Thermodynamic and Kinetic Aspects of Binding and Recognition in the Cytochrome c/Cytochrome c Peroxidase Complex

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Abstract: Quantitative static and time-resolved singlet energy transfer measurements as well as fluorescence anisotropy measurements are reported for the specific complex formed between the luminescent derivative of CcP, (MgCcP), and Cc from yeast and higher eukaryotes. Significant differences were found in the ionic strength dependent binding of yeast and horse Cc, suggesting a different balance of interactions for these species within the binding domain. Small differences are also apparent in the bound MgCcP/Cc singlet-state energy transfer dynamics as monitored by fluorescence quenching. Dynamic measurements show energy transfer is more efficient for yeast Cc than for horse Cc, also consistent with some differences in the docking geometry with CcP between horse Cc and yeast Cc. Overall results suggest some plasticity in the binding of Cc and CcP, with a distribution of complex conformations that depends on species specific interactions. Evidence for a dynamic redistribution among these conformations includes the observation of energy transfer kinetics that appear quite complex at 77 K, but are significantly simplified by dynamic averaging at 300 K.

Introduction

Molecular recognition and binding between redox proteins control both the rate and specificity of the electron transfer step.[1] One paradigm for exploring binding is the redox active complex between cytochrome *c* (Cc) and cytochrome *c* peroxidase (CcP).[2] This system is notable for several reasons. The structues of both Cc[3] and CcP[4] are known at high resolution, and a detailed stereochemical model for the complex has been proposed.[5a] While this manuscript was in review, the detailed structure of the CcP:Cc complex was reported in a structural tour de force by Pelletier and Kraut.[5b] In this structure,[5] a balance of specific hydrogen bonds and hydrophobic interactions comprise the Cc:CcP binding site. This balance differs between the horse Cc:CcP, where "salt bridges" appear more important, and the yeast Cc:CcP system.

Aspects of binding have been probed both by direct and differential[6,7] chemical modification and by physical measurements like energy transfer.[8] In general, these experiments are consistent with the general features of the recent structure, as well as by earlier "binding domain" models suggested by Margoliash and others.[8,9] However, theoretical studies have proposed that binding may not be restricted to a single stereospecific site,[10] in seeming contradiction with the structural data.

A wealth of kinetic data is also available for the reactions of Cc with CcP.[11-15] Among the interesting findings of the kinetic studies is the fact that the kinetic parameters[11-15] which characterize the reaction of CcP with fungal Cc differ significantly from those of other eukaryotic Cc complexes. The structural or dynamic basis for these differences, however, is unclear. Static and nanosecond time-resolved singlet singlet energy transfer measurements are used here to examine structural and dynamic effects in the complexes formed between CcP and a variety of Cc complexes: yeast iso-1, horse, and manduca.

Experimental Section

Cytochrome *c* peroxidase was isolated by a modification of the procedure of Nelson,[16] as described by Conklin.[17] Yeast iso-1 and iso-2 Cc complexes were prepared as described by Stewart.[18] Horse and tuna cytochromes were purchased from Sigma and purified by ion exchange chromatography. Manduca Cc was a gift from E. Margoliash. MgCcP and H_2 porphyrin CcP were made by a modification of the procedure of Yonetani.[19] All buffers were prepared using 2× distilled deionized water, and other reagents were the highest purity commercially available.

Steady-state fluorescence measurements were made on a Perkin-Elmer MPF 44A fluorescence spectrometer. Fluorescence intensities were corrected for inner filter effects, but at the low concentrations and consequent low absorbances ($A \leq 0.02$), these effects were minimal. Time-resolved measurements at Rochester were made using a mode-locked YAG pumped cavity dumped dye laser Quantronix as previously described.[20] The system used has a system response time of ca. 50 ps (fwhm in pump curve), which can be deconvoluted to >10 ps. At Northwestern, fluorescence lifetime measurements were made as previously described.[21] Briefly, 600-nm excitation pulses were obtained from

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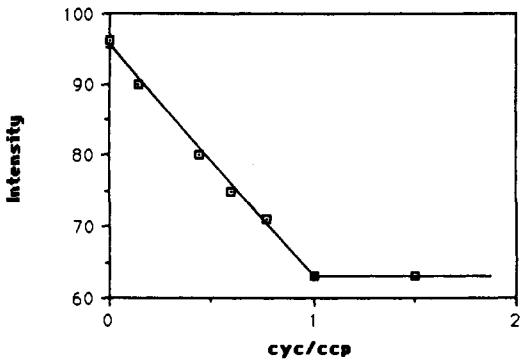

Figure 1. Steady-state fluorescence binding curve of MgCcP with yeast Fe(III) iso-1-cytochrome *c* at 10 mM phosphate pH 7 buffer. There is a clear break at 1:1 stoichiometry. The excitation and emission wavelengths for MgCcP were 556 nm and 600 nm.

the cavity-dumped pulse train taken from an argon ion pumped cavity-dumped dye laser. Emission was passed through a Corning 2-60 filter ($\lambda > 620$ nm). The instrument response function had a fwhm of ca. 350 ps. Data were fit using iterative deconvolution.[21] Experiments were repeated on multiple, independently prepared samples, and in both laboratories.

Solutions for fluorescence experiments were deoxygenated in any of three ways: gentle bubbling with N_2 or Ar gas, stirring in inert atmosphere followed by addition of the glucose oxidase deoxygenation reagents,[22] or degassing under gentle vacuum, after the procedure of Hazzard.[23] Identical results were obtained by all three methods. (Indeed, for short counting times, identical results were obtained for aerated samples, showing that O_2 does not directly quench the emissive singlet state.)

Results

Steady-State Experiments. In order to obtain meaningful time-resolved data, it was first necessary to examine the stoichiometry and binding constants of the Cc:CcP complexes, which have been a matter of some dispute.[24-28] Both 1:1 and 2:1 complexes have been postulated, and a wide (10^3) range of binding constants reported.[24-28]

The present experiments show that H_2CcP or MgCcP strongly bind one molecule of Cc (horse Cc or yeast) (Figure 1, Table I). Competition titrations between MgCcP and Fe^{3+} CcP for binding Fe^{3+} Cc show that MgCcP and Fe^{3+} CcP compete for this binding site (Figure 2).[27] We therefore believe that MgCcP is a faithful analogue of Fe CcP for binding experiments. The values obtained here for horse Cc agree well with recent values reported by Vitello and Erman,[28] using a similar approach. The results for yeast Cc show a *much* weaker dependence on ionic strength than the horse Cc system. The present result agrees well with a previous report by Leonard and Yonetani,[6] where fluorescence energy transfer measurements revealed relatively strong binding of yeast Cc to yeast CcP, even at an ionic strength of 0.2 M. The Leonard and Yonetani result was criticized by Erman[28] as possibly reflecting a trivial inner filter effect. However, Leonard reported in the same paper[6] *lifetime* quenching, which cannot be affected by inner filter effects. The present data resolve the apparent discrepancy between the data of Vitello and Erman and those of Leonard and Yonetani. We confirm that mammalian Cc and yeast Cc indeed are affected differently by supporting electrolyte, with special ion effects (e.g., phosphate) playing an additional role in modulating binding,[25] suggesting differences must exist in the precise mode of binding between the highly homologous yeast iso-1 and horse Cc. Such differences are, in fact, observed in the structure;[5b] horse Cc appears to form additional "salt bridges" in the structure. Among the few differences between yeast Cc and mammalian Cc is the post-translational modification of Lys 72 in yeast to trimethyl lysines. This same modification is obtained when pigeon Cc is expressed in yeast. In the crystal structure, Pelletier and Kraut find evidence for a salt bridge between Lys 72 and Glu 290. Obviously, no equivalent interaction can occur when Lys 72 is trimethylated. We therefore compared the binding of native pigeon Cc and Me_3 Lys 72 pigeon Cc to assess the effect of trimethylation on binding. As Table I shows, there is no effect of trimethylation on binding, so this interaction, observed in the structure, cannot substantially contribute to binding. These results are consistent with recent NMR data,[31,32] which also suggest that the small differences in binding of CcP to horse Cc versus yeast Cc are reflected in the sensitive hyperfine shifted heme resonances of Cc.

A final aspect of these data requires comment. The relatively high binding constant, K_b, of yeast Cc with CcP places tight constraints on kinetic parameters. Specifically, $K_b = 10^7\ M^{-1} = k_{on}/k_{off}$. It is reported that $k_{on} \geq 10^8\ M^{-1}\ s^{-1}$ under similar conditions,[2b] which gives $k_{off} \leq 10\ s^{-1}$. However, the observed limiting catalytic rate is[2b] $k_{cat} = 400\ s^{-1}$. Thus, it appears that product dissociation is slower than steady-state turnover. Two possible explanations might be appropriate. The first assumes that substrate and product might bind independently, given the apparent differences in the binding (sub)domains observed for Fe^{2+}Cc and Fe^{3+}Cc. This is not the case, as shown by a simple NMR experiment in which 1 equiv of Fe^{2+}Cc is added to a 1:1 [Fe^{3+}Cc:CcP] complex. At equilibrium, CcP is equally partitioned between Fe^{2+}Cc and Fe^{3+}Cc complexes (Figure 3). This is consistent with the equivalent binding constants shown in Table I,[25] and argues that Fe^{2+}Cc occupies a very similar structural site to Fe^{3+}Cc.[5b]

A second, more subtle explanation follows from data like those in Figure 3.[31,32] While Figure 3 shows Fe^{3+}Cc bound and Fe^{3+}-Cc free are in slow-intermediate exchange ($k_{exch} \sim 300\ s^{-1}$), it has long been observed[31,32] that at higher concentrations (Cc > 10^{-3} M) *fast* exchange ($k_{exch} > 1000\ s^{-1}$) is observed. Thus, the rate of Cc(III) dissociation must include a concentration dependent term, in addition to the concentration independent) rate constant established by the (low concentration) equilibrium measurement. Using data from our labs,[32] an apparent second-

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Table I. Binding Constants for the Cytochrome *c* Peroxidase/Cytochrome *c* Complex

CcP/Cc	ionic strength (M)	K_b (M^{-1})	ref
MgCcP/yeast iso-1-Cc/Fe(III)	pH = 7.0 10 mM K phosphate	$(1.9 \pm 0.4) \times 10^7$	this work
MgCcP/yeast iso-1-Cc/Fe(III)	pH = 6.0 50 mM K phosphate	$(2.0 \pm 0.3) \times 10^7$	this work
MgCcP/yeast iso-1-Cc/Fe(II)	pH = 6.0 50 mM phosphate	$(2.0 \pm 0.5) \times 10^7$	this work
MgCcP/yeast iso-1-Cc/Fe(III)	pH = 6.0 80 mM phosphate	$(2.5 \pm 0.5) \times 10^5$	this work
MgCcP/yeast	pH = 6.0 110 mM phosphate	$(4 \pm 1) \times 10^4$	this work
H_2CcP/horse Cc/Fe(IV)	pH = 6.0 20 mM ionic strength	$(4.5 \pm 0.9) \times 10^5$	27
H_2CcP/horse Cc/Fe(IV)	pH = 6.0 50 mM ionic strength	$(6.3 \pm 1.6) \times 10^4$	27
MgCcP/pigeon	pH = 6.0 50 mM phosphate	$(3.3 \pm 0.5) \times 10^4$	this work
MgCcP/Me_3Lys72 pigeon	pH = 6 50 mM phosphate	$(3.2 \pm 0.5) \times 10^4$	this work

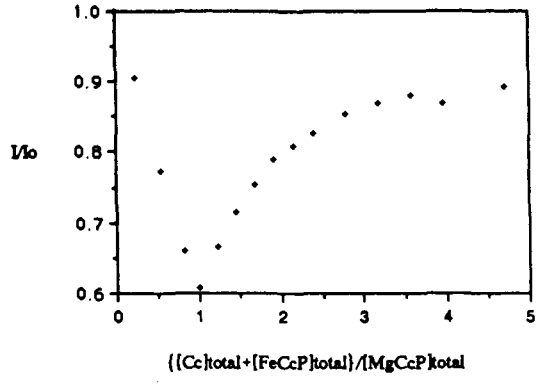

Figure 2. Fluorescence competition titration between MgCcP and FeCcP for binding Fe(III) iso-1-cytochrome *c* at 10 mM phosphate pH 5.7. The excitation and emission wavelengths for MgCcP were 556 nm and 600 nm. An equivalent of Cc was first added to the MgCcP solution where $\{[Cc]_{total} + [FeCcP]_{total}\} / [MgCcP]_{total} = 1.0$, and then FeCcP was added; the released MgCcP showed recovery back in the intensity.

order rate constant for assisted displacement of "k_{off}^{app}" $\sim 10^6$ M^{-1} s^{-1} is observed. This appears to be in agreement with the elegant detailed studies recently reported by Satterlee et al.[31] If we assume a similar displacement occurs in the presence of substrate, then, under conditions of maximum turnover, a catalytically competent rate can be obtained by "substrate-assisted product displacement." We note that a similar phenomenon of "assisted displacement" has been observed in the enzymatic turnover of dihydrofolate reductase.[33]

Time-Resolved Energy Transfer. With the steady-state results available to define conditions for complete 1:1 binding, time-resolved measurements were undertaken. The fluorescence of MgCcP is characterized, at 293 K in 10 mM pH 7 phosphate, by a double exponential. A fast component, $\tau_1 \sim 1 \times 10^{-9}$ s, accounts for ca. 10% of the total decay. The major decay occurs in a slower component, $\tau_2 = 8.2 \times 10^{-9}$. On lowering the temperature to 77 K, the decay of MgCcP remains biexponential, and both lifetimes predictably increase, to 2.1 and 8.9 ns, respectively. On adding equimolar portions (or a slight excess) of either oxidized (Fe^{3+}Cc) or reduced (Fe^{2+}Cc) Cc to MgCcP, the lifetime of the major decay component, τ_2, of MgCcP decreases, indicative of energy transfer quenching by the heme chromophore of Cc. Consistent with their absorption spectra, Fe^{3+}Cc is a more effective quencher than Fe^{2+}Cc. Typical data are shown in Figures 4–7, and the values of τ_2 and the calculated quenching rate constant, k_Q, for the complexes studied are given in Table II. The magnitude and lifetime, τ_1, of the minor fast component were found to be unchanged within experimental error upon complexation with either Fe^{3+}Cc or Fe^{2+}Cc. Consequently, we limit our discussion to the effects of Cc on the major component, τ_2.

Interestingly, the value of $I/I_0 = 0.4$ obtained from static fluorescence quenching (Figure 1) of MgCcP by yeast iso-1 Fe^{3+}-Cc differs from the corresponding value of $\tau/\tau_0 = 0.6$ obtained from the time-resolved measurements. This discrepancy suggests that the binding of MgCcP to Cc not only leads to energy transfer quenching but also affects the yield of the emissive singlet state (for example, by enhancing singlet → triplet intersystem crossing). Similar effects have been observed by Vanderkooi.[22] The emission decay profile of H_2porph CcP is even more complex, requiring at least three exponentials to fit the data. Therefore, experiments on H_2porph CcP were not pursued.

Species Dependence. Cytochrome *c* variants examined show similar quenching of the lifetime of MgCcP, but the fungal cytochromes, yeast iso-1 and candida, quench more effectively than do the other eukaryotic Cc's examined (Table II). The "higher" eukaryotes examined (horse, tuna, rat, manduca) all give similar decay rates, despite the wide phylogenetic range represented. We conclude that, although all the bound CcP/Cc complexes are similar, some specificity of interaction exists. This conclusion seems supported by the recent comparison of structures of the Cc:CcP complexes involving horse Cc and yeast Cc.

The variation among all the complexes may be quantitatively assessed by considering the magnitude of a structural change necessary to produce a change in the energy transfer rate of the magnitude observed. Since $k \propto (1/r)^6$ (assuming no change in orientation or spectral overlap), a change of ΔR(horse–yeast) $\cong$ 2 Å could account for the observed differences in quenching.[34] The observed complex structures[5b] support such a shift from R = 26 Å (yeast) to R = 30 Å (horse). This 4-Å shift *should* give an even larger rate effect than observed. Whether the observed effects involve additional small structural differences or dynamic averaging between structures, remains unclear. These studies support a model in which the binding of CcP to Cc is somewhat plastic and can be modulated by species specific contacts at the binding surface.[5b,29] Interestingly, this variation is only clear

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(34) In general, distance, R, and angle orientation factor $(\kappa)^2$ appear as offsetting parameters: an increase in κ results in a (smaller) decrease in R. Taking R = 26 Å from ref 5b and setting the overlap integral as 8.4×10^{-34} cm^5, from the known spectra of MgCcP and Fe^{3+}Cc, gives $(\kappa)^2 \approx 3$. For this high value, small changes in κ will not materially affect R.

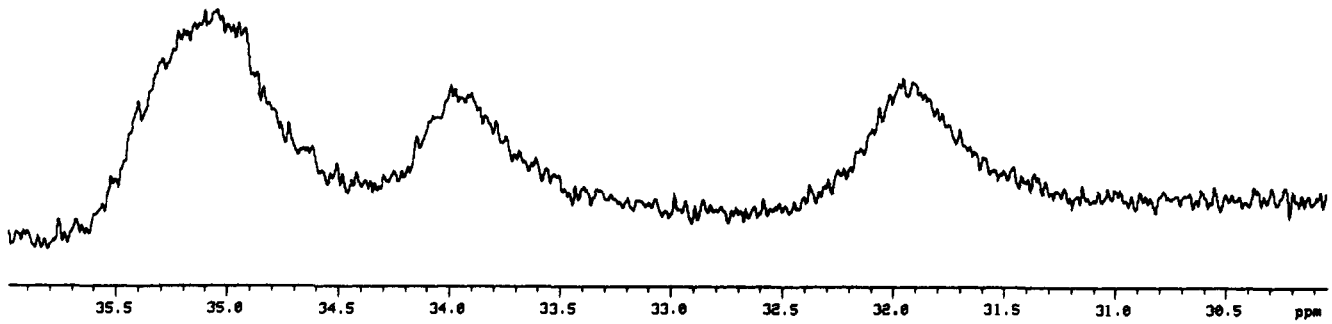

Figure 3. NMR spectrum of 3×10^{-4} M CcP plus 3×10^{-4} M yeast Fe(III) Cc plus 3×10^{-4} M yeast Fe(II) CC. Note the equal magnitude of the bound peak at 34 ppm and the free peak at 32 ppm.

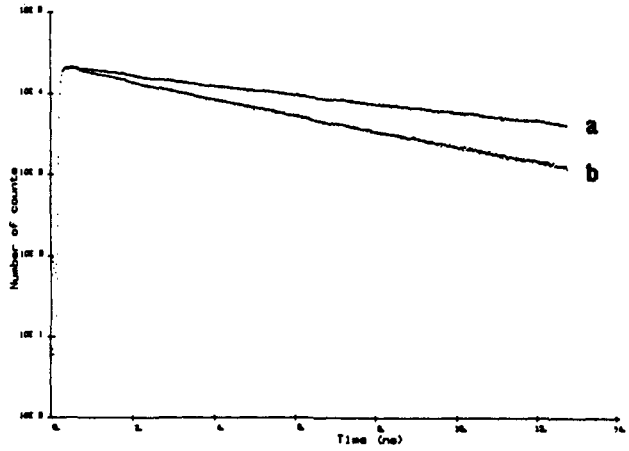

Figure 4. SPC decay profiles of MgCcP (a) and MgCcP:Fe(III) Cc complex (b) at 300 K. The excitation and emission wavelengths for MgCcP were 556 nm and 600 nm.

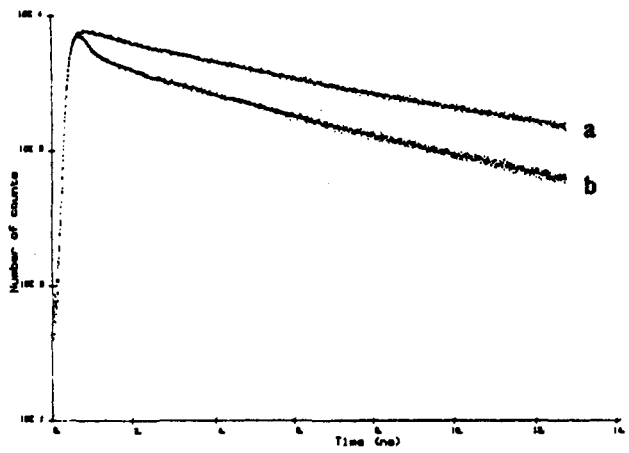

Figure 5. SPC decay profiles at 77 K of (a) MgCcP and (b) MgCcP:Fe(III) cytochrome complex. The MgCcP/Fe(III) Cc complexes was dissolved in a buffer (0.01 M phosphate, pH 7.0) and cooled to 77 °K in an optical dewar.

Figure 6. SPC decay profile for MgCcP/Fe(II) Cc complex, as in Figure 4.

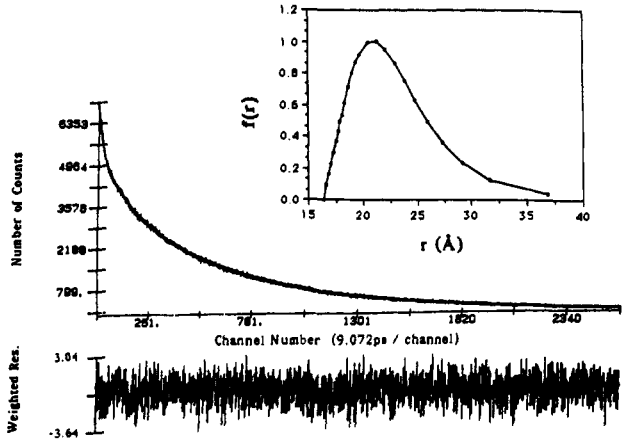

Figure 7. Fit of the data in Figure 4b to a distance distribution function (shown in the inset). This distribution assumes that the distribution in rates reflects the distribution in distance[34] (via the Forster equation, holding $\kappa = 1$). If this oversimplification were relaxed, a somewhat narrower distance distribution could result: in particular, for a heme–heme angle of ~65°,[5b] a small change in angle should not markedly affect $\langle r \rangle$.

when oxidized Cc is the acceptor. For reduced Cc, all the quenching rates agree within experimental error.

Low-Temperature Measurements. The data above do not address whether any *individual* Cc species can dynamically access different binding conformers within the general binding domain, as previously suggested.[10] In principle, energy transfer measurements might detect such heterogeneity of binding. Since each conformer of the CcP:Cc complex might have a characteristic heme–heme (donor–acceptor) distance and angle, each might also have a characteristic energy tranfer rate. Prelminary results for energy transfer under such conditions have been presented elsewhere.[29] In brief, three limits can be imagined. If the dynamics of interconversion between conformers is fast with respect to energy transfer, a single exponential decay will be observed. If interconversion is slow with respect to energy transfer, then multiexponential behavior will be observed, with the amplitude of each decay component weighed by the population of that component.

Finally, if interconversion occurs on the same time scale as energy transfer, a complex decay line shape can result, which on casual inspection can resemble the fast interchange case. At ambient temperature, the decay of the MgCcP:yeast iso-1 Cc complex shows no more kinetic complexity than MgCcP alone. This result suggests *either* that a single binding site exists for this

Table II. Fluorescence Lifetimes and Quenching Rates

sample	τ_2 (ns)	k_0 ($\times 10^{-8}$ s^{-1})
	Mg/Fe(III)	
MgCcP/horse	5.4 ± 0.1	0.63 ± 0.05
MgCcP/rat	5.5 ± 0.1	0.61 ± 0.04
MgCcP/manduca	5.2 ± 0.1	0.72 ± 0.05
MgCcP/saccharomyces	4.9 ± 0.2	0.80 ± 0.06
MgCcP/candida	4.8 ± 0.1	0.90 ± 0.05
MgCcP/T102	4.8 ± 0.2	0.90 ± 0.06
	Mg/Fe(II)	
MgCcP/horse	7.1 ± 0.1	0.18 ± 0.01
MgCcP/rat	7.2 ± 0.1	0.17 ± 0.01
MgCcP/manduca	6.9 ± 0.1	0.22 ± 0.02
MgCcP/T102	7.0 ± 0.05	0.20 ± 0.01
MgCcP/candida	6.8 ± 0.1	0.23 ± 0.02
MgCcP control	8.20 ± 0.05	
	8.90 ± 0.05 (77 K)	

complex or that, if multiple sites exist, they rapidly equilibrate. In order to distinguish between these possibilities, the energy transfer measurements were repeated at 77 K. For MgCcP alone, although the lifetime predictably increases on cooling, no additional complexity in the decay curve was found on cooling to 77 K. (This result argues against dynamic conformers being responsible for the biexponential decay at room temperature.) However, the MgCcP:Cc complex does exhibit a qualitatively different line shape when comparing 300 K and 77 K data. At 77 K, at least three components are necessary to fit the data, suggesting a distribution of quenching rate constants (and an associated distribution of complex structures). Fitting to a distribution in distance recovers the appropriate value of R_{D-A}[34] (but does *not* reproduce the correct distribution width in R, since the decay function must include both the donor-acceptor distance *and* angle).

In comparing yeast Fe(III) with the horse protein, both species show multiexponential decays. However, the horse protein shows a broader distribution of rates, relatively more weighted toward longer time components (e.g., longer distances). For reduced Fe(II) Cc far less complexity is induced on freezing (Figure 6). These comparisons underscore the room-temperature results in suggesting that binding involves a family of closely related structures which can distinguish between phylogenetically distinct Cc, as well as distinguishing between the different oxidation states of a given species of Cc.

The qualitative difference between the MgCcP decay line shape in the Cc:CcP complex between room temperature and low temperature is most easily explained if several closely related binding conformers can exist between Cc and CcP and that these modes exhibit different quenching. These individual modes would necessarily be of similar energy and likely occupy similar overlapping positions within the binding domain. At low temperature (77 K), the distribution of complexes would be frozen out and could not rearrange on the time scale of fluorescence (10^{-8} s). At ambient temperature (ca. 300 K), small amplitude motions at the interface would allow these conformers to equilibrate. If equilibration occurs on a nanosecond time scale, the distributed kinetics found at low temperatures would be largely or completely washed out.

Fluorescence Anisotropy. Anisotropy measurements can provide information complementary to energy transfer measurements. While the latter depend on the donor–acceptor distance and/or orientation (or their distributions) in the complex, anisotropy decay is particularly sensitive to the overall shape of the complex. Measurements of anisotropy for MgCcP and the MgCcP:Cc complex both show two components (τ_1 = 91 ns at 295 K; τ_2 = 0.5 ns at 295 K). The fast decay time depends strongly on temperature and that reflects a photophysical process of the chromophore alone, which is not yet understood. However, metalloporphyrin excited states exhibit a 2-fold degeneracy or near-degeneracy, and this rapid anisotropy decay might represent thermal equilibration of the two Jahn-Teller-split singlet substates. Consistent with this suggestion, the t = 0 amplitude of the fast component requires that the emitting molecule exhibit only 2-fold symmetry.

The long-time component, however, has a simple physical interpretation: it represents rotation of the molecule or complex as a whole. The rotational correlation time obtained from the anisotropic decay of CcP alone, τ_r = 19 ns at 295 °C, is in reasonable agreement with the value expected for a protein of MW 34 000, when treated as a prolate ellipsoid of axial ratio $a/b \cong 1.35$. This value of (a, b) is that predicted from the known crystal structure of CcP. On forming the complex, the rotational correlation time increases to 27 ns, as expected for a larger species. Somewhat suprisingly, this increase is nearly what would be anticipated if the complex had essentially the *same* shape as the CcP alone, but only a larger volume, i.e., $(a/b)_{complex} \cong (a/b)_{CcP}$. This appears hard to immediately understand in terms of the static crystal structure[5b] but might be understood if dynamic averaging occurs about the emission axes. Of course, such analysis is complicated by several factors. First, fluorescence anisotropy measurements are actually sensitive to the relative orientation of the transition moment dipole(s) for emission, with respect to the principal axes of the ellipsoid. These orientations will change on changing the direction of the long axis. Second, it is possibly true that, when Cc binds to CcP, it releases a number of tightly bound solvent molecules which contribute to τ_r of free CcP. Thus, comparison of the molecular volumes of CcP alone to the model complex probably overestimate the increase in (hydrodynamic) volume which accompanies complex formation.

Summary

The present results in toto suggest a motif for molecular recognition in the Cc:CcP complex which is generally consistent with crystallographic data. However, to explain all the results, it appears necessary to include dynamic interconversion among structurally similar complexes which can be modulated by differences in either primary structure or solution conditions. Binding may occur to (sub)sites of overall similar structures but with small differences in Fe–Fe distance (±2 Å) or heme–heme angle, which interconvert rapidly at high temperatures but not at lower temperatures. The distribution of these sites is sensitive to species variation, so that yeast Cc binds not only more strongly but also with more effective overlap than does horse Cc.

These results complement electron- and energy-transfer measurements on the slower time scale of the triplet-state decay.[15,19,30] Electron-transfer measurements suggest that the complex can interconvert among forms with different reactivity[15] where triplet energy transfer measurements show that cooling the complex freezes out the interconversion processs in what appears to be a cooperative transition to a low-temperature state.[19a,30]

Although a complete picture of the interfacial dynamics within the now structurally characterized complex[5b] is not yet within reach, it seems likely that such a picture can be expected from the combination of approaches being employed.

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