^{1}H NMR Evaluation of Yeast Isozyme-1 Ferricytochrome *c* Equilibrium Exchange Dynamics in Noncovalent Complexes with Two Forms of Yeast Cytochrome *c* Peroxidase

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Abstract: Solutions consisting of 2:1 mole excess of yeast isozyme-1 (iso-1) ferricytochrome *c* in combination with either resting-state cytochrome *c* peroxidase (CcP) or cyanide-ligated cytochrome *c* peroxidase (CcPCN) consist of equimolar concentrations of the noncovalent 1:1 complex (peroxidase/ferricytochrome *c*) and ferricytochrome *c* free in solution. This work reveals that in these solutions the ferricytochrome *c* is in dynamic exchange between the peroxidase bound (b) environment and the free (f) environment. The exchange is in the slow-intermediate regime on the NMR time scale because solutions of these mixtures simultaneously display an iso-1 ferricytochrome *c* heme 3-CH_3 resonance from ferricytochrome *c* molecules in both free and bound environments. Two types of magnetization-transfer experiments have been used to measure the preexchange lifetimes (τ) of iso-1 ferricytochrome *c* in the free and bound environments: saturation transfer and inversion transfer. The results show that τ_b and τ_f range between 1 and 4 ms, depending upon temperature and concentration. The temperature dependence is not great. Arrhenius graphs show that the activation energy for exchange is 7 ± 2 kcal/mol. Furthermore, the concentration dependence of the lifetime indicates that the exchange process is not governed by unimolecular dissociation and that τ is related to a bimolecular rate constant. These results imply that a peroxidase mechanism must incorporate concepts such as (i) "substrate assisted" product dissociation from the intermediate electron-transfer complexes or (ii) two cytochrome *c* binding sites on CcP.

Introduction

Yeast cytochrome *c* peroxidase (CcP) and ferricytochromes *c* (ferricyt *c*) spontaneously form noncovalent complexes in low-salt solutions and these complexes are thought to essentially represent the structure of the "active intermediate" electron-transfer complexes involved in the catalytic cycle of CcP.[1-5] The motivation for studying these complexes arises not solely from information that is being gained for these specific proteins but because these complexes are a paradigm for other biologically active electron-transfer complexes in which one of the partner proteins is cytochrome *c*. Consequently, ^{1}H NMR work on these complexes continues to define the types of experiments and the experimental conditions that can be useful in studying related complexes of respiratory proteins. ^{1}H NMR spectroscopy has specifically proven to be an advantageous technique for studies of cytochrome *c* peroxidase/ferricytochrome *c* complexes in solution because of its unique ability to report directly on specifically identified protons in each protein. For example, the discovery of significant changes in proton hyperfine shifts of ferricyt *c* upon complex formation with either resting-state CcP or cyanide-ligated CcP (CcPCN) has allowed the extent of complex formation and the apparent stoichiometry of the predominant complexes that are formed in NMR solutions to be directly determined, has provided limited information concerning the orientation of the two proteins in the interface region of the complexes, and has provided preliminary information on the exchange dynamics of ferricyt *c* in these complexes.[6-10]

In terms of the work that we report here, there have already been two seminal observations for mixtures of CcP or CcPCN with yeast isozyme-1 (iso-1) ferricyt *c*. In dilute solutions, at low salt concentration and near room temperature, and for which [iso-1 ferricyt c] > [peroxidase], it was discovered that one could simultaneously detect separate iso-1 ferricyt *c* heme 3-CH_3 proton resonances for those molecules of iso-1 ferricyt *c* that were either CcP-bound (b) or free (f) in solution.[7-9] These data indicated that the equilibrium dynamic exchange of iso-1 ferricyt *c* was in the slow-intermediate NMR time scale.[11,12] It was pointed out in those studies that this situation resulted uniquely from the unusually large complex-induced iso-1 ferricyt *c* heme 3-CH_3 shift (over 1000 Hz at 500 MHz) in each of these complexes. Whereas in both peroxidase complexes used here other resonances in iso-1 ferricyt *c* exhibit complex induced shifts,[6,8-10] none match the magnitude of the iso-1 ferricyt *c* heme 3-CH_3 shift, so it is only for this resonance in each complex that the criterion of slow exchange on the NMR time scale is met: rate of exchange $\ll (\omega_b - \omega_f)$.[11,12] Therefore, as demonstrated below, the iso-1 ferricyt *c* heme 3-methyl hyperfine resonances have been used as a unique means for gaining information on its equilibrium rate of exchange between peroxidase-bound and free environments using a set of one-dimensional proton magnetization-transfer experiments.

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In this work we exclusively treat the two mixtures, CcP/yeast iso-1 ferricyt *c* and CcPCN/yeast iso-1 ferricyt *c*, at equilibrium. These are mixtures of the physiological partner proteins, since both occur in yeast mitochondria. The noncovalent complexes contained in these mixtures are models that closely approximate the biological electron-transfer complexes.[7,9,10] Although other ferricytochromes *c* spontaneously form complexes with CcP and CcPCN, the magnitude of the complex-induced shifts is quite species specific, being much smaller for, e.g., horse and tuna ferricytochromes *c* than for yeast iso-1 ferricyt *c*.[6,7,9] For the horse and tuna proteins the magnitude of the corresponding complex-induced shifts obtained under conditions similar to those of the experiments reported here are not large enough to satisfy the criterion for slow exchange noted above, and only single, averaged heme 3-CH_3 resonances are observed.

Methods and Materials

Yeast iso1 cytochrome *c* was purchased from Sigma Chemical Co. It was fully oxidized with $K_3Fe(CN)_6$ (Baker Analyzed) prior to use, and excess $K_3Fe(CN)_6$ was removed by passing the oxidized cytochrome *c* through a short Dowex 8× (Bio-Rad) column prior to use. Cytochrome *c* peroxidase was isolated and purified as previously described.[13,14] Protein concentrations were measured by visible spectroscopy using extinction coefficients of 106 mM^{-1} cm^{-1} at 409 nm for yeast iso1 ferricyt *c*, 93 mM^{-1} cm^{-1} at 408 nm for native, resting-state CcP, and 103 mM^{-1} cm^{-1} at 414 nm for CcPCN. All protein solutions for NMR spectroscopy were made up in a standard, uniform solution consisting of 10 mM KNO_3 in D_2O (99.9%; Isotec), pH' 6.5. The pH' refers to the uncorrected meter reading in this D_2O solution using a Fisher 910 meter and a calibrated combination electrode. The pH was adjusted, where necessary, using diluted DCl (MSD Isotopes).

1D ^1H NMR, saturation-transfer, and inversion-transfer experiments were run on a Varian VXR500S spectrometer operating at a nominal proton frequency of 500 MHz. The calibrated 5-mm probe temperature was regulated at 20 °C. The residual water resonance, used as an internal reference and assigned a chemical shift of 4.70 ppm, was suppressed by irradiation at all times except during acquisition. A brief background and essential details of the implementation of these experiments to fast-relaxing paramagnetic molecules is given in the *Background* section.

Data were analyzed on an IBM workstation using an iterative fitting and Monte Carlo method until the unweighted mean-squared difference between the predicted intensities and observed intensities was a minimum.

Background

Due to the spontaneity of the electron-transfer reaction and for other reasons previously described, one cannot study equilibria associated with the actual electron-transfer complex of CcP with cytochrome *c*.[9] Rather, our approach has been to approximate the properties of this active complex using both the peroxidase and cytochrome *c* in nonreactive spin and oxidation states. Several of these complexes could be considered, as previously described,[9] but in this initial work we have used two different CcP complexes that differ only by the the axial ligation state and the spin state of the CcP heme iron ion: (i) a mixture of CcP and yeast iso-1 ferricyt *c*, and (ii) a mixture of CcPCN and yeast iso-1 ferricyt *c*. In both cases the solutions contained low salt concentration (10 mM KNO_3) to facilitate noncovalent complex formation, and the total iso-1 ferricyt *c* concentration was twice as large as the CcP (or CcPCN) concentration. These experimental conditions guaranteed that the concentration of the noncovalent complex was equal to the concentration of free iso-1 ferricyt *c*. Under these conditions we have previously shown[7-10] that very dilute mixtures of peroxidase and iso-1 ferricyt *c* display individual iso-1 ferricyt *c* heme 3-CH_3 proton resonances for the free and peroxidase-bound populations of iso-1 ferricyt *c* (Figures 1 and 2). Therefore, in either mixture, the exchange-coupled iso-1 ferricyt *c* heme 3-CH_3 proton resonances are suitable NMR spectroscopic probes for quantitating the chemical exchange of iso-1 ferricyt *c* between its two chemically and magnetically inequivalent environments.

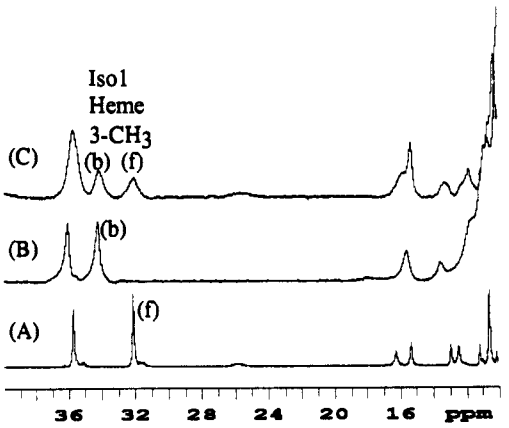

Figure 1. High-frequency hyperfine shift regions of 500-MHz 1D ^1H NMR spectra of (A) yeast iso-1 ferricytochrome *c*, (B) 1:1 CcP/yeast iso-1 ferricyt *c* complex, and (C) 1:2 CcP/yeast iso-1 ferricyt *c* mixture. All samples were in 10 mM KNO_3/D_2O, pH' 6.5, and spectra were obtained at 20 °C.

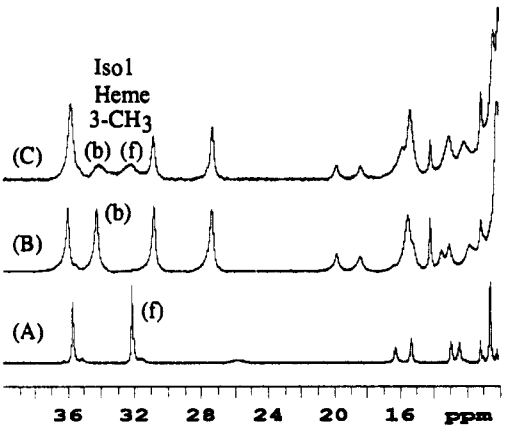

Figure 2. High-frequency hyperfine shift regions of 500-MHz 1D ^1H NMR spectra of (A) yeast iso-1 ferricytochrome *c*, (B) 1:1 CcPCN/yeast iso-1 ferricyt *c* complex, and (C) 1:2 CcPCN/yeast iso-1 ferricyt *c* mixture. All the samples were in 10 mM KNO_3/D_2O, pH' 6.5, and spectra were obtained at 20 °C.

According to the experiments described below, the equilibrium exchange process in both mixtures is dynamic. The observed iso-1 ferricyt *c* exchange occurs between the peroxidase-bound population (b) and the population of iso-1 ferricyt *c* free in solution (f).

There are three common NMR methods for quantitating this type of dynamic two-site exchange in the slow–intermediate exchange regime: (A) NOESY; (B) saturation transfer and (C) inversion transfer. On the NMR time scale, the slow exchange regime occurs when the rate of exchange between the two magnetically inequivalent sites is less than the frequency separation of the resonances in the two sites. The two-dimensional exchange (NOESY)[15] experiment has in many cases supplanted the previously used one-dimensional saturation-transfer and the inversion-transfer experiments. However, we have so far been unable to apply the NOESY experiment in this work because we are restricted to very dilute solutions. Thus, as previously shown,[7] simultaneous observation of proton resonances from the free and peroxidase-bound iso-1 ferricyt *c* populations occurs when the peroxidase concentration is well under 1.0 mM. In this work we used peroxidase concentrations of either 0.25 or 0.50 mM. The

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necessity of using these dilute solutions to achieve slow chemical exchange, limitations on available instrument time, and performance limitations on available hardware precluded two-dimensional experiments. Instead we employed the one-dimensional experiments.

Simple bases for understanding equilibrium chemical two-site exchange and NMR are the suitably modified Bloch equations.[11,12] For the cases at hand, during chemical exchange the z-component macroscopic magnetizations ($\mathbf{M}_z$) for the peroxidase-bound iso-1 ferricyt c (b) and free iso-1 ferricyt c (f) are described by the following equations:

$$d\mathbf{M}_{zb}/dt = \{-(\mathbf{M}_{zb} - \mathbf{M}_{0b})/T_{1b}\} - \{\mathbf{M}_{zb}/\tau_b\} + \{\mathbf{M}_{zf}/\tau_f\} \quad (1)$$

$$d\mathbf{M}_{zf}/dt = \{-(\mathbf{M}_{zf} - \mathbf{M}_{0f})/T_{1f}\} - \{\mathbf{M}_{zf}/\tau_f\} + \{\mathbf{M}_{zb}/\tau_b\} \quad (2)$$

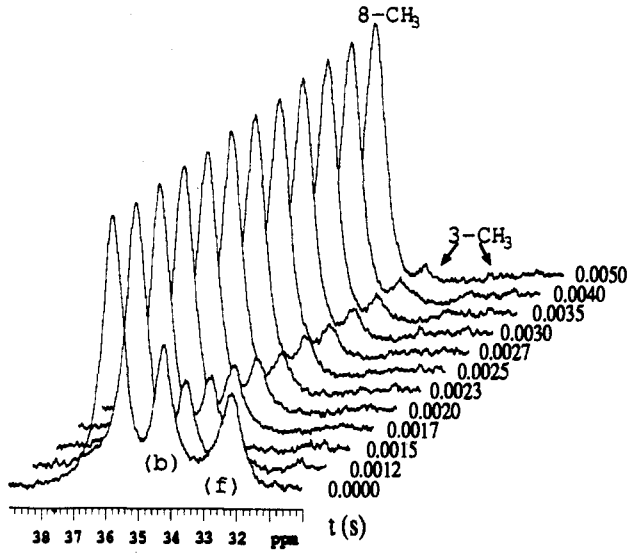
The last two terms in each expression include the preexchange lifetimes or residence times (τ_i; i = b or f) and the spin–lattice relaxation time (T_1) of the iso-1 ferricyt c in each of the magnetically inequivalent sites.

The underlying principles of both types of magnetization-transfer experiments have been previously described,[11,16-18] and we reiterate only details unique to experiment implementation for rapidly relaxing paramagnetic molecules and for data analysis.

A suite of saturation-transfer experiments is carried out by selectively saturating one of the exchange-coupled peaks for varying times, t, as shown in Figure 3A (iso-1 ferricyt c 3-CH_3-(f)). Individual one-dimensional spectra are acquired for each irradiation time. During the irradiation time, the saturation is transferred to the nonsaturated site via chemical exchange. Exchange is manifested in a loss of intensity for the exchange-coupled resonance in the nonsaturated site (iso-1 ferricyt c 3-CH_3-(b) in Figure 3A). The extent of saturation transfer and comcomitant loss of intensity of the nonirradiated resonance increase as the exchange time, t, increases. For saturation-transfer data it can be shown[11,16,17] that a plot of $\ln\{\mathbf{M}_{zi}(t) - \mathbf{M}_{zi}(\infty)\}$ vs t should be a straight line with a slope of $-1/t_{1i}$, where $1/t_{1i} = 1/T_{1i} + 1/\tau_i$.[11] In terms of Figure 3A, the $\log_e$ term represents the progressive difference in intensity between the iso-1 ferricyt c 3-CH_3(b) peak at various irradiation times, t, and the iso-1 ferricyt c 3-CH_3 (b) peak intensity at very long irradiation time ($t = \infty$). As expected, the semilog$_e$ graph of the data from Figure 3A, shown in Figure 3B, is indeed linear. From the relationship $1/t_{1i} = 1/T_{1i} + 1/\tau_i$,[11] one can see that for this data the slope allowed the preexchange lifetime, τ_b, to be determined once an independent measurement of T_{1b} had been made (data not shown). However simple in principle, this method is subject to experimental constraints[18] and to a limitation on the relative magnitudes of the preexchange lifetime (τ_i) and T_{1i}. Qualitatively, one can see that if the rate of exchange is much more rapid than the nuclear spin–lattice relaxation rate (or τ_i is much shorter than T_{1i}), then saturation of one of the two exchange-linked resonances will lead to exponential decay of the intensity at the other site. The result will be complete loss of the nonirradiated peak's intensity. Thus, a criterion for successful application of the saturation transfer method is the following:[11,16]

$$0.1 \leq T_{1i}/\tau_i \leq 40 \quad (3)$$

Using the appropriate nonselective spin–lattice relaxation time for the iso-1 ferricyt c heme 3-methyl resonance (100 ms) and representative values of the several preexchange lifetimes ultimately determined (1–4 ms; Table 1) leads to $T_{1i}/\tau_i = 25$–100 for the specific protein system studied here. This range encompasses the upper bound of eq. 3, so to increase confidence in our results we have also utilized inversion-transfer measurements to quantitate the preexchange lifetimes.



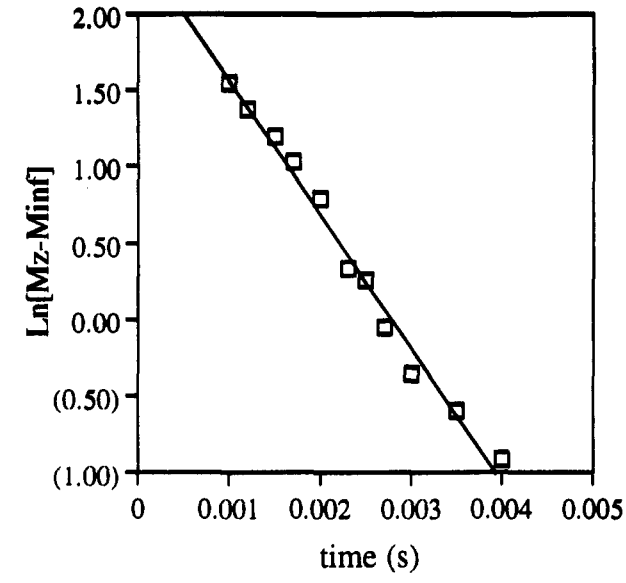


Figure 3. (A, top) Stack plot of a complete saturation-transfer experiment for a 0.25 mM/0.50 mM mixture of CcP/yeast iso-1 ferricyt c at 20 °C. The free-form yeast iso-1 ferricyt c heme 3-CH_3 was seletively saturated for various times t (in s) shown on the plot. (B, bottom) Plot of $\ln\{\mathbf{M}_{zi}(t) - \mathbf{M}_{zi}(\infty)\}$ vs t for the experimental data showing linear behavior.

Table 1. Results from Saturation-Transfer Experiments on 1:2 (mol/mol) Mixtures of CcP/Yeast Iso-1 Ferricyt c and CcPCN/Yeast Iso-1 Ferricyt c

mixture	conc (mM)	T (°C)	no.[a]	τ_b (ms)[b]
CcP/iso-1 ferricyt c	0.23/0.45	20	5	1.49 ± 0.11
		5	5	4.00 ± 0.36
CcPCN/iso-1 ferricyt c	0.25/0.50	20	3	1.48 ± 0.25

[a] Number of determinations. [b] Error was determined from t test at the 90% confidence level.

Implementing an inversion-transfer experiment is similar to the saturation-transfer method described above except that in this case one of the exchange-related resonances is inverted by a π-pulse. It is the inverted magnetization that is transferred to the second site by exchange of the iso-1 ferricyt c. Several descriptions of the inversion-transfer experiment for diamagnetic molecules have appeared,[11,19,20] and recently a more extensive analysis of related applications has been made.[21] We have chosen to follow the procedure of Alger and Prestegard.[19] What is

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different here is that the magnetic properties of paramagnetic molecules (broad lines, short relaxation times),[9] like ferricytochrome *c*, complicate the application of this method to our chosen system, as follows.

Whereas for slowly relaxing nuclei in diamagnetic molecules, highly selective, long-duration, low-power inversion pulses can be used to achieve a high degree of inversion selectivity,[19-21] for rapidly relaxing resonances in an exchanging paramagnetic molecule (i.e., ferricyt *c*), much shorter pulses at higher power levels must be used. This results in a significant loss of selectivity for direct inversion attempts. For the system studied here, the nonselective spin–lattice relaxation of the ferricyt *c* heme 3-CH_3-(b) in the absence of exchange was 100 ms, and, anticipating the results, the preexchange lifetime, τ_b, was on the order of 4 ms, or less. These experimental conditions demanded a π-pulse duration on the order of tens of microseconds, at most. Although direct resonance inversion was attempted using both the transmitter and decoupler channels for direct generation of π-pulses (or by using Dante sequences), we were unable to achieve a suitable balance between rapid inversion and selectivity in this manner.

Subsequently, we employed an "on-resonance" nonselective inversion sequence with the transmitter frequency corresponding to either the free or bound iso-1 ferricyt *c* heme 3-CH_3 resonance (Figures 4A and 5A). This sequence, shown in eq 4, varies from

$$\pi/2(x)—1/(2\Delta)—\pi/2(x)—t—\pi/2(\phi) \qquad (4)$$

that used in related studies[9,21] in that here the phase of the second $\pi/2$ pulse is identical to that of the first, resulting in "on-resonance" inversion.

Figure 4A, for example, demonstrates that transfer of inverted magnetization from the iso-1 ferricyt *c* 3-CH_3(f) occurred via exchange of iso-1 ferricyt *c* between the bound and free environments during the variable exchange time, *t* (eq 4). Data such as those shown in Figures 4 and 5 reveal that reasonable levels of selectivity and inversion can be achieved even for these very broad, rapidly relaxing resonances. However, this experiment is not completely without flaws, the most serious being that with rapidly relaxing resonances such as we have here, some relaxation takes place during the evolution period that precedes the exchange interval, *t*, in eq 4.

Data from the suite of inversion transfer experiments carried out here were analyzed by the procedure of Alger and Prestegard[19] based upon the solutions to the exchange-modified Bloch equations,[11,16,19-21] and as noted by those authors, the problem is underdetermined. Lacking independent measurements of the spin–lattice relaxation times for iso-1 ferricyt *c* in both the free and bound environments in the absence of exchange, there are five unknowns, as before.[19] Although we initially calculated our data with five unknowns, we have also reduced this problem to one of three unknowns by measuring T_{1b} (possible in 1:1 complexes) and T_{1f} (in solutions of iso-1 ferricyt *c* alone). These spin–lattice relaxation time measurements were made nonselectively using the standard inversion–recovery experiment, and values for T_1 were calculated from the early, linear portion of semilog$_e$ intensity difference plots, as previously described.[22-25] Independent determination of these spin–lattice relaxation times was made at each temperature for which exchange experiments were performed. Both five-parameter and three-parameter fits

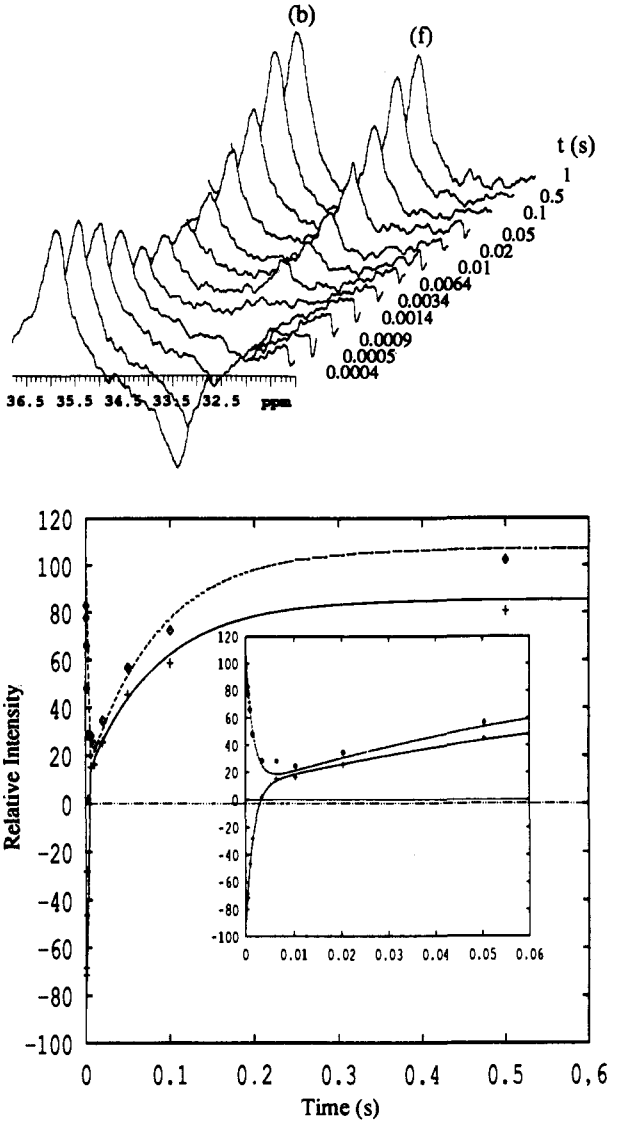

Figure 4. (A, top) Stack plot of a complete inversion-transfer experiment for a 0.25 mM/0.50 mM mixture of CcP/yeast iso-1 ferricyt *c* at 5 °C. The free-form yeast iso-1 ferricyt *c* heme 3-CH_3 was inverted by the pulse sequence presented in eq 4. (B, bottom) Relaxation profile of this inversion-transfer experiment plotted as peak intensity vs magnetization-transfer mixing time (*t* in eq 4). ♦, observed intensities of the bound-form heme 3-CH_3; +, observed intensities of the inverted free-form heme 3-CH_3. The dashed and solid lines are calculated from the Monte Carlo/least-squares data fitting. Inset depicts the behavior of both peaks at early exchange times (where chemical exchange dominates), which is not shown clearly at the larger scale.

were carried out employing a nonlinear least-squares minimization method. In the five-parameter fit, the best values for T_{1b} and T_{1f} differed from the directly measured values. However, in both the five-parameter and three-parameter fits, nearly identical values for the preexchange lifetimes were obtained, indicating the relative insensitivity of the results in this system to the specific value of T_{1i}. This is a consequence of the fact that $\tau \ll T_1$, as described above with respect to eq 3. Brute force (i.e., incrementally–iteratively varying each variable) and Monte Carlo[26] /least-squares minimization methods were used to achieve the "best" values of the unknowns. Fits to the experimental data appear to be very good as shown in Figures 4B and 5B.

Results

Previous NMR studies have shown that both CcP and CcPCN form stoichiometric 1:1 complexes with iso-1 ferricyt *c* at

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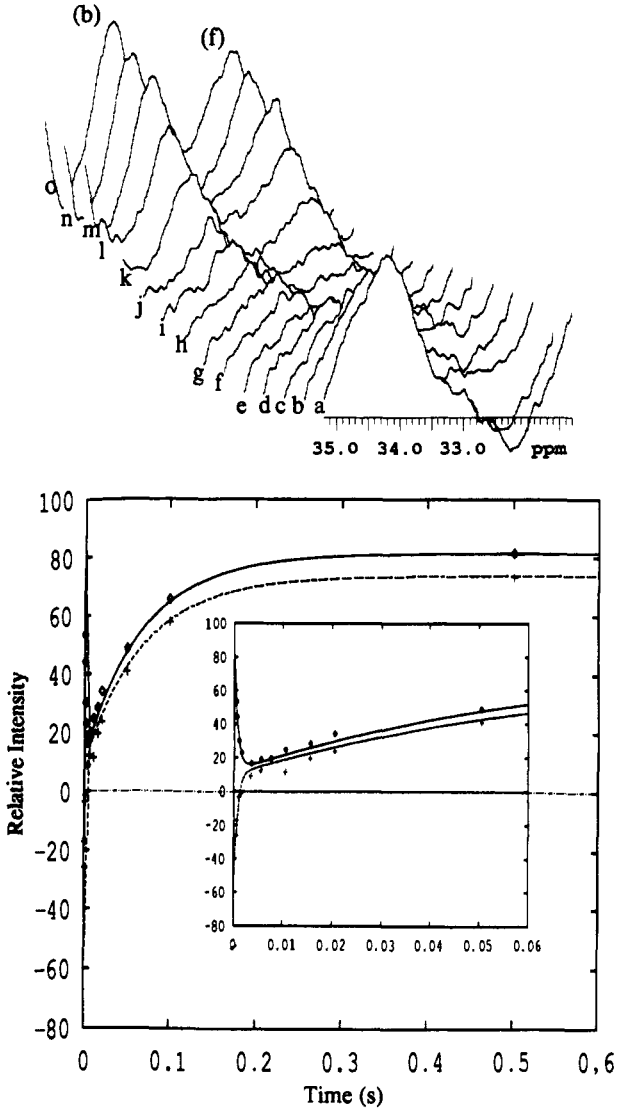

Figure 5. (A, top) Stack plot of a complete inversion-transfer experiment for a 0.50 mM/1.0 mM mixture of CcPCN/yeast iso-1 ferricyt *c* at 20 °C. The free-form yeast iso-1 ferricyt *c* heme 3-CH_3 was inverted by the pulse sequence presented in eq 4. Letters a–o refer to the exchange time, *t*, in eq 4 (*t* in s: a, 0.00053; b, 0.00063; c, 0.0010; d, 0.0015; e, 0.0035; f, 0.0055; g, 0.0075; h, 0.011; i, 0.016; j, 0.021; k, 0.051; l, 0.10; m, 0.50; n, 1.0; o, 3.0). (B, bottom) Relaxation profile of this inversion-transfer experiment plotted as peak intensity vs magnetization-transfer mixing time (*t* in eq 4). ♦, observed intensities of the bound-form heme 3-CH_3; +, observed intensities of the inverted free-form heme 3-CH_3. The dashed and solid lines are calculated from the Monte Carlo/least-squares data fitting. Inset depicts the behavior of both peaks at early exchange times (dominated by chemical exchange), which is not shown clearly at the larger scale.

millimolar protein concentrations that result in extensive complex-induced proton resonance shifts.[7-10] Figure 1 illustrates this by comparing the high-frequency proton hyperfine shift regions of iso-1 ferricyt *c* alone (Figure 1A) and of two mixtures of yeast iso-1 ferricyt *c* with CcP. For the experiment whose result is presented in Figure 1B, the mole ratio of iso-1 ferricyt *c* to peroxidase was 1:1, and only peaks of the complex are apparent,[7] whereas for the experiment depicted in Figure 1C, the mole ratio of iso-1 ferricyt *c* to peroxidase was 2:1, and resonances assignable to both the 1:1 complex and free iso-1 ferricyt *c* occur.[7] Figure 1C represents the situation where the concentration of the noncovalent 1:1 complex is equal to the concentration of the free iso-1 ferricyt *c*. These spectra are typical of the results found in this work, where the concentrations of CcP used were either 2.5 × 10^{-4} or 5.0 × 10^{-4} M.

Figure 2 shows the corresponding spectra for iso-1 ferricyt *c* (Figure 2A) and the complexes made using CcPCN (Figure 2B and C). As with the native enzyme, the 2:1 mixture (Figure 2C) shows resonances assignable to the 1:1 noncovalent complex as well as free iso-1 ferricyt *c*.[8-10] Comparing Figure 1B with Figure 2B shows that the observed shifts of iso-1 ferricyt *c* in both complexes are virtually identical. Furthermore, comparing Figure 1C with Figure 2C, one sees virtually identical iso-1 ferricyt *c* heme 3-CH_3 resonance patterns, with peaks belonging to both free and bound environments present. The comparable line widths and shifts of the iso-1 ferricyt *c* heme 3-CH_3 resonances shown in Figures 1C and 2C lead to the preliminary conclusion that complex formation and the equilibrium exchange rate of iso-1 ferricyt *c* are not significantly dependent upon the peroxidase heme iron ion ligation and spin state.

The simultaneous appearance of significantly broadened free and bound heme 3-CH_3 resonances for yeast iso-1 ferricyt *c* shown in Figures 1C and 2C, combined with previous preliminary studies, indicated that chemical exchange of iso-1 ferricyt *c* occurs in these mixtures.[7,9,10] The subsequent magnetization-transfer experiments confirmed this and quantitated the lifetimes of iso-1 ferricyt *c* in the free and peroxidase-bound environments. To simplify data analysis, the solutions used in making these measurements were constructed so that, within experimental error, the concentration of the noncovalent 1:1 complex was equal to that of the free ferricyt *c* (i.e., those reported in Figures 1C and 2C). It can be shown that in such mixtures $\tau_b = \tau_f$, due to the relationship between the population of a site, p_i, and the preexchange lifetime of that site, τ_i, $\{p_b/\tau_b\} = \{p_f/\tau_f\}$.[11]

Saturation Transfer. Figure 3A presents a set of saturation-transfer experiments as stack plots for the 2:1 mixture of CcP and iso-1 ferricyt *c*. This figure shows that progressively longer saturation of the iso-1 ferricyt *c* heme 3-CH_3(f) resonance (indicated in seconds along the right axis in Figure 3A) leads to the expected intensity decrease of the 3-CH_3(b) resonance. Such behavior directly confirms that chemical exchange is present in this mixture. The extent of saturation transfer increases as the application time of the irradiating field increases. One clearly sees that by 5-ms saturation time, magnetization transfer has reduced the intensity of the nonirradiated peak to nearly zero. Longer irradiation produces no further intensity loss at the heme 3-CH_3(b) resonance, and so this intensity represents the equilibrium intensity under the condition of continuous irradiation ($t = \infty$). From these data one can estimate the appropriate intensity ratio $(M_{0b}/M_{zb}(\infty))$[16] as approximately 50. Since in the limit of continuous irradiation it can be shown that eq 5 holds, and

$$\{M_{0b}/M_{zb}(\infty)\} = \{T_{1b}/t_{1b}\} \quad (5)$$

remembering that $1/t_{1i} = 1/T_{1i} + 1/\tau_i$,[11] we can conclude from the relative equilibrium peak intensities (Figure 3A) in the absence and presence of continuous saturation that τ_b is much shorter than T_{1b}. These qualitative results are confirmed by the experimental values of τ_b shown in Table 1. These numbers represent the first directly measured values for the preexchange lifetimes of CcP-bound iso-1 ferricyt *c*, but have associated with them a fairly high level of uncertainty, due to the error associated with measuring the small equilibrium intensity of the heme 3-CH_3 resonance under the condition of continuous saturation (Figure 3A).

Table 1 summarizes the results from several independent experiments in which each of the exchange-coupled iso-1 ferricyt *c* heme 3-CH_3 peaks was saturated in both of the peroxidase mixtures. Although not shown for the sake of succinctness, a suite of saturation-transfer data for CcPCN/yeast iso-1 ferricyt *c* (results reported in Table 1) is very similar to that shown in Figure 3. This further confirms that dynamic chemical exchange of iso-1 ferricyt *c* is also present in the CcPCN/iso-1 ferricyt *c* mixture.

Table 2. Results from Inversion-Transfer Experiments on 1:2 (mol/mol) Mixtures of CcP/Yeast Iso-1 Ferricyt *c* and CcPCN/Yeast Iso-1 ferricyt *c*

mixture	concn (mM)	T (°C)	no.[a]	τ_b (ms)[b]
CcP/iso-1 ferricyt *c*	0.25/0.50	20	2	1.76 ± 0.22
		16	1	2.11
		13	2	2.44 ± 1.61
		9	2	2.61 ± 2.50
		5	1	3.53
CcPCN/iso-1 ferricyt *c*	0.25/0.50	20	2	2.12 ± 0.04
		9	4	2.50 ± 0.57
	0.50/1.00	20	2	1.47 ± 0.45
		16	2	0.99 ± 0.41
		9	2	1.35 ± 0.00

[a] Number of determinations. [b] Error was determined from *t* test at the 90% confidence level.

Inversion Transfer. In order to refine the accuracy of the measured preexchange lifetimes, τ_i, inversion-transfer experiments were carried out as described above. Results for both mixtures are shown as stack plots in Figures 4A and 5A. As in the case of the saturation-transfer experiments, these stack plots are representative of several repeated experiments, as summarized in Table 2.

In Figure 4A these spectra show that the inverted peak's (3-CH_3(f)) intensity behaves as if it were undergoing an inversion–recovery experiment, with negative intensity at short exchange times and progressive recovery of positive intensity as the exchange times lengthen. The noninverted, exchanged-coupled partner resonance (3-CH_3(b)) experiences a decrease in intensity at short exchange times, due to the chemical exchange, but then it too recovers to maximal amplitude via spin–lattice relaxation as the exchange times increase. These results are similar for Figure 5A.

The digitized peak intensities for each exchange-coupled pair of iso-1 heme 3-CH_3 resonances in both mixtures are plotted in Figures 4B and 5B. Although the experiments were carried out to exchange times $t = 10\,T_1$, only data out to ~600 ms are shown in Figures 4B and 5B. The solid and dashed lines drawn through these data points are the best fits derived from the least-squares/Monte Carlo calculation described above. The quality of fits can be seen to be very good, leading to values for the parameters that quite adequately describe the data. These experiments were carried out at several temperatures as indicated in Table 2, and the data and fits were, in all cases, comparable to what is shown in Figures 4 and 5.

Discussion

The results presented in Tables 1 and 2 show that differences in the spin state and the ligation state of the CcP heme iron ion do not measurably affect the iso-1 ferricyt *c* preexchange lifetimes. In this respect, then, there is no significant effect on the iso-1 ferricyt *c* exchange dynamics caused by local structural changes at the heme ligand binding site in CcP.

Over the temperature range used in this study, 5–20 °C, the preexchange lifetime decreases with increasing temperature, as expected for an activated process. The most extensive data set was obtained at 0.25 mM CcP (or CcPCN)/0.5 mM iso-1 ferricyt *c* using the inversion-transfer technique, Table 2. Fitting these data to the Arrhenius equation[27] gives an activation energy of 7 ± 2 kcal/mol for the exchange process (Figure 6).

The data in Table 2 also show that the preexchange lifetime is dependent upon protein concentration. Doubling the protein concentration leads to a 41 ± 15% decrease in the preexchange lifetime, averaged over the three temperatures at which data were obtained at both concentrations used in this study. The concentration dependence of the preexchange lifetime is consistent

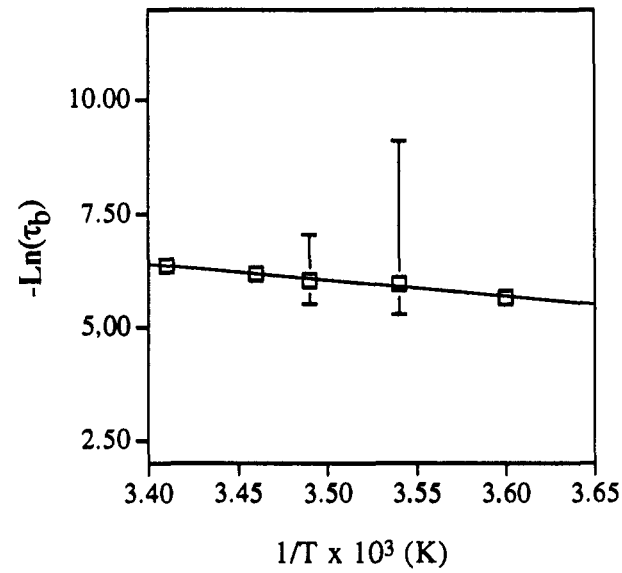

Figure 6. Arrhenius graph of the CcP/iso-1 ferricytochrome *c* exchange data from Table 2.

with previous observations[7] where a solution of 0.1 mM Ccp and 0.26 mM iso-1 ferricyt *c* was found to be in slow exchange, while a solution of 1.7 mM CcP and 4.1 mM iso-1 ferricyt *c* was found to be in fast exchange. The solution conditions employed in those previous experiments were essentially identical to those used in this study, except that the previous temperature was 27 °C.

The simplest mechanism for complex formation between CcP and iso-1 ferricyt *c* is shown in eq 6:

$$\text{CcP} + \text{cyt } c \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} \text{CcP–cyt } c \qquad (6)$$

The preexchange lifetimes for this mechanism are $\tau_b = 1/k_{d1}$ and $t_f = 1/k_{a1}[\text{CcP}]$, where [CcP] is the equilibrium concentration of free peroxidase. This mechanism predicts that τ_b should be independent of protein concentration and is clearly inconsistent with our data.

The exchange mechanism can be extended by including the reaction in eq 7:

$$\text{CcP–cyt } c + \text{cyt } c^* \underset{k_{a2}}{\overset{k_{a2}}{\rightleftharpoons}} \text{CcP–cyt } c^* + \text{cyt } c \qquad (7)$$

The asterisk is only used to illustrate the dynamic exchange of the iso-1 ferricytochrome *c*. Equation 7 suggests that an approaching iso-1 ferricytochrome *c* can facilitate the dissociation of a bound iso-1 ferricytochrome *c*. Combining eqs 6 and 7, the preexchange lifetimes are given by eqs 8 and 9:

$$\tau_b^{-1} = k_{d1} + k_{a2}[\text{cyt } c] \qquad (8)$$

$$\tau_f^{-1} = k_{a1}[\text{CcP}] + k_{a2}[\text{cyt } c] \qquad (9)$$

In these equations, [CcP] and [cyt. *c*] are the equilibrium concentrations of free peroxidase and iso-1 ferricytochrome *c*, respectively. For this extended mechanism, τ_b or τ_f is predicted to decrease with increasing concentrations of free iso-1 ferricytochrome *c*, as observed in this and previous[7] studies.

Corin et al. have determined the equilibrium association constant for the binding of CcP and yeast iso-1 ferricytochrome *c*.[28] Values of 2×10^7 M^{-1} were obtained at both pH 6 in 50 mM phosphate buffer and pH 7 in 10 mM phosphate buffer. The pH and ionic strength of the latter buffer are similar to those used

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in our NMR investigations. If we combine the value of K_A obtained by Corrin et al.[28] with the preexchange lifetimes at 0.25 and 0.50 mM free iso-1 ferricytochrome *c* (extrapolated to 25 °C), we obtain values of 180 s^{-1}, 3.6×10^9 M^{-1} s^{-1}, and 1.8×10^6 M^{-1} s^{-1} for k_{d1}, k_{a1}, and k_{a2}, respectively.

McLendon et al.[29] have recently reported an apparent second-order rate constant for "assisted displacement" of cytochrome *c* for the CcP–cytochrome *c* complex of $\sim 10^6$ M^{-1} s^{-1} as well as k_{on} (our k_{a1}) $\geq 10^8$ M^{-1} s^{-1} and k_{off} (our k_{d1}) ≥ 10 $s,^{-1}$,[30] consistent with the analysis presented here.

The value of 3.6×10^9 M^{-1} s^{-1} for k_{a1} derived from our data is quite large, and one can ask whether it is reasonable. An upper limit for the association rate constant can be estimated from the Smoluchowski equation[31] for diffusion-limited reactions. A value of 6×10^9 M^{-1} s^{-1} is obtained for *neutral* molecules the size of CcP and cytochrome *c*. The electrostatic attraction between these two proteins should enhance the diffusion-limited rate. Using the analysis of Alberty and Hammes[31] and the net charges on CcP and cytochrome *c* at pH 7, a value of 6×10^{10} M^{-1} s^{-1} is obtained at zero ionic strength. These two calculations provide an *upper limit* for k_{a1} varying between 6×10^9 and 6×10^{10} M^{-1} s^{-1}, depending upon ionic strength. A *lower limit* for k_{a1} can be obtained from transient-state kinetic studies on the oxidation of yeast iso-1 ferrocytochrome *c* by CcP compound I. Geren et al.[32] obtain a value of 4.8×10^8 M^{-1} s^{-1} at pH 7 in approximately 250 mM ionic strength buffer, and we have obtained a value of 1.3×10^9 M^{-1} s^{-1} at pH 7, 70 mM ionic strength buffer (A. Matthis and J. Erman, unpublished data). Lowering the ionic strength from 70 to 10 mM could easily increase the value of k_{a1} by a factor of 3, and we therefore conclude that 3.6×10^9 M^{-1} s^{-1} is a reasonable value for k_{a1}.

The rate constants determined from the NMR exchange experiments have an important bearing on the catalytic mechanism of CcP and the maximum turnover rate for the enzyme. A number of kinetic investigations on the CcP-catalyzed oxidation of yeast iso-1 ferrocytochrome *c* by hydrogen peroxide have been carried out near neutral pH in low ionic strength buffer.[33-35] Maximum enzyme turnover rates vary from 400 s^{-1} in pH 6, 50 mM phosphate buffer[35] to 780 s^{-1} between 10 and 100 mM ionic strength at pH 7.5.[34] A product dissociation rate of 180 s^{-1}, as determined from the NMR data presented here, is too slow to support a mechanism which involves a single cytochrome *c* binding site and sequential binding, oxidation, and dissociation of two cytochrome *c* molecules per enzyme turnover.

Our studies do not eliminate *all* single binding site mechanisms. Equations 6 and 7 show a single binding site, substrate-assisted dissociation model which is compatible with the NMR exchange data and could be compatible with both transient- and steady-state kinetic studies of the CcP-catalyzed oxidation of ferrocytochrome *c* by hydrogen peroxide. In this model, cytochrome *c* binds to a single binding site on CcP, eq 6. Interaction of the 1:1 complex with a second cytochrome *c*, eq 7, is a true bimolecular reaction, with a ternary complex (two cytochromes *c* and one CcP) existing only in the transition state. The bimolecular reaction causes the exchange of cytochrome *c* to be concentration dependent as predicted by eq 8 and demonstrated by the NMR data presented here. This bimolecular process may be the reason why the CcP-catalyzed steady-state oxidation of yeast iso-1 ferrocytochrome *c* by hydrogen peroxide does not saturate at low ionic strength.[33,34]

A two binding site mechanism is also compatible with our data. A two binding site mechanism, involving both 1:1 and 2:1 cytochrome *c*–CcP complexes, was first proposed by Margoliash and co-workers in 1977.[33] A two binding site mechanism is consistent with the NMR data, with k_{a1} and k_{d1} being the rate of binding and dissociation, respectively, from the high-affinity binding site in the absence of binding at the low-affinity site. The association rate for binding at the low-affinity site is k_{a2}. A value of k_{d2} is not required to fit the NMR exchange data since the exchange rate does not saturate under the conditions of our experiments. The nonsaturation of the exchange rate is an important observation in and of itself since it indicates that only a small concentration of the 2:1 complex can be present in solution, if it exists at all. Assuming that the high-affinity site is saturated with cytochrome *c* and that the low-affinity site could be up to 50% occupied at the highest cytochrome *c* concentrations used in this study (a very generous assumption), a lower limit of 0.25 mM is predicted for the equilibrium dissociation constant for the low-affinity site. To explain the observed concentration dependence of the exchange rate for cytochrome *c* bound at the high-affinity site, binding of cytochrome *c* to the low-affinity site has to significantly increase the rate of dissociation from the high-affinity site, implying strong interaction between sites.

Since it is impossible to prove the nonexistence of a 2:1 cytochrome c–CcP complex, ultimately resolving the question of whether one or two molecules of cytochrome *c* can bind to CcP will require the unequivocal demonstration of a 2:1 complex. To our knowledge, this has not been accomplished for native CcP and cytochrome *c* in solution under any experimental conditions. The importance of the present study is that we have been able to make precise estimates of the association and dissociation rate constants for binding at the high-affinity site under equilibrium conditions. These estimates are independent of one or two binding site models for the interaction of cytochrome *c* and CcP. In addition, we can state that if the 2:1 complex exists, the equilibrium dissociation constant must be greater than 0.25 mM under our experimental conditions. These results put additional constraints upon any proposed mechanism for electron transfer within the cytochrome *c*–CcP system.

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