degree of vacuum, and exposure gas. The spin-lattice relaxation time likely has a relatively large temperature dependence, because echo signals were not obtained at room temperature. This temperature dependence probably arises from the motion of the terminal methyl group and, accordingly, corresponds to the large ellipsoids for methyl carbon in the crystal structure analysis.

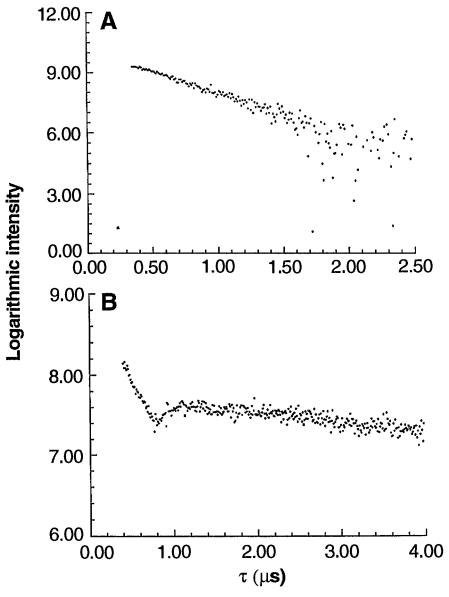

Fig. 4. Envelopes against the interval between the first and second pulses, τ, of (**A**) two-pulse spin echo and (**B**) three-pulse spin echo for atomic hydrogen in γ-irradiated $[(CH_3)_3Si]_8Si_8O_{20}$ at 77 K. Conditions: (A) ν = 8.952155 GHz, magnetic field (H) = 342.733 mT, pulse width = 40 ns; (B) ν = 8.95167 GHz, H = 342.711 mT, pulse width = 40 ns, interval between the second and third pulses (τ) = 400 ns.

We could not encapsulate hydrogen atoms in D3R silicate cages as mentioned earlier and we have yet to study the larger D5R, D6R, and D7R silicate cages (9). These cages provide a chemically shielded space in which an atom or atoms can behave just as in the free state. The construction of new cages should lead to novel developments in the study of atoms and atom-cluster interactions.

REFERENCES

1. G. E. Pake and T. L. Estle, *The Physical Principles of Electron Paramagnetic Resonance* (Benjamin, Reading, MA, ed. 2, 1973), pp. 233–240.
2. R. A. Weeks and M. Abraham, *J. Chem. Phys.* **42**, 68 (1965).
3. T. Cole and A. H. Silver, *Nature* **200**, 700 (1963).
4. D. Hoebbel and W. Wieker, *Z. Anorg. Allg. Chem.* **384**, 43 (1971).
5. Yu. I. Smolin, *ACS Symp. Ser.* **194**, 329 (1982).
6. G. T. Trammell, H. Zeldes, R. Livingston, *Phys. Rev.* **110**, 630 (1958); M. Bowman, L. Kevan, R. N. Schwartz, *Chem. Phys. Lett.* **30**, 208 (1975); B. L. Bales and E. S. Lesin, *J. Chem. Phys.* **65**, 1299 (1976).
7. J. E. Wertz and J. R. Bolton, *Electron Spin Resonance: Elementary Theory and Applications* (McGraw-Hill, New York, 1972), pp. 434–445.
8. S. N. Foner, E. L. Cochran, V. A. Bowers, C. K. Jen, *J. Chem. Phys.* **32**, 963 (1960).
9. P. A. Agaskar, V. W. Day, W. G. Klemperer, *J. Am. Chem. Soc.* **109**, 5554 (1987).

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Stern-Volmer in Reverse: 2:1 Stoichiometry of the Cytochrome c–Cytochrome c Peroxidase Electron-Transfer Complex

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A reverse protocol for measurements of molecular binding and reactivity by excited-state quenching has been developed in which the quencher, held at a fixed concentration, is titrated by a photoexcitable probe molecule whose decay is monitored. The binding stoichiometries, affinities, and reactivities of the electron-transfer complexes between cytochrome c (Cc) and cytochrome c peroxidase (CcP) were determined over a wide range of ionic strengths (4.5 to 118 millimolar) by the study of photoinduced electron-transfer quenching of the triplet excited state of zinc-substituted Cc (ZnCc) by Fe^{3+}CcP. The 2:1 stoichiometry seen for the binding of Cc to CcP at low ionic strength persists at the physiologically relevant ionic strengths and likely has functional significance. Analysis of the stoichiometric binding and rate constants confirms that one surface domain of CcP binds Cc with a high affinity but with poor electron-transfer quenching of triplet-state ZnCc, whereas a second binds weakly but with a high rate of electron-transfer quenching.

Measurements of excited-state quenching have been important for no less than the 75 years since the original Stern-Volmer report (*1*) and have been used to study the binding of one biomolecule to another for nearly half a century (*2–6*). In particular, recent quenching studies of protein-protein electron-transfer complexes, which are representative of even more complicated assemblies in photosynthesis and respiration, give information about binding (*7*, *8*) and interfacial dynamics and recognition (*9–12*) as well as the electron-transfer process itself (*13*, *14*). Despite the wide use of quenching methods, the basic experiment has remained unchanged: A probe molecule at fixed concentration is examined while being titrated with the quencher. Even our recent study (*8*), which showed that CcP can bind two molecules of Cc under low-salt conditions, although unusual in that it involved a titration of a "substrate," Cc, with an enzyme, CcP, used this basic procedure. Here we describe a "reverse" quenching experiment: The quencher at fixed concentration is titrated by the probe molecule. Through the use of this protocol, we demonstrate that the 2:1 stoichiometry seen for the binding of Cc to CcP at low ionic strength persists at the physiologically relevant ionic strengths and thus is likely to have functional significance. The results further provide the means for analyzing the dependence of the electron-transfer rate constant on ionic strength in terms of changes in the distribution among multiple structures of a complex.

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Before discussing the use of the reverse titration procedure to examine the stoichiometry of the binding of Cc to CcP at high ionic strength, we compare the predicted results when the normal and reverse protocols are applied to a simple 1:1 binding equilibrium. Consider a protein, S, that functions as a probe while forming a complex with a quencher protein, Q

$$Q + S \rightleftharpoons QS \quad (1)$$

where the association constant is K_1 and the intracomplex quenching constant is k_1. In the rapid-exchange limit (*15*), the excited state of S decays exponentially, with a quenching constant (*16*) given by

$$k_q = k_1[QS]/[S]_0 = k_1 f_1 \quad (2)$$

Here $[S]_0$ and $[Q]_0$ are the total concentrations of S and Q, and [QS] is the concentration of the complex as calculated from the association equilibrium. Thus, f_1 is the fraction of the total S that is bound to Q.

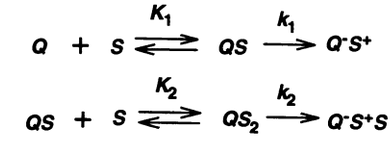
In a conventional titration where the concentration of S is fixed and that of Q varies, k_q increases smoothly from 0 at [Q] = 0 to a plateau value of $k_q = k_1$ in the limit $K_1[Q] \gg 1$ (*17*). However, the limit is difficult to achieve for weakly bound complexes (those with small values of K_1). In contrast, in a reverse titration with the quencher at fixed concentration $[Q]_0$ and the probe S as titrant, the observed quenching constant has a well-defined, nonzero limit (intercept) as $[S]_0 \rightarrow 0$, denoted as k_q^0, that is readily measured for any K_1:

$$k_q^0 = k_1K_1[Q]_0/(1 + K_1[Q]_0) = k_1f_1^0 \quad (3)$$

where f_1^0 is the fraction of bound S in the extrapolated limit as the ratio $R = [S]_0/[Q]_0 \rightarrow 0$. This relation (Eq. 3) between K_1 and k_1 can be used to eliminate one parameter in the fitting of a full titration curve. The quenching constant necessarily falls monotonically with increasing $[S]_0$ according to Eq. 2, the dependence being essentially linear for $K_1[S]_0 \ll 1$. The shape of the curve provides data for determining the second fitting parameter.

Measurements of complex formation by the physiological redox partners, Cc and CcP (*18*, *19*), have been carried out by study of the photoinduced electron-transfer quenching of Zn-substituted Cc (ZnCc) (*20*) as the probe protein S, whose excited state is monitored, and Fe^{3+}CcP as the quencher Q (*21*). The replacement of Fe(II) in Cc by Zn(II) yields Zn-porphyrin in ZnCc as the photoactive probe (*22*) without perturbing the conformation (*23*) of Cc or its association with other proteins (*24*). We showed that a normal quenching titration is advantageously done with this Q, S pair, namely by titrating the "substrate" probe ZnCc with the enzyme Fe^{3+}CcP (*8*, *25*) rather than by titrating ZnCcP with the substrate quencher Fe^{3+}Cc, and that the complex formed by these species is in rapid exchange (*7*, *15*, *21*). We first discuss normal titrations for ionic strength from 4.5 to 118 mM (Fig. 1) and then present the corresponding results for the reverse protocol.

At low ionic strength (4.5 mM), the normal titration of Fe^{3+}CcP with ZnCc does not yield the hyperbolic curve for k_q that is expected for 1:1 binding (Fig. 1A). Instead, k_q exhibits a maximum at a ZnCc:Fe^{3+}CcP concentration ratio of $R \approx 2:1$ and then decreases upon addition of more quencher. Such a result cannot occur for a 1:1 binding stoichiometry and unambiguously shows that at low ionic strength (4.5 mM) the CcP can bind Cc simultaneously at two distinct domains. The dependence of k_q on [Fe^{3+}CcP] at this ionic strength can be quantitatively described by Eq. 4, which is based on a thermodynamic electron-transfer mechanism (Scheme 1)



Scheme 1

involving complexes with 1:1 (QS) and 2:1 (QS_2) stoichiometries in the rapid-exchange limit (*8*, *26*):

$$k_q = k_1[QS]/[S]_0 + k_2[QS_2]/[S]_0 = k_1f_1 + k_2f_2 \quad (4)$$

Here, f_1 and f_2 represent the fractions of total S that exist in the form of 1:1 and 2:1 complexes, respectively; these quantities are readily derived as functions of the total concentrations of the ZnCc and Fe^{3+}CcP and of the stoichiometric binding constants K_1 and K_2. The parameters k_1 and k_2 are stoichiometric rate constants that, respectively, correspond to the net quenching in complexes with 1:1 and 2:1 stoichiometries. If one considers a microscopic version of the 2:1 thermodynamic (stoichiometric) Scheme 1 (Fig. 2), then the concentration of the 1:1 complex in Eq. 4, [QS], is seen to be the sum of the concentrations of the two distinct complexes {[1, 0] and [0, 1]} with a Cc bound at one domain on Fe^{3+}CcP.

The parameters to Scheme 1 obtained from the fit (Table 1) show that the first Cc binds to CcP with a low affinity but with a low net rate constant for electron-transfer quenching, whereas the second does so with a low affinity but yields a 2:1

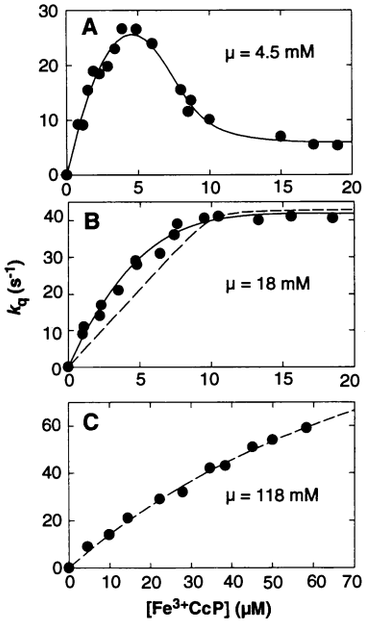


Fig. 1. Normal quenching titration of ^{3}ZnCc by Fe^{3+}CcP, plotted as k_q versus $[Fe^{3+}CcP]_0$. The solid lines are calculated from Eq. 4 with the parameters given in Table 1. (**A**) Quenching at 4.5 mM ionic strength. Conditions: $[ZnCc]_0$ = 8.5 μM in potassium phosphate buffer (pH 7.0) at 20°C. (**B**) Quenching at 18 mM ionic strength. The dashed line is the fit to a 1:1 isotherm (Eq. 2) with $k_1 = 43\ s^{-1}$ and $K_1 = 5 \times 10^7\ M^{-1}$. Conditions: $[ZnCc]_0$ = 10 μM in potassium phosphate buffer (pH 7.0) at 20°C. (**C**) Quenching at 118 mM ionic strength. The dashed line is the fit to a 1:1 isotherm (Eq. 2) with parameters given in Table 1. Conditions: $[ZnCc]_0$ = 10 μM in potassium phosphate buffer (pH 7.0) at 20°C. Uncertainties in k_q are $\pm 2\ s^{-1}$.

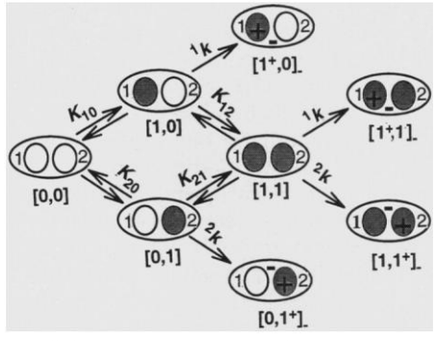


Fig. 2. Scheme that describes the nonexclusive binding by CcP (outer oval) of two Ccs at two distinct domains (1 and 2). The presence of a bound Cc is indicated by shading. The K_{ij} (i = 1, 2 and j = 0, 1, 2) are domain binding constants (Eqs. 5 and 6), and the ^ik (i = 1, 2) are domain electron-transfer rate constants (Eqs. 7 and 8). The product of electron transfer from ZnCc to Fe^{3+}CcP is represented by a (+) on the reactive Cc and by a (−) on the CcP.

complex that is highly reactive. In terms of the microscopic mechanism of Fig. 2, this result indicates that one domain binds well but reacts poorly and that the second binds poorly but reacts well. When the normal titration is done at slightly higher ionic strength (18 mM), the titration curve again departs from the hyperbola expected for a 1:1 binding stoichiometry, but the difference between titration curves for 1:1 and 2:1 binding models is sharply reduced (Fig. 1B).

At 118 mM ionic strength, however, the normal titration gives no evidence for a 2:1 complex (Fig. 1C). Instead, the dependence of k_q on Fe^{3+}CcP quencher concentration can be described extremely well by Eq. 2 (Fig. 1C), on the basis of electron transfer involving only a 1:1 complex in rapid exchange; the resulting values for K_1 and k_1 are given in Table 1. Thus, binding studies of the Cc:CcP complex by the traditional quenching approach might suggest that the 2:1 binding stoichiometry seen at low ionic strength is not of functional significance because it is not seen at physiological ionic strengths of ~100 mM or greater.

Now let us consider reverse titrations in which ZnCc is added to a fixed concentration of the quencher Fe^{3+}CcP. Such a titration at 4.5 mM ionic strength also is dramatically different from the prediction for a 1:1 binding mechanism (Fig. 3). According to Eq. 1, for such a model the quenching constant should decrease monotonically with [ZnCc] (Fig. 3A). Instead the data show a lag, with k_q changing minimally from the intercept k_q^0 until R = [ZnCc]/[Fe^{3+}CcP] ~1, and then an increase with increasing [ZnCc]. This lag occurs because with Fe^{3+}CcP in excess ($R < 1$), essentially all ZnCc molecules bind to Fe^{3+}CcP at the strongly binding but poorly reactive domain. In the context of Fig. 2, this result indicates that the [1, 0] form dominates and that the [0, 1] form is negligibly populated. As R increases beyond 1, the additional ZnCc begins to bind to Fe^{3+}CcP at the weakly binding, highly reactive domain. This leads to increasing amounts of the reactive 2:1 complex [1, 1], which causes the quenching constant to increase. Finally, a maximum is reached and k_q decreases. The decrease past the maximum occurs despite a continued increase in the concentration of [1, 1] because there is a decrease in the fraction of the total ZnCc that is bound in the reactive [1, 1] complex; according to Eq. 4, this decreases the quenching constant. Equation 4 also yields Eq. 3 for a reverse titration in the limit $R \rightarrow 0$, with the attendant benefit.

The reverse titration at 4.5 mM ionic strength is well described by the theoretical curve for 2:1 binding based on Eq. 4 (Fig. 3A) and yields binding and kinetic parameters (Table 1) in agreement with those used for the normal titration (Fig. 1A). However, in this instance the values of stoichiometric constants obtained from the normal titration are more reliable because of the constraints imposed by the unusual shape of the normal titration curve. Thus, both protocols agree in showing that there are two distinctive binding sites on CcP and that at low ionic strength CcP can bind two Ccs simultaneously.

At 18 mM ionic strength, the normal titration gives marginal evidence for a 2:1 binding (Fig. 1B). However, a reverse titration unambiguously disproves the model of a 1:1 binding stoichiometry (Fig. 3B). The parameters for the 1:1 model that best describe the normal titration curve (Fig. 1B) require that k_q exhibit a monotonic, fourfold decrease during the reverse titration of 10 μM Fe^{3+}CcP by ZnCc. Instead, k_q increases during the initial phase of the reverse titration, achieves a maximum increase of ~50%, and then decreases. The explanation for this result is the same as for the titration at 4.5 mM ionic strength, and the experimental data can be fit well with the 2:1 model embodied in Eq. 4 (Table 1). Table 1 also includes the fitting parameters for the normal titration at 18 mM ionic strength; in this case, they are the less reliable because at 18 mM ionic strength the normal titration curve deviates so little from a 1:1 binding isotherm.

At 118 mM ionic strength, the normal titration does not deviate from the curve for a 1:1 binding model (Fig. 1C). However, the reverse titration again unambiguously disproves this model: It shows an increase of k_q with increasing [ZnCc] as predicted by the 2:1 model rather than the decrease required in the 1:1 binding model (Fig. 3C). The data again are well fit by Eq. 4, based on the thermodynamic 2:1 model (Scheme 1), and the fit gives the parameters in Table 1. Thus, the 2:1 model is required to explain the data at all ionic strength values used.

Of the four stoichiometric constants

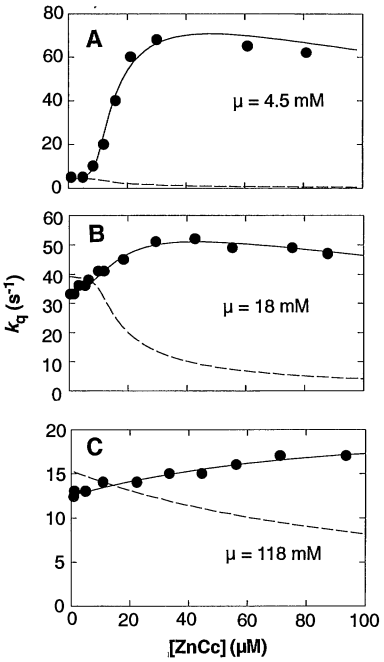


Fig. 3. Reverse quenching titration of Fe^{3+}CcP by ZnCc, plotted as k_q versus $[ZnCc]_0$. The solid lines are calculated from Eq. 4 with the parameters given in Table 1. (**A**) Quenching at 4.5 mM ionic strength. The dashed line is the theoretical curve of a 1:1 binding model (Eq. 2) with $k_1 = 4\ s^{-1}$ and $K_1 = 5 \times 10^7\ M^{-1}$. Conditions: $[Fe^{3+}CcP]_0 = 10$ μM in potassium phosphate buffer (pH 7.0) at 4.5 mM ionic strength and 20°C. (**B**) Quenching at 18 mM ionic strength. The dashed line is the theoretical curve of a 1:1 binding model (Eq. 2) with $k_1 = 40\ s^{-1}$ and $K_1 = 5 \times 10^7\ M^{-1}$. Conditions: $[Fe^{3+}CcP]_0 = 10.4$ μM in potassium phosphate buffer (pH 7.0) at 18 mM ionic strength and 20°C. (**C**) Quenching at 118 mM ionic strength. The dashed line is the theoretical curve of a 1:1 binding model (Eq. 2) with $k_1 = 170\ s^{-1}$ and $K_1 = 1.0 \times 10^4\ M^{-1}$. Conditions: $[Fe^{3+}CcP]_0 = 10$ μM in potassium phosphate buffer (pH 7.0) at 118 mM ionic strength and 20°C. Uncertainties in k_q are $\pm 2\ s^{-1}$.

Table 1. Stoichiometric constants for the 2:1 binding of Cc by CcP. Stoichiometric constants are defined in Scheme 1. Parameters obtained from the best fit of data from a normal titration to Eq. 4 are indicated as *N* and those from the reverse titration are indicated as *R*. When both types of data are presented, the more reliable is given first. The most realistic estimate of errors for the data at 4.5 and 18 mM ionic strength is given by the difference between the *N* and *R* parameters; a similar error is assigned for the k_1 and K_1 values at 118 mM. The values of k_2 and K_2 at 118 mM ionic strength have uncertainties of ±50%.

Parameter	Ionic strength					
	4.5 mM		18 mM		118 mM	
	N	*R*	*R*	*N*	*R*	*N*
K_1 (M^{-1})	7.7×10^6	9.8×10^6	1.2×10^6	4.9×10^5	6.6×10^3	1.0×10^4
K_2 (M^{-1})	7.5×10^3	7.7×10^3	4.6×10^3	4×10^3	1.4×10^3	—
k_1 (s^{-1})	4.0	4.5	37	40	200	170
k_2 (s^{-1})	1630	1490	1460	1620	2000	—

for the 2:1 thermodynamic binding Scheme 1, three (K_1, K_2, and k_1) depend on ionic strength but k_2 does not (Table 1). The K_1 and K_2 constants decrease at higher ionic strength, with K_1 changing more. This difference arises because K_1 describes the binding of the positively charged Cc to the negatively charged CcP, forming either [1, 0] or [0, 1], but K_2 describes the binding of Cc to a weakly charged 1:1 complex (Fig. 2). The fact that k_1 depends strongly on ionic strength but k_2 does not cannot be used to infer that electron transfer within the 1:1 complex is sensitive to ionic strength but that within the 2:1 complex this transfer is not sensitive. Instead, it can be understood by recalling that the stoichiometric constants defined by Scheme 1 and measured by a titration experiment are composites of the microscopic constants associated with the two binding domains on CcP, as defined in Fig. 2 (*17*). The relations are as follows:

$$K_1 = K_{10} + K_{20} \quad (5)$$

$$K_1K_2 = K_{10}K_{12} = K_{20}K_{21} \quad (6)$$

$$k_1 = {}^1k[K_{10}/(K_{10} + K_{20})] + {}^2k[K_{20}/(K_{10} + K_{20})] \quad (7)$$

$$k_2 = {}^1k + {}^2k \quad (8)$$

The stoichiometric rate constants k_1 and k_2 for 1:1 and 2:1 complexes, respectively, both depend on the two microscopic single-domain rate constants, ${}^1k$ and ${}^2k$, but only k_1 further depends on the two microscopic binding constants K_{10} and K_{20} through the functions $K_{i0}/\Sigma K_{i0}$, with $i = 1, 2$. These functions respectively describe the fraction of 1:1 complexes that have Cc bound at domain 1 {$i = 1$; [1, 0] in Fig. 2} and at domain 2 {$i = 2$; [0, 1]}. The two stoichiometric binding constants change differentially with ionic strength (Table 1), which requires an accompanying change in the distribution of a single bound Cc between the two binding domains on CcP, namely a change in the relative amounts of the [1, 0] and [0, 1] forms of the 1:1 complex (Fig. 2). However, Eqs. 7 and 8 show that this alteration forces a change in the stoichiometric rate constant k_1, even if the domain rate constants do not.

Analogous results would be obtained if one protein bound another in 1:1 stoichiometry at two overlapping sites within a single domain, where the two sites had different electron-transfer rate constants. In this case, there would still be two 1:1 forms of the complex in formal equivalence to [1, 0] and [0, 1]. A shift in their relative populations with ionic strength would change the measured stoichiometric rate constant even without any changes in the site rate constants. This discussion in terms of Fig. 2 and Eqs. 5 to 8 gives a precise form to ideas discussed by Hazzard and colleagues (*27*) and by McLendon (*28*), who reported ionic strength–dependent, electron-transfer rate constants.

The reverse-titration protocol reported here accentuates the differences between 1:1 and 2:1 binding models and has shown that a functionally significant 2:1 complex of Cc and CcP occurs at physiological ionic strengths of >100 mM. Thus, we must revise the view held for almost a quarter of a century that CcP binds Cc in a 1:1 stoichiometry (*6*, *29*–*33*). Analysis of the stoichiometric binding and rate constants over a wide range of ionic strength confirms that one domain on CcP binds Cc with a high affinity but in a fashion such that the electron-transfer quenching of ^{3}ZnCc by Fe^{3+}CcP is poor, whereas the second domain has weak binding but a high rate of electron-transfer quenching. As a result, in this experiment the minority 2:1 complex provides the bulk of the reactivity. The likely identity of the first domain 1 is given by the x-ray study of Pelletier and Kraut (*31*), whereas the calculations of Northrup and co-workers give a plausible location for the second domain (*33*). Clearly, it is difficult to probe contributions from two forms of a complex when the reactive one is in the minority, and this condition is particularly true for bulk physical probes such as nuclear magnetic resonance and x-ray crystallography. Thus, the CcP-Cc system may not be unique in presenting such a situation. Finally, the above discussion of site (or domain) versus stoichiometric rate constants provides a means of analyzing an ionic strength–dependent, interprotein electron-transfer rate constant in terms of changes in binding affinities.

REFERENCES AND NOTES

1. O. Stern and M. Volmer, *Phys. Z.* **20**, 183 (1919).
2. D. J. Laurence, *Biochem. J.* **51**, 168 (1952).
3. S. F. Velick, in *Light and Life*, W. D. McElroy and B. Glass, Eds. (Johns Hopkins Univ. Press, Baltimore, MD, 1961), p. 108.
4. G. Weber and F. W. J. Teale, in *The Proteins*, H. Neurath, Ed. (Academic Press, New York, 1965), vol. 3, p. 445.
5. H. N. Eisen and J. E. McGuigan, in *Methods in Immunology and Immunochemistry*, C. A. Williams and M. W. Chase, Eds. (Academic Press, New York, 1971), vol. III, p. 395.
6. J. J. Leonard and T. Yonetani, *Biochemistry* **13**, 1465 (1974).
7. E. D. A. Stemp and B. M. Hoffman, *ibid.* **32**, 10848 (1993).
8. J. S. Zhou and B. M. Hoffman, *J. Am. Chem. Soc.* **115**, 11008 (1993).
9. J. M. Nocek *et al.*, *ibid.* **113**, 6822 (1991); S. A. Wallin *et al.*, *ibid.*, p. 1842.
10. G. McLendon *et al.*, *ibid.* **115**, 3665 (1993).
11. Q. Zhang, J. Marohn, G. McLendon, *J. Phys. Chem.* **94**, 8628 (1990).
12. J. S. Zhou and N. M. Kostić, *J. Am. Chem. Soc.* **114**, 3562 (1992); *ibid.* **115**, 10796 (1993).
13. D. N. Beratan, J. N. Onuchic, J. R. Winkler, H. B. Gray, *Science* **258**, 1740 (1992).
14. M. J. Clarke *et al.*, Eds., *Struct. Bonding (Berlin)* **75**, 1 (1991); H. Sigel and A. Sigel, Eds., *Met. Ions Biol. Syst.* **27** (no. 33), 1 (1992).
15. The rapid-exchange limit means that the complex formation and dissociation are faster than the intracomplex quenching.
16. The quenching rate constant, k_q, is the difference between the observed decay rate constant of the ^{3}ZnCc and the intrinsic decay rate constant of the ^{3}ZnCc.
17. I. M. Klotz, *Introduction to Biomolecular Energetics, Including Ligand-Receptor Interactions* (Academic Press, Orlando, FL, 1986); _____ and D. L. Hunston, *Arch. Biochem. Biophys.* **193**, 314 (1979); I. M. Klotz, *Q. Rev. Biophys.* **18**, 227 (1985).
18. T. Yonetani and G. S. Ray, *J. Biol. Chem.* **241**, 700 (1966).
19. C. H. Kang, S. Ferguson-Miller, E. Margoliash, *ibid.* **252**, 919 (1977).
20. The ZnCc compound was prepared from horse heart Cc, type VI (Sigma), as in (*19*), and purified by cation-exchange chromatography on a column of CM-52 sized at 2.5 cm by 40 cm. The column was equilibrated with a 10 mM phosphate buffer at pH 7.0; an 85 mM phosphate buffer at pH 7.0 was the eluent. The major band (second band) was used in further experiments.
21. The decay of the ^{3}ZnCc is monitored at a wavelength of 460 nm. Experiments reported here were performed at pH 7.0 and 20° ± 0.2°C with standard procedures (*9*). The ionic strength higher than 18 mM of the phosphate buffer (KPi) was obtained by the addition of KCl, and the strength lower than 18 mM was obtained by dilution. In titration experiments, each data point was obtained with a new fresh sample to avoid the extended exposure of the ZnCc sample to the laser. The observed decay of ^{3}ZnCc in the presence of Fe^{3+}CcP was exponential. Electron-transfer products, the ZnCc cation radical, and Fe^{3+}CcP, were detected by time-resolved transient absorption spectroscopy.
22. In the absence of Fe^{3+}CcP, the decay of ^{3}ZnCc is exponential and the intrinsic rate constant of $k_D = 67 \pm 2\ s^{-1}$ is independent of ionic strength from 4.5 to 118 mM and independent of ZnCc concentration from 0.5 to 95 μM.
23. G. Moore *et al.*, *J. Inorg. Biochem.* **12**, 1 (1980).
24. J. M. Vanderkooi, F. Adar, M. Erecinska, *Eur. J. Biochem.* **64**, 381 (1976).
25. K. T. Conklin and G. McLendon, *Inorg. Chem.* **25**, 4804 (1986).
26. A kinetic mechanism with >2:1 stoichiometry of course could fit the data equally well, but this fit is wholly improbable on physical grounds.
27. J. T. Hazzard, G. McLendon, M. A. Cusanovich, G. Tollin, *Biochem. Biophys. Res. Commun.* **151**, 429 (1988).
28. G. McLendon, *Struct. Bonding (Berlin)* **75**, 159 (1991).
29. E. Mochan, *Biochim. Biophys. Acta* **216**, 80 (1970); E. Nicholls and P. Mochan, *ibid.* **267**, 309 (1972); E. Mochan, *Biochem. J.* **121**, 69 (1971).
30. R. K. Gupta and T. Yonetani, *Biochim. Biophys. Acta* **292**, 502 (1973).
31. H. Pelletier and J. Kraut, *Science* **258**, 1748 (1992).
32. J. E. Erman and L. B. Vitello, *J. Biol. Chem.* **255**, 6224 (1980); Q. Yi, J. E. Erman, J. D. Satterlee, *J. Am. Chem. Soc.* **116**, 1981 (1994).
33. S. H. Northrup, J. O. Boles, J. C. L. Reynolds, *Science* **241**, 67 (1988).
34. Support for the 2:1 stoichiometry, first proposed in (*19*), also has been obtained from measurements of proton uptake and release upon complex formation (M. R. Mauk, J. C. Ferrer, A. G. Mauk, *Biochemistry*, in press).

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