Kinetics of Reduction by Free Flavin Semiquinones of the Components of the Cytochrome *c*–Cytochrome *c* Peroxidase Complex and Intracomplex Electron Transfer†

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ABSTRACT: The kinetics of reduction by free flavin semiquinones of the individual components of 1:1 complexes of yeast ferric and ferryl cytochrome *c* peroxidase and the cytochromes *c* of horse, tuna, and yeast (iso-2) have been studied. Complex formation decreases the rate constant for reduction of ferric peroxidase by 44%. On the basis of a computer model of the complex structure [Poulos, T. L., & Finzel, B. C. (1984) *Pept. Protein Rev. 4*, 115–171], this decrease cannot be accounted for by steric effects and suggests a decrease in the dynamic motions of the peroxidase at the peroxide access channel caused by complexation. The orientations of the three cytochromes within the complex are not equivalent. This is shown by differential decreases in the rate constants for reduction by neutral flavin semiquinones upon complexation, which are in the order tuna $\gg$ horse $>$ yeast iso-2. Further support for differences in orientation is provided by the observation that, with the negatively charged reductant FMNH$^{\bullet}$, the electrostatic environments near the horse and tuna cytochrome *c* electron-transfer sites within their respective complexes with peroxidase are of opposite sign. For the horse and tuna cytochrome *c* complexes, we have also observed nonlinear concentration dependencies of the reduction rate constants with FMNH$^{\bullet}$. This is interpreted in terms of dynamic motion at the protein–protein interface. We have directly measured the physiologically significant intracomplex one electron transfer rate constants from the three ferrous cytochromes *c* to the peroxide-oxidized species of the peroxidase. At low ionic strength these rate constants are 920, 730, and 150 s^{-1} for tuna, horse, and yeast cytochromes *c*, respectively. These results are also consistent with the contention that the orientations of the three cytochromes within the complex with CcP are not the same. The effect on the intracomplex electron-transfer rate constant of the peroxidase amino acid side chain(s) that is (are) oxidized by the reduction of peroxide was determined to be relatively small. Thus, the rate constant for reduction by horse cytochrome *c* of the peroxidase species in which only the heme iron atom is oxidized was decreased by only 38%, indicating that this oxidized side-chain group is not tightly coupled to the ferryl peroxidase heme iron. Finally, it was found that, in the absence of cytochrome *c*, neither of the ferryl peroxidase species could be rapidly reduced by flavin semiquinones. This is consistent with a facilitation of electron transfer by cytochrome *c* as proposed in the Poulos and Finzel model, as well as suggestive of a change in the dynamics of the peroxidase structure following the binding and reduction of H_2O_2.

Much interest has recently been focused on the study of electrostatically stabilized intermediate complexes formed by biochemical redox couples in order to elucidate the factors that govern the rates and specificity of biological electron-transfer processes. Computer models of several such electron-transfer complexes have been proposed (Salemme, 1976; Poulos & Kraut, 1980; Simondsen et al., 1982; Poulos & Mauk, 1983), on the basis of crystallographic structures of the individual components, since direct structure determinations of the native complexes has not yet been accomplished. The theoretical models involve electrostatically determined protein–protein orientations that enable essentially parallel alignment of the macrocycles (Salemme, 1978). A nonpolar interprotein interface and close approach of the parallel macrocycles allows electron transfer via an outer-sphere mechanism or, as in the case of the cytochrome *c*–CcP[1] complex (Poulos & Kraut, 1980; Poulos & Finzel, 1984), via a combination of aromatic residues and a hydrogen-bonding network connecting the two redox centers.

In order to examine the validity of the proposed model complexes, kinetic experiments have been performed to measure the effects of complexation on ligand binding and on oxidation–reduction reactions utilizing exogenous reagents. In some cases, covalent cross-linking was used. This latter methodology has been applied to two electron transfer complexes, horse cytochrome *c*–*Azotobacter vinelandii* flavodoxin (Dickerson et al., 1985) and horse cytochrome *c*–yeast cytochrome *c* peroxidase (Pettigrew & Seilman, 1982; Waldmeyer et al., 1982; Waldmeyer & Bosshard, 1985; Bechtold & Bosshard, 1985). In the latter complex, a 16-fold decrease

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[1] Abbreviations: cytochrome *c*(III) and *c*(II), ferric and ferro cytochrome *c*, respectively; CcP(III) and CcP(II), ferric and ferro cytochrome *c* peroxidase, respectively; CcP(IV,R$^{\bullet+}$), peroxidase species oxidized by H_2O_2 yielding an Fe(IV) and an oxidized amino acid, R$^{\bullet+}$ (i.e., compound I); CcP(IV), peroxidase species oxidized to the Fe(IV) state without R group oxidation; EDTA, ethylenediaminetetraacetic acid; LFH$^{\bullet}$, RFH$^{\bullet}$, FMNH$^{\bullet}$, and 5-DRFH$^{\bullet}$, neutral semiquinone species of lumiflavin, riboflavin, flavin mononucleotide, and 5-deazariboflavin, respectively; NADP$^+$, oxidized nicotinamide adenine dinucleotide phosphate; $E_{m,7}$, midpoint reduction potential measured at pH 7.

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in the rate constant for ascorbate reduction of complexed cytochrome *c* was observed, as well as a 95% decrease in peroxidase activity toward exogenous cytochrome *c*(II). Inherent problems with kinetic studies utilizing covalently cross-linked complexes are the uncertainty as to what structural perturbations might occur within the individual components as a result of the establishment of covalent bonds, heterogeneity with respect to orientation of the two proteins within the complex, and the introduction of constraints on dynamic processes occurring within the complex.

Several kinetic studies on the electrostatically stabilized cytochrome *c*–CcP complex have been performed. Mochan and Nicholls (1972) found that, as a result of complex formation, ascorbate reduction of cytochrome *c* was inhibited by 90% relative to the free protein, whereas the reaction of CcP with H_2O_2 was not affected. In agreement with these results is a recent report by Hoth and Erman (1984), which showed that the rate constant for CN^- binding to cytochrome *c* was decreased by 90%, whereas the reaction of fluoride with CcP(III) was not affected. In both of these studies, however, the effect of ionic strength was not investigated, and therefore it is difficult to differentiate between electrostatic and steric factors.

There have also been recent measurements of nonphysiological electron-transfer processes occurring within modified cytochrome *c*–CcP complexes, involving either triplet Zn-substituted peroxidase to cytochrome *c*(III) transfer (Ho et al., 1985; Peterson-Kennedy et al., 1985) or transfer from an anion radical porphyrin cytochrome *c* to CcP(III) (Cheung et al., 1986). It is interesting to note that, in both cases, significant differences have been observed in the rate constants between various species of cytochrome *c*, with reactions involving yeast cytochrome giving larger rate constants than those obtained with horse or tuna cytochromes.

Another kinetic method of investigation has been successfully used in our laboratory to study the reduction of individual components, and intramolecular electron-transfer processes, within noncovalent protein–protein complexes. This involves rapid formation (<1 μs) of free flavin semiquinones by laser flash photolysis, which produces small reductants in situ that react with the prosthetic groups of the individual proteins comprising the complexes. Differences in reductant size, electrostatic charge, and reduction potential permit the study of both the structural and the electrostatic environments of the protein prosthetic groups within the complexes, in addition to providing information concerning the intramolecular electron-transfer process. This methodology has been applied to flavocytochromes (Cusanovich & Tollin, 1980; Tollin et al., 1984; Cusanovich et al., 1985; Bhattacharyya et al., 1985), the cytochrome *c*–cytochrome *c* oxidase complex (Ahmad et al., 1982), the *Clostridium pasteurianum* rubredoxin–spinach ferrodoxin–$NADP^+$ reductase complex (Przysiecki et al., 1985), the spinach ferrodoxin–spinach ferrodoxin–$NADP^+$ reductase complex (Bhattacharyya et al., 1986), and the horse cytochrome *c*–*C. pasteurianum* flavodoxin electron-transfer complex (Hazzard et al., 1986).

In this study, we report the effects of complexation on the kinetics of reduction by flavin semiquinones of the components of the cytochrome *c*–cytochrome *c* peroxidase complex. We have also directly measured the rate constants for the physiologically relevant one-electron intracomplex electron transfers from cytochrome *c*(II) to the peroxide-oxidized CcP species, CcP(IV,$R^{\cdot+}$) and CcP(IV). By utilizing the cytochromes *c* from horse, tuna, and yeast (iso-2), we have obtained evidence that there are significant species-dependent differences both in the reactivities of the cytochromes themselves within the complex toward free flavin semiquinones and in the rates of the intracomplex electron-transfer reactions. These results suggest that the complexes that are formed between these three cytochromes and CcP are not structurally equivalent and that extrapolation from the hypothetical electron-transfer complex proposed by Poulos and Kraut (1980) and Poulos and Finzel (1984), which utilized the known structures for tuna cytochrome *c* and yeast peroxidase, to other *c*-type cytochromes may not be accurate.

Materials and Methods

Horse cytochrome *c* was obtained from Sigma (type VI) and used without further purification. Tuna cytochrome *c* (Sigma) was purified by ion-exchange chromatography on CM-cellulose. Yeast iso-2 cytochrome *c* was the generous gift of Drs. Susan Moench and James Satterlee. Cytochrome *c* concentration was calculated with ϵ_{550} = 30.8 and 8.16 mM^{-1} cm^{-1} for the ferrous and ferric states, respectively. Cytochrome *c* peroxidase was purified from commercial bakers' yeast (Red Star) by a modification of the procedure of Yonetani (1967) and Nelson et al. (1977), developed by Moench (1984). The protein was purified to a ratio of A_{408}/A_{280} = 1.21. CcP(III) concentration was determined with ϵ_{409} = 93 mM^{-1} cm^{-1} (Yonetani, 1967). Preparation of lumiflavin and purification of FMN were performed as previously described (Simondsen & Tollin, 1983). Riboflavin was obtained from Sigma and used without further purification. 5-Deazariboflavin was generously donated by Drs. William McIntire and Thomas P. Singer.

For reactions with peroxide-oxidized CcP, two procedures were used. In order to generate CcP(IV,$R^{\cdot+}$), the ferric peroxidase was titrated with a buffered 5 mM H_2O_2 solution in the absence or presence of equimolar cytochrome *c*(III). CcP(IV) was obtained by steady-state photoreduction of CcP(III), with either lumiflavin or 5-DRF plus EDTA, followed by titration with H_2O_2 (Ho et al., 1983). A stoichiometric amount of cytochrome *c*(III) was then added to this solution. Kinetic experiments were performed immediately following the production of both forms of the ferryl peroxidase.

Laser flash photolysis reduction studies were performed as described previously (Ahmad et al., 1982; Simondsen & Tollin, 1983). The free flavin concentration for kinetic experiments at μ = 8 mM was 50–70 μM in 3 mM phosphate buffer at pH 7.0 containing 0.5 mM EDTA. A 2-fold dilution of this buffer resulted in an ionic strength of 4 mM. The high ionic strength buffers were prepared by addition of the appropriate amount of KCl. All reactions were carried out under anaerobic conditions at 24 °C. The reduction of cytochrome *c*(III), CcP(III), and CcP(IV,$R^{\cdot+}$) or CcP(IV) was measured by the loss of absorbance at 575, 505, and 550 nm, respectively (see Figure 1 below).

Under these experimental conditions, the concentration of free flavin semiquinone generated by each laser flash was ≤0.1 μM (Simondsen et al., 1982). For the determination of rate constants, protein concentrations were always ≥5 μM, and hence, flavin semiquinone disproportionation did not effectively compete with protein reduction, and pseudo-first-order conditions were obtained. Semilog plots of kinetic data (δ signal vs. time) were linear over at least four half-lives; the slopes of these plots were used to calculate k_{obsd}. The second-order rate constants, k_2, were calculated from the slopes of linear plots of k_{obsd} vs. protein concentration. The error in the rate constants presented below is estimated to be ±10%. Nonlinear plots of k_{obsd} vs. protein concentration were fit by computer modeling as described in Hazzard et al. (1986).

Table I: Reduction Potentials for the Free Flavins and Proteins

flavin or protein	redox couple	$E_{m,7}$ (mV)	footnote
lumiflavin	oxidized/semiquinone	−231	*a*
riboflavin	oxidized/semiquinone	−231	*b*
FMN	oxidized/semiquinone	−238	*b*
5-DRF	oxidized/semiquinone	−630	*c*
horse cytochrome *c*	Fe(III)/Fe(II)	260	*d*
tuna cytochrome *c*	Fe(III)/Fe(II)	260	*d*
yeast iso-2 cytochrome *c*	Fe(III)/Fe(II)	260	*e*
yeast CcP	Fe(III)/Fe(II)	−190	*f*
	Fe(IV)/Fe(III)	1087	*g*

[a] Draper and Ingraham (1968a). [b] Draper and Ingraham (1968b). [c] Blankenhorn (1976). [d] Tollin et al. (1984). [e] Henderson and Rawlinson (1961). [f] Conroy et al. (1978). [g] The $E_{m,7}$ has not been reported; value given was determined at pH 5.2 (Purcell & Erman, 1976).

Steady-state oxidation of ferrous cytochrome *c* by CcP in the presence of excess peroxide was performed by the procedure of Kang et al. (1977). Cytochrome *c* was reduced in situ under anaerobic conditions by illumination in the presence of 1 μM lumiflavin and EDTA. H_2O_2 was added giving a concentration of 180 μM, and the reaction was begun upon addition of 2 nM CcP. Buffer conditions were as in the laser flash experiments with μ = 8 mM. Initial velocity was calculated from the decrease in absorbance at 550 nm. The turnover number was obtained from the intercept on the abscissa of plots of v_0/[cytochrome *c*] vs. v_0.

Electrostatic fields of tuna cytochrome *c*, CcP, and the CcP–cytochrome *c* complex were computed from the atomic coordinates by a modification of the original Tanford–Kirkwood theory (Tanford & Roxby, 1972). This modified treatment scales the local dielectric constant of the charged groups according to their solvent-accessible surface areas (Shire et al., 1974; Matthew et al., 1979; Matthew & Richards, 1982). Fields were displayed as color-coded dots on an Evans and Sutherland PS300 graphics system supporting the model building program FRODO (Jones, 1978).

Computer graphics representations of the cytochrome *c*–CcP complex were displayed with the Evans and Sutherland PS300 system and the graphics display program INSIGHT (Biosym Technologies, Inc.). Coordinates for the lumiflavin molecule were obtained from the FMN structure of *Clostridium MP* flavodoxin (Smith et al., 1977) deposited in the Brookhaven Data Bank. For purposes of docking lumiflavin with CcP or cytochrome *c*, van der Waals surfaces were utilized to ensure proper positioning of the molecules.

Results and Discussion

Steady-State and Flash Photolysis Redox Difference Spectra. It has been previously shown (Meyer et al., 1983; Tollin et al., 1984) that, for reduction of a wide variety of electron-transfer proteins by neutral flavin semiquinones, there is a direct correlation between the second-order reduction rate constants and the difference in midpoint reduction potentials between the reactants ($\Delta E_{m,7}$). We have previously utilized this property to selectively reduce specific proteins within protein–protein electron-transfer complexes (Hazzard et al., 1986; Bhattacharyya et al., 1986). In order to facilitate an understanding of the protocol employed in these studies, we have listed the midpoint reduction potentials of the relevant free flavins and proteins in Table I. The higher potential flavins (e.g., LF, RF, and FMN) have been used to study the reduction of free and complexed cytochrome *c*, whereas the lower potential 5-deazariboflavin (5-DRF) has been employed for reduction of CcP(III), both free and in the complex with cytochrome *c*.

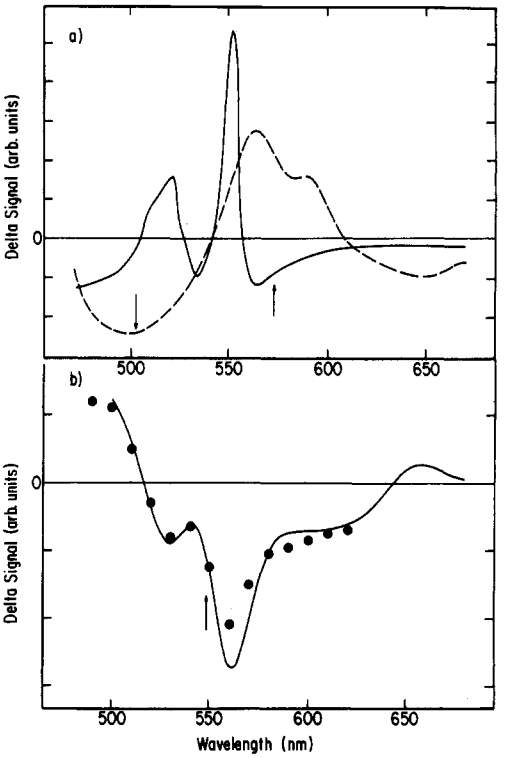

FIGURE 1: Redox difference spectra (reduced minus oxidized) of cytochrome *c* and CcP. (a) Steady-state difference spectra of horse cytochrome *c*(II) minus *c*(III) in the presence of equimolar CcP(III) (solid line) and CcP(II) minus CcP(III) in the presence of equimolar cytochrome *c*(II) (broken line), obtained by exposure to white light in the presence of LF or 5-DRF, respectively. Protein concentrations were 30 μM. (b) Steady-state difference spectrum for reduction of CcP(IV,$R^{\bullet+}$) in the presence of 5-DRF (solid line). Laser flash transient difference spectrum measured at t = 30 ms (●) of a solution containing equimolar (40 μM) cytochrome *c*(III) and CcP(IV,$R^{\bullet+}$) and 50 μM LF. The two spectra were normalized at ~530 nm. In all experiments, buffer contained 3 mM phosphate and 50 μM EDTA (μ = 8 mM) at pH 7.

Figure 1 shows redox difference spectra (reduced minus oxidized) for horse cytochrome *c* and CcP. In each case, the wavelength at which the reduction reaction was monitored for flash photolysis kinetic analysis (see below) is designated by an arrow. Figure 1a shows the redox difference spectrum obtained by irradiation with white light in the presence of lumiflavin and EDTA (solid line) for the reduction of a solution containing equimolar amounts of cytochrome *c*(III) and CcP(III) at low ionic strength (μ = 8 mM). Under these ionic strength conditions, complex formation is expected to be essentially complete, on the basis of an association constant of ~10^6 M^{-1} (Nicholls & Mochan, 1971; Mochan & Nicholls, 1972). The isosbestic points at 504, 526, and 541 nm, as well as the absorbance maxima at 520 and 550 nm, agree well with those of free cytochrome *c* [cf. Figure 1 in Hazzard et al. (1986)]. There was no indication of ferric peroxidase reduction within the complex with ferric cytochrome *c*, which is to be expected on the basis of the midpoint redox potentials for cytochrome *c* (270 mV; Meyer et al., 1983) and CcP(III) (−190 mV; Conroy et al., 1978). Also shown in Figure 1a is the steady-state redox difference spectrum obtained by photoreduction with white light of a 1:1 mixture of cytochrome *c*(II) and CcP(III) at μ = 8 mM with the lower potential 5-DRF and EDTA. The isosbestic points at 540 and 609 nm, as well as the absorbance maximum at 560 nm and large shoulder at ~590 nm, agree well with previously reported spectra of the ferric and ferrous peroxidases (Wittenberg et al., 1968; Ho et al., 1983). These results demonstrate that,

Table II: Second-Order Rate Constants for Reduction of Free and CcP(III)-Complexed Cytochromes *c*(III) by LFH• and RFH•

	k_2 ($\times 10^{-7}$ M^{-1} s^{-1})[a]			
	LFH•		RFH•	
cytochrome	free	complex	free	complex
horse	7.2	1.9	5.4	0.6
tuna	7.1	5.7	5.5	3.5
yeast iso-2	5.3	0.5		

[a] Rate constants were obtained from the slopes of the plots of Figure 2a,b.

under the appropriate experimental conditions, it is possible to separately photoreduce the individual components of cytochrome *c*–CcP mixtures.

Figure 1b shows the steady-state redox difference spectrum obtained upon reduction of free CcP(IV,R•+) by irradiation with white light in the presence of lumiflavin and EDTA (solid line). The data points correspond to a laser flash transient difference spectrum (measured at t = 30 ms after the flash) for a 1:1 mixture of CcP(IV,R•+) and horse cytochrome *c*(III) at low ionic strength (μ = 8 mM) reduced by LFH•. Within the limits of experimental error, the two spectra agree well; the truncation of the large absorption band at ~564 nm in the transient spectrum is commonly observed and can be ascribed to the greater slit width of the laser flash apparatus, relative to that of the spectrophotometer. Thus, the species that was reduced at t = 30 ms after the flash was CcP(IV,R•+). In the absence of cytochrome *c*(III) and on the time scale used to obtain the transient spectrum of Figure 1b, CcP(IV,R•+) was reduced to a much smaller extent, with kinetics that were consistent with reduction primarily by fully reduced lumiflavin (produced by disproportionation of LFH•) and to a much lesser degree by LFH• (see below). Similar results were obtained with the lower potential 5-DRF semiquinone. These results demonstrate that the reduction of CcP(IV,R•+) by flavin semiquinones is greatly enhanced by the presence of ferric cytochrome *c*, the kinetic data for which shall be addressed in more detail below (cf. Figure 4).

Reduction Kinetics of Free and Complexed Cytochrome c(III). Plots of k_{obsd} vs. concentration for laser-induced reduction of tuna, horse, and yeast iso-2 cytochromes *c*, both free and in 1:1 mixtures with ferric cytochrome *c* peroxidase (at μ = 8 mM), by LFH• are shown in Figure 2a. The second-order rate constants, k_2, obtained from the slopes of these plots are listed in Table II. The rate constants for the free horse and tuna cytochromes are in good agreement with values previously reported (Meyer et al., 1983, 1984; Hazzard et al., 1986). In the case of free yeast cytochrome *c*, there is a 25% decrease in the reduction rate constant relative to either horse or tuna cytochromes. Since the reduction potentials for all three cytochromes are essentially the same, this suggests that the accessibility of the heme edge in the yeast cytochrome is less than in the other two cytochromes (Meyer et al., 1984; Tollin et al., 1986). Complex formation with CcP(III) decreased the second-order reduction rate constant of the cytochrome heme to varying degrees depending on the cytochrome species. The magnitude of the rate constant decrease was of the order tuna < horse < yeast iso-2, with decreases in k_2 of 20, 74, and 91%, respectively, relative to the values obtained for the free cytochromes. These results demonstrate that in the complexes with the peroxidase the steric environments near the cytochrome *c* heme edges were markedly different. Whereas the rate constant for tuna cytochrome reduction was only slightly decreased by complex formation, suggesting relatively unhindered access to the heme

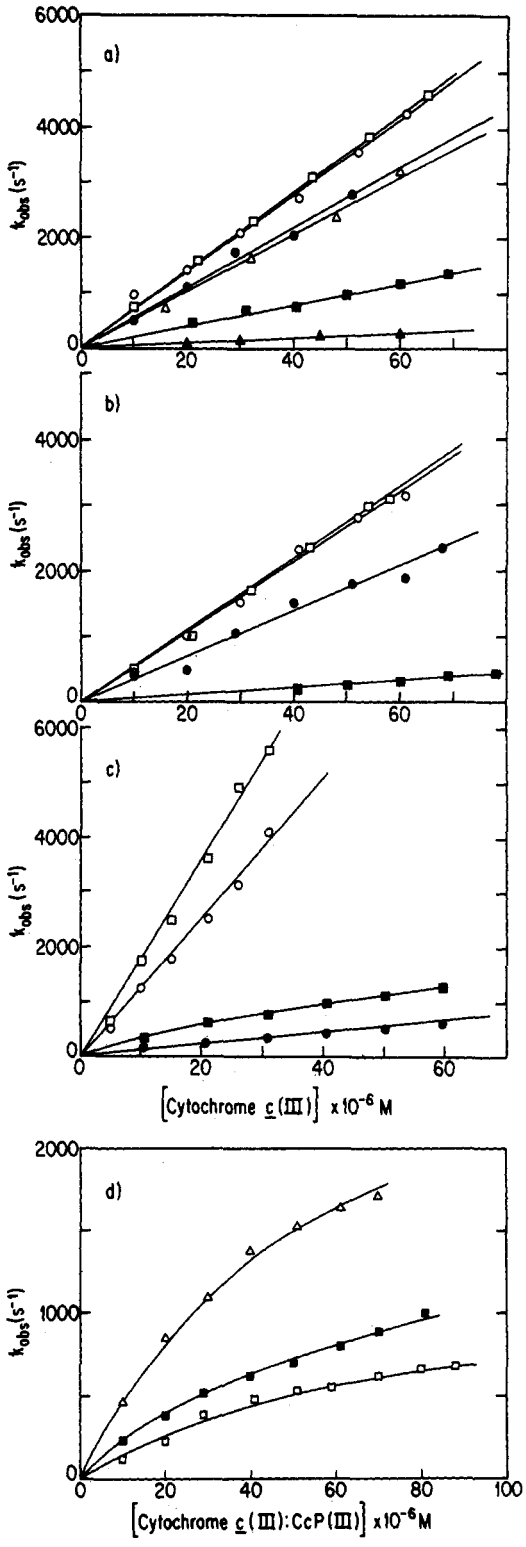

FIGURE 2: Plots of k_{obsd} vs. concentration for reduction of free and CcP(III)-complexed horse, tuna, and yeast iso-2 cytochromes *c*(III) by LFH•, RFH•, and FMNH•. (a) LFH• reduction of free horse (□), tuna (○), and yeast iso-2 (△) cytochromes; LFH• reduction of CcP(III)-complexed horse (■), tuna (●), and yeast iso-2 (▲) cytochromes. All experiments were at μ = 8 mM. (b) RFH• reduction of free horse (□) and tuna (○) cytochromes *c*; RFH• reduction of CcP(III)-complexed horse (■) and tuna (●) cytochromes *c*. All experiments were at μ = 8 mM. (c) FMNH• reduction of free horse cytochrome *c*(III) at μ = 4 (□) and 8 mM (○) and FMNH• reduction of complexed horse cytochrome *c*(III) reduction at μ = 4 (■) and 8 mM (●). (d) FMNH• reduction of complexed tuna cytochrome *c*(III) at μ = 4 mM (□), 8 mM (■), and 15 mM (△). The buffer conditions were the same as in Figure 1, except for the changes in ionic strength.

in the complex, the yeast iso-2 cytochrome heme, and to a lesser extent that of the horse cytochrome, was rendered markedly less accessible to the incoming LFH• reductant by complexation.

In order to further probe the steric environments near the heme edges for the various cytochromes, both free and in the complex with the peroxidase, we performed experiments analogous to those above using riboflavin semiquinone as the reductant. Due to the replacement of the N10 methyl group of lumiflavin with the ribityl side chain, the second-order reduction rate constants for RFH• have been shown to be 20–30% smaller than those for LFH• for a wide variety of free *c*-type cytochromes (Meyer et al., 1984). Plots of k_{obsd} vs. concentration for reduction of horse and tuna cytochromes *c*, both free and in 1:1 mixtures with CcP(III) at low μ, are shown in Figure 2b, and the rate constants derived from these plots are given in Table II. For the free cytochromes, decreases in k_2 of 24% relative to the values obtained for LFH• reduction were observed, in good agreement with the previous findings. For tuna cytochrome *c*, a decrease in k_2 of 36% upon complexation with CcP(III) was observed, whereas for horse cytochrome *c* the decrease was 89%. (Due to the large magnitude of the decrease in k_2 for LFH• reduction of yeast cytochrome within the complex, determination of the rate constant for RFH• reduction was not experimentally feasible, since the time scale of the reaction was so slow that virtually all of the riboflavin semiquinone reacted by disproportionation before it could react with the complexed yeast iso-2 cytochrome.) These effects of complexation were in the same direction as those obtained with LFH•, although the magnitude of the difference between the horse and tuna cytochromes was considerably larger. Thus, there are also significant species-dependent differences in the reactivity of RFH• with the complexes cytochromes, which were not found for the free cytochromes. On the basis of the 91% decrease in k_2 that we observed for yeast iso-2 cytochrome using LFH•, we can conclude that the most sterically occluded heme in these three cytochrome *c*–cytochrome *c* peroxidase complexes is that of the yeast iso-2, followed by that of horse cytochrome *c*. Tuna cytochrome *c* is only moderately affected in its reactivity by complex formation, suggesting that in the complex this cytochrome heme is the most exposed.

Utilization of the semiquinone of FMN permits us to study the electrostatic environment near the prosthetic groups of redox proteins, due to the interaction of the negatively charged FMNH• and charged amino acid residues at the electron-transfer site [cf. Meyer et al. (1984)]. The results for the reduction of free and complexed horse cytochrome *c*(III) by FMNH• are shown in Figure 2c, and the second-order rate constants obtained from these plots are listed in Table III. For free cytochrome *c*, the reduction rate constant decreased from a value of 1.9×10^8 M^{-1} s^{-1} to a value of 1.3×10^8 M^{-1} s^{-1} with an increase in ionic strength from 4 to 8 mM. The magnitude of the decrease in k_2 with the increase in ionic strength is consistent with the results of Meyer et al. (1984), although the data in the earlier work were obtained at much higher ionic strengths than used in these studies. Similar ionic strength dependencies have been observed for the reduction of free tuna cytochrome *c* by FMNH• (Meyer et al., 1984), with the magnitude of the electrostatic charge in the vicinity of the heme edge being essentially the same as that of horse cytochrome *c*. The inverse change of k_2 relative to the change in μ illustrates that the electrostatic interaction of FMNH• with these free cytochromes *c* is of an attractive (or minus/plus) nature, which has been attributed to the interaction of the negatively charged phosphate group of FMNH• with lysine residues surrounding the cytochrome heme edge (Simondsen et al., 1982; Meyer et al., 1984).

Table III: Rate Constants for Reduction of Free Horse Cytochrome *c* and CcP(III)-Complexed Horse and Tuna Cytochromes *c*(III) by FMNH•

	μ (mM)	k_2 ($\times 10^{-7}$ M^{-1} s^{-1})
cytochrome *c*(III)[a]	4	19
	8	13

complexed cytochrome(III)	μ (mM)	k_1 (s^{-1})	k_2 ($\times 10^{-7}$ M^{-1} s^{-1})	K_1
horse *c*[b]	4	1500	5.6	1.4
	8		1.1	
tuna *c*[b]	4	955	4.0	.6
	8	1235	5.1	.8
	15	1820	9.0	1.0

[a] Rate constants were obtained from the slopes of the plots of Figure 2c. There were no significant differences in the rate constants for free horse or tuna cytochrome reduction. [b] Rate constants were obtained from computer fits to the nonlinear kinetic plots of Figure 2c,d. At μ = 8 mM for the horse cytochrome *c*, nonlinear kinetics were not observed; cf. Figure 2c.

In the complex with the peroxidase the ionic strength dependencies of the reaction of FMNH• with horse and tuna cytochromes *c* were markedly different. Panels c and d of Figure 2 show the plots of k_{obsd} vs. concentration for horse and tuna cytochromes complexed to CcP at various ionic strengths. There are two significant features of the results obtained for the reduction of the complexed cytochromes relative to the free proteins. For tuna cytochrome *c* at μ = 4, 8, and 15 mM and horse cytochrome *c* at μ = 4 mM, the kinetic plots are nonlinear with k_{obsd} values becoming independent of the protein concentration. For horse cytochrome at μ = 8 mM, the kinetic plot is essentially linear over the concentration range of the experiment. Nonlinear concentration dependencies have previously been observed for the reduction of horse cytochrome *c* by FMNH• in a 1:1 complex with *C. pasteurianum* flavodoxin (Hazzard et al., 1986). For this system it was suggested that the tendency of k_{obsd} to approach a limiting value could be attributed to an isomerization of the protein complex producing a sterically and electrostatically favorable state for the reduction of the cytochrome heme by FMNH•. The data for the cytochrome *c*–CcP complexes were, therefore, modeled with an analogous mechanism:

$$\text{Cyt } c\text{(III)–CcP(III)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Cyt } c\text{(III)–CcP(III)*} \quad (1)$$

$$\text{Cyt } c\text{(III)–CcP(III)*} + \text{FMNH}^\bullet \xrightarrow{k_2} \text{Cyt } c\text{(II)–CcP(III)} + \text{FMN} \quad (2)$$

where Cyt *c*(III)–CcP(III)* represents the state of the complex with which FMNH• interacts via electron transfer. On the basis of this mechanism, the rate-limiting first-order isomerization process is characterized by k_1 and the second-order reduction of the complexed cytochrome by k_2. The values for these rate constants obtained from computer fits to the data at the different ionic strengths [cf. Hazzard et al. (1986)] are listed in Table III. For complexed tuna cytochrome, as μ increases both the rate-limiting isomerization rate constant, k_1, and the isomerization equilibrium constant, $K_1 = k_1/k_{-1}$, increase, suggesting a weakening of the tuna cytochrome *c*–CcP complex. In the case of horse cytochrome *c*, we were unable to ascertain this aspect of the ionic strength dependence, although it is noteworthy that at μ = 4 mM both k_1 and K_1 are larger for horse cytochrome than for tuna cytochrome, suggesting a weaker complex.

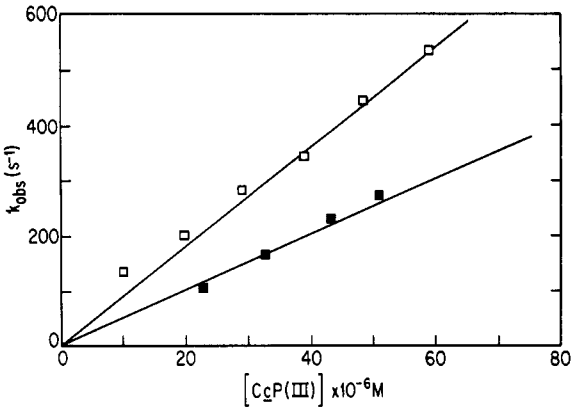

FIGURE 3: Plots of k_{obsd} vs. concentration for reduction of free (□) and horse cytochrome *c*(II) complexed CcP(III) (■) by 5-DRFH• at μ = 8 mM.

Table IV: Second-Order Rate Constants for Reduction of Free and Cytochrome *c*(II) Complexed CcP(III) and Cytochrome c_3 by 5-DRFH•

	k_2 ($\times 10^{-6}$ M^{-1} s^{-1})
CcP(III)	9[a]
CcP(III)–horse cytochrome *c*(II)	5[a]
cytochrome c_3	2400[b]

[a] Values are from the slopes of the plots of Figure 3 (μ = 8 mM). [b] Value from Meyer et al. (1983) (μ = 100 mM).

The second significant feature is that for complexed horse cytochrome *c* the second-order rate constant, k_2 (cf. Table III), decreases with increasing ionic strength, whereas for the tuna cytochrome *c* there is an increase in k_2 with increasing ionic strength. These results indicate that the electrostatic environments near the electron-transfer site which the approaching FMNH• experiences are opposite in sign for these two cytochromes. Thus, for complexed horse cytochrome, there is an apparent positive electrostatic environment, whereas for tuna the environment is negatively charged. Differences in the electrostatic environments of the two cytochromes at the electron-transfer site have been observed previously. Although the electrostatic environment near the heme edge for free tuna and horse cytochromes are essentially the same when a small reductant such as FMNH• is employed (Meyer et al., 1984), when the two cytochromes are reduced by the larger, and more negatively charged, *C. pasteurianum* flavodoxin, there is a larger apparent number of positively charged groups influencing the kinetics of horse cytochrome than are found for tuna cytochrome (Tollin et al., 1984). The difference in electrostatic environments that the FMNH• experiences when approaching the two cytochrome *c*–CcP complexes indicates that the orientations of the cytochromes in the complex may not be equivalent, resulting in different charge cancellation effects at the complex interface (see below for further discussion).

Reduction Kinetics of Free and Complexed CcP(III). Plots of k_{obsd} vs. concentration for reduction of CcP(III), both free and in a 1:1 complex with horse cytochrome *c*(II), by 5-DRFH• are shown in Figure 3, and the second-order rate constants obtained from these plots are listed in Table IV. For free CcP(III), a value of 9×10^6 M^{-1} s^{-1} was obtained. This rate constant is quite small when compared with the value of $\sim 2 \times 10^9$ M^{-1} s^{-1} obtained by Meyer et al. (1983) for cytochrome c_3, which has a reduction potential of −250 mV for the first reduction step (Postgate, 1956; Hori & Kamen, 1961), which is similar to that of CcP(III) (−190 mV). Although the rate constant for cytochrome c_3 was measured at a higher ionic strength, we found that the value for peroxidase was not significantly altered by increases in ionic strength. There are two important structural differences between the heme environments in cytochrome c_3 and in CcP. First, the methyl groups on pyrrole rings A[2] and B of the c_3 heme are highly exposed to the solvent (Higuchi et al., 1981), whereas the peroxidase heme is deeply buried within the interior of the protein (Poulos et al., 1980). Second, the c_3 heme Fe has two axial histidine ligands (Higuchi et al., 1981), whereas the peroxidase heme Fe has one axial histidine and one displaceable water ligand (Poulos et al., 1980). It has been suggested (Crutchley et al., 1985) that, for heme proteins of equivalent redox potentials, reduction rate constants may be markedly influenced by the nature of the proximal (or sixth) ligand of the heme such that reactions involving the loss of a ligated H_2O would have a greater activation barrier, and hence a smaller rate constant, than reactions in which there is no change in Fe coordination. Therefore, the ~500-fold difference in the reduction rate constants for CcP(III) and cytochrome c_3 probably reflects both the relative accessibility of the hemes to incoming reductants (Tollin et al., 1986) and any structural changes that may occur in the peroxidase heme ligation sphere upon reduction.

In the complex with horse cytochrome *c*(II), the second-order rate constant for reduction of CcP(III) had a value of 5×10^6 M^{-1} s^{-1} (Table IV), which corresponds to a 44% decrease relative to the value obtained for the free peroxidase. The magnitude of the rate constant decrease for complexed vs. free CcP is significantly smaller than that found for horse cytochrome *c* reduction by RFH• (89%), which has steric properties equivalent to 5-DRFH•. Thus, we conclude that access to the peroxidase heme is less affected by complex formation than is access to the horse and yeast cytochrome hemes; this will be addressed in more detail below.

Reduction Kinetics of Free and Complexed CcP(IV,R•+) and CcP(IV). Addition of H_2O_2 to the ferric peroxidase produces the well-known compound I species in which the iron has been oxidized to the Fe(IV) state and an amino acid residue(s) of uncertain identity has (have) been oxidized (Peisach et al., 1968; Wittenberg et al., 1968; Yonetani, 1976; Hoffman et al., 1981; Ho et al., 1983; Hori & Yonetani, 1985; Myers & Palmer, 1985; Goodin et al., 1986). Although the reduction potential of CcP(IV,R•+) has not been measured, it should be close to that of horseradish peroxidase compound I, ~1 V (Yamazaki, 1974), which is appreciably higher than that of cytochrome *c* (260 mV) and of CcP(III) (−190 mV). Therefore, we would expect to observe a rapid reduction of CcP(IV,R•+) by both LFH• and 5-DRFH•. Figure 4a shows the transient decay curve obtained upon laser flash photolysis of a lumiflavin solution containing EDTA in the absence of protein monitored at 550 nm. A rapid increase in signal, due to the formation of LFH•, is followed by a nonexponential (second-order) decay back to the preflash base line due to semiquinone disproportionation. In the presence of CcP(IV,R•+), as shown in Figure 4b, there is a small change in the shape of the decay curve, which suggests that there is slow reduction of CcP(IV,R•+) by a reduced lumiflavin species. On the basis of the time scale involved, the predominant reductant is fully reduced lumiflavin, with a small amount of reduction due to the lumiflavin semiquinone also occurring. We can also place an upper limit on the second-order rate constant for reduction of CcP(IV,R•+) by LFH• of 10^6 M^{-1} s^{-1}. Addition of horse cytochrome *c*(III) dramatically changes the characteristics of the transient decay curve, as illustrated in Figure

[2] The designation of the heme pyrrole rings A–D, in accord with Brookhaven Protein Data Bank nomenclature, is synonymous with the older designation of rings I–IV.

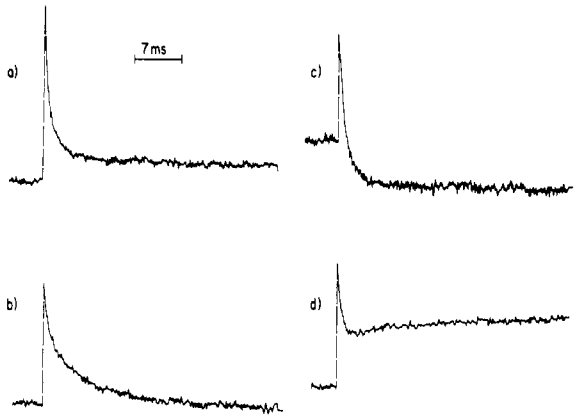

FIGURE 4: Laser flash transient decay curves for LFH• disproportionation and cytochrome *c* and CcP(IV,R•+) reduction monitored at 550 nm. (a) Transient decay curve of a solution containing 50 μM lumiflavin and 500 μM EDTA in phosphate buffer (μ = 8 mM) at pH 7. (b) Decay curve for the solution of (a) to which had been added CcP(IV,R•+) (40 μM). (c) Decay curve for the solution of (b) to which had been added horse cytochrome *c*(III) (40 μM). (d) Decay curve for the solution of (a) to which had been added horse cytochrome *c*(III) (40 μM).

4c. Within 3 ms after the laser flash, the decay curve goes markedly below the preflash base line, indicating a much greater extent of reduction than observed with CcP(IV,R•+) alone. A net decrease in signal below the preflash base line at 550 nm is consistent with the redox difference spectrum of CcP(III) minus CcP(IV,R•+) (Figure 1b). Note also that the reaction in the case of the cytochrome *c*–CcP(IV,R•+) mixture is essentially complete within 10 ms, whereas the reaction with CcP(IV,R•+) alone continues even after t = 40 ms. This indicates that virtually all of the reaction proceeds with the semiquinone form of lumiflavin in the presence of cytochrome *c*. For comparison, Figure 4d shows the transient decay curve obtained at this wavelength in the presence of cytochrome *c* alone. There is a net increase in absorbance over the 40-ms time interval, relative to the preflash base line and to the transient curve obtained with lumiflavin alone, consistent with the cytochrome *c* reduction difference spectrum shown in Figure 1a. The slow rise in this transient curve can be attributed to the further reaction of cytochrome *c* with fully reduced lumiflavin (Ahmad et al., 1981, 1982). On the basis of Figure 4c,d, we can conclude that the reduction of CcP(IV,R•+) by LFH• is greatly facilitated by the presence of cytochrome *c*. Similar results have also been obtained with 5-DRFH• (data not shown). Two mechanisms for peroxidase reduction in these experiments can be proposed. First, direct electron transfer from the flavin semiquinone to CcP(IV,R•+) could occur via a pathway that is facilitated by the complexation with cytochrome *c*(III). Second, an initial reduction of cytochrome *c* by the flavin semiquinone could occur, followed by a rapid intracomplex one-electron transfer to CcP(IV,R•+). These two mechanisms would have quite different kinetic characteristics. The former would obey simple second-order kinetics, whereas the latter would show limiting first-order kinetics under conditions in which the rate constant for intracomplex electron transfer was smaller than the (pseudo-first-order) rate constant for cytochrome *c* reduction by the flavin semiquinone. Furthermore, the limiting reaction in the second mechanism would not show any kinetic dependency on the particular flavin used as the reductant.

In order to test these mechanisms, we measured the concentration dependencies for reduction of CcP(IV,R•+) in a 1:1 complex with horse cytochrome *c* by 5-DRFH• and LFH•. The kinetic plots from these experiments are shown in Figure

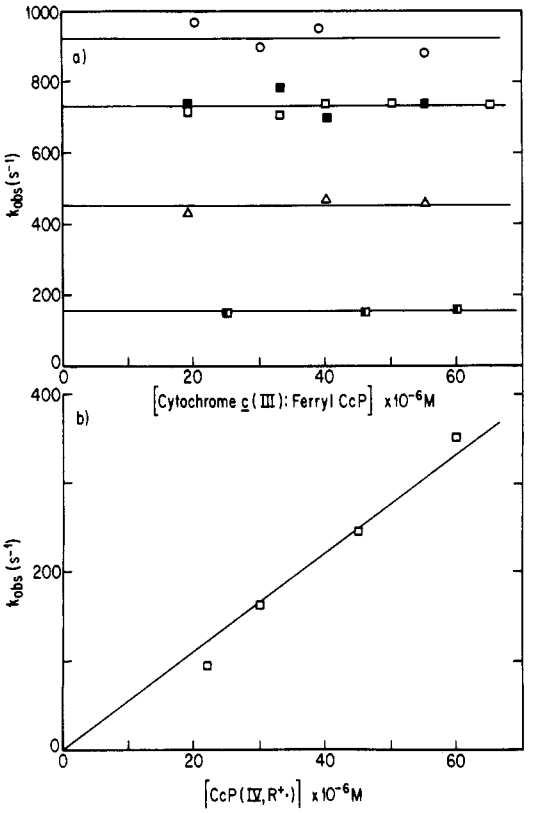

FIGURE 5: Plots of k_{obsd} vs. concentration for reduction of CcP(IV,R•+) and CcP(IV) in 1:1 complexes with cytochromes *c*(III) by 5-DRFH• and LFH•. (a) 5-DRFH• (■) and LFH• (□) reduction of CcP(IV,R•+) in the complex with horse cytochrome *c*(III). LFH• reduction of CcP(IV,R•+) in 1:1 complexes with tuna (○) and yeast iso-2 (◧) cytochromes. LFH• reduction of CcP(IV) (△) in 1:1 complex with horse cytochrome *c*(III). (b) Reduction of CcP(IV,R•+) in the presence of horse cytochrome and LFH• at μ = 200 mM and pH 7.0. Horse cytochrome concentration was held constant at 20 μM.

Table V: First-Order Rate Constants for Intracomplex Electron Transfer from Cytochrome *c*(II) to CcP(IV,R•+) and CcP(IV)

	k_1 (s^{-1})[a]	
	LFH•	5-DRFH•
CcP(IV,R•+)–horse cyt *c*(II)	730	730
CcP(IV,R•+)–tuna cyt *c*(II)	920	
CcP(IV,R•+)–yeast iso-2 cyt *c*(II)	150	
CcP(IV)–horse cyt *c*(II)	450	

[a] Rate constants were obtained from the plots of Figure 5.

5. For both flavin species, the value of k_{obsd} at low ionic strength (Figure 5a) did not change over a wide range of protein concentrations. Furthermore, the value of k_{obsd} was independent of the chemical nature of the flavin semiquinone. These results provide strong evidence that one-electron reduction of complexed CcP(IV,R•+) occurred via an intracomplex first-order transfer from the initially reduced cytochrome *c* and not by a direct interaction of the flavin with the peroxidase.[3] Similar concentration-independent first-order

[3] In discussing the results obtained above for the intramolecular reduction reaction, we have made the assumption that the initial electron transfer to the cytochrome was a second-order process. Although we have some flash photolysis data indicating a rapid initial reduction of the cytochrome, we have not been able to directly measure the rate constant for reduction of cytochrome *c*(III) in the CcP(IV,R•+) complex. Because of the relatively large intracomplex rate constants and the relatively small values of k_2 for the complexed cytochromes, in order to observe distinctly biphasic kinetics it would have been necessary to use protein concentrations too high to permit accurate data acquisition and analysis with our present flash apparatus.

kinetics were observed for complexes prepared with tuna and yeast iso-2 cytochromes and are also shown in Figure 5a. The rate constants obtained from these data are given in Table V. As was observed for the reduction of the three cytochromes within the complex, there are species-dependent differences in the values for the intracomplex electron-transfer rate constants as well. Remarkably, the smallest value, 150 s^{-1}, was obtained with yeast iso-2 cytochrome, whereas for tuna cytochrome the largest value, 920 s^{-1}, was observed. These differences between the intracomplex electron-transfer rate constants for yeast vs. tuna or horse cytochromes are consistent with the steady-state results of Kang et al. (1977, 1978), which showed that at low ionic strengths the turnover number for horse cytochrome reduction of CcP(IV,$R^{\bullet+}$) is larger than that of yeast. It has also been reported recently that intracomplex rate constants for electron transfer from the triplet state of Zn-substituted CcP to ferric cytochrome *c* (Ho et al., 1985; Peterson-Kennedy et al., 1985), and CcP(II) reduction of ferric cytochrome *c* (Cheung et al., 1986) within the 1:1 complex, exhibit species dependencies. However, in these reverse electron-transfer reactions, relative to the physiological reaction, the rate constants for yeast cytochrome *c* reduction are approximately 10 times larger than those obtained for horse or tuna cytochrome *c*.

In order to obtain a turnover number under the same conditions as in our transient kinetic experiments, we performed a steady-state kinetic analysis of the reduction of CcP(IV,$R^{\bullet+}$) by ferrous yeast cytochrome *c* according to the procedure of Kang et al. (1977). A value of 90 s^{-1} for the turnover number was obtained at μ = 8 mM, which is very similar to the value of 100 s^{-1} calculated from the data of Kang et al. (1977; cf. Figure 1b) obtained at μ = 10 mM and pH 7. These workers have also shown that the value of V_{max} varies markedly with ionic strength and the anion species. Furthermore, as Kang and Erman (1982) have pointed out, in the steady-state experiment the interpretation of the turnover number is complicated by the existence of (at least) two rate constants corresponding to the two electron-transfer steps and the dissociation of two cytochrome *c* molecules from CcP. In the laser flash photolysis experiments described above, the intracomplex one electron transfer rate constant was directly obtained from an electrostatically stabilized preformed 1:1 complex. Therefore, complications in the interpretation of kinetic data arising from multiple electron-transfer steps and from the association and dissociation steps were eliminated.

As shown by Kang et al. (1977), in order to obtain the turnover number commonly cited for both horse and yeast cytochrome *c*, 1500 s^{-1}, it is necessary to carry out the experiments at pH 6.0 and at ionic strengths ≥200 mM. At 200 mM and pH 7, we found in the laser flash experiment that reduction of CcP(IV,$R^{\bullet+}$) could again be achieved only in the presence of cytochrome *c*. As shown in Figure 5b, the linear increase in k_{obsd} with increasing CcP concentration indicated that the reduction reaction was a second-order process with a bimolecular rate constant of 6×10^6 M^{-1} s^{-1}. On the basis of the small concentration of horse cytochrome *c* reduced during each flash (<1 μM), this rate constant must correspond to the one-electron reduction of CcP(IV,$R^{\bullet+}$) by cytochrome *c*(II). The change in the reduction kinetics from first to second order with an increase in ionic strength indicates that at μ = 200 mM the protein complex was dissociated into its component parts. Therefore, we are unable to directly relate the intracomplex electron-transfer rate constant measured by the laser flash technique with the maximal turnover number obtained at high ionic strengths by steady-state analysis, although since in our experiments the kinetics remain linear in cytochrome *c* concentration we can conclude that the limiting first-order rate constant must be greater than 400 s^{-1} under these conditions (Figure 5b).

Another aspect of the peroxide-oxidized CcP species that has received a great deal of interest is the nature and role of the amino acid(s) from which a reducing equivalent can be obtained. In order to determine what effect the oxidized protein residue(s) might have on the intracomplex reduction rate constant, we performed similar reduction experiments on a cytochrome *c*–CcP complex in which the peroxidase heme had been reduced to the Fe(II) state prior to addition of H_2O_2. Ho et al. (1983) have shown that by this procedure a direct two-electron oxidation of the Fe(II) occurs, without oxidation of any amino acid group(s). As shown in Figure 5a (triangles), reduction of CcP(IV) in a 1:1 complex with horse cytochrome *c* again occurred by an intracomplex process, with a rate constant of 450 s^{-1}. Inasmuch as less than a 2-fold decrease in the intracomplex rate constant for CcP(IV) relative to CcP(IV,$R^{\bullet+}$) was found, the oxidized R group(s) is (are) apparently not tightly coupled to the peroxidase heme and does (do) not have a strong influence on the kinetics of the one-electron transfer process from cytochrome *c* to the ferryl heme of CcP. This is consistent with the relatively small heme electronic spectral perturbation caused by the R group oxidation (Ho et al., 1983; Goodin et al., 1985). Our results also complement the recent studies by Goodin et al. (1985) using site-directed mutagenesis, which have attempted to determine whether Met-172 of the peroxidase is the protein residue that undergoes oxidation, as suggested by Hoffman and co-workers (Hoffman et al., 1979, 1981) on the basis of low-temperature EPR measurements. These workers found that conversion of Met-172 to Ser-172 or Cys-172 had no significant effect on the steady-state kinetics for cytochrome *c* reduction of CcP(IV,$R^{\bullet+}$). Furthermore, very little change in the visible or EPR spectrum of CcP(IV,$R^{\bullet+}$) resulted from the Ser-172 substitution. Taken together, the directly measured intracomplex rate constant, the steady-state measurements, and the spectral studies indicate, as suggested by Goodin et al. (1985), that the loss of an electron from the protein residue(s) surrounding the heme may be delocalized over more than one amino acid residue.

Relationship between Reduction Kinetics and the Hypothetical Cytochrome c–CcP Complex. One of the purposes of this study was to compare the experimental kinetic data with the hypothetical complex derived from computer graphics modeling (Poulos & Kraut, 1980; Poulos & Finzel, 1984). In general, the structure of the cytochrome *c*–CcP model is consistent with our results, although the kinetic data illustrate the necessity of taking into account such factors as protein dynamics and species-dependent changes in cytochrome structure that may affect the orientations of the two proteins within the electron-transfer complex. Specific comparisons are as follows.

Accessibility of the CcP(III) heme was much less affected by complexation than was generally found for reduction of the complexed cytochromes. In the case of horse cytochrome *c*, only a 44% decrease in the rate constant for reduction of CcP(III) was observed. The simplest explanation for this is that the site of electron transfer is accessible in both the free and the complexed CcP(III). Whereas the heme in CcP is well removed from the molecular surface, a channel connects the surface with the peroxide binding site at the Fe atom and very likely provides the closest approach to the heme for both peroxide and the flavins used in this study (Poulos & Finzel,

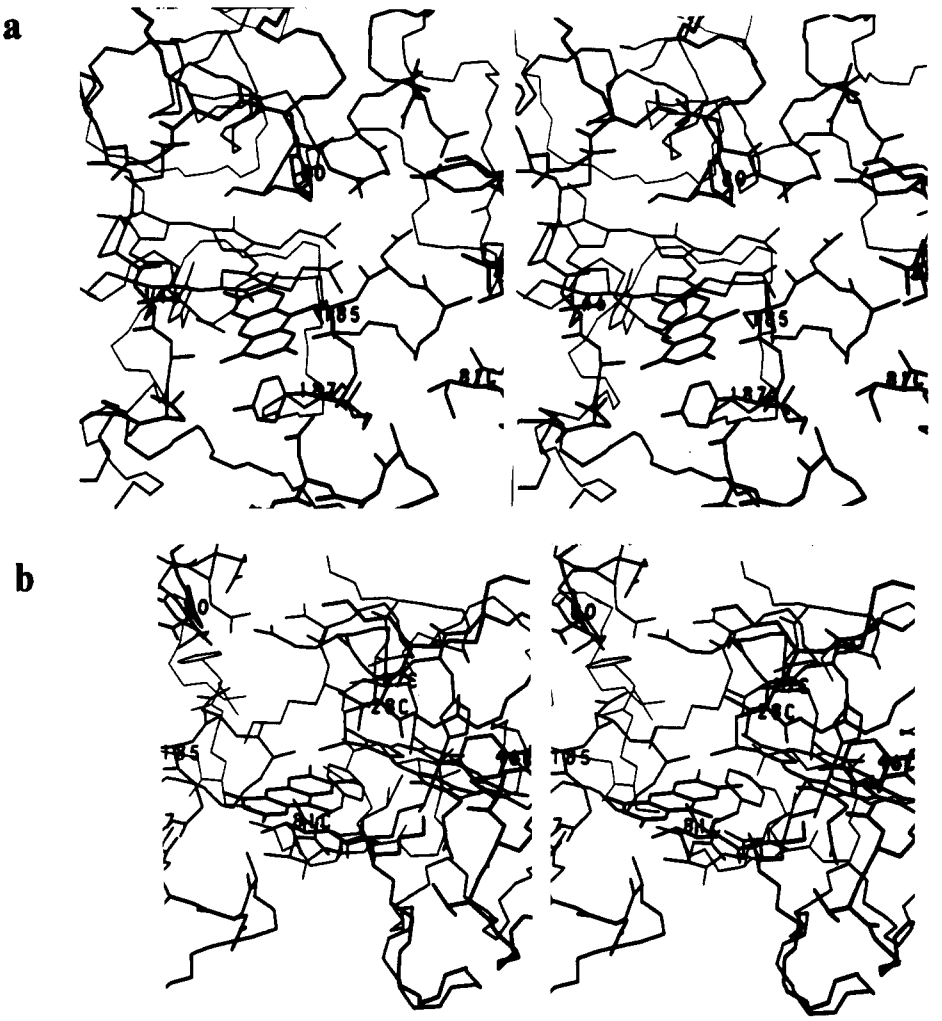

FIGURE 6: Stereoviews of the interaction of LFH• with the hypothetical tuna cytochrome *c*–yeast CcP complex. Cytochrome *c* and CcP are displayed as α-carbon, carbonyl carbon, and peptide nitrogen backbones with key residues near the peroxidase and cytochrome *c* heme edges displayed. In both representations, CcP is to the left and the cytochrome is to the right. Key residues of the cytochrome are denoted with the appended letter "C". (a) Insertion of LFH• into the CcP peroxide access channel. In this orientation, the distance from the 7- and 8-methyl groups of the dimethylbenzene ring of LFH• to the peroxidase heme edge is 4.3 Å. (b) Interaction of LFH• with the exposed heme edge of tuna cytochrome *c*. The distance from the 7-methyl group of LFH• to the thioether bridging sulfur of Cys-17C is 3.8 Å.

1984). Figure 6a shows the placement of the lumiflavin isoalloxazine ring within the peroxide access channel of CcP, accomplished by computer graphics docking procedures. Tuna cytochrome *c* is located to the right of the access channel. Since this channel is not blocked by cytochrome *c* and thus remains accessible in the hypothetical complex, the interaction with reductants via the access channel would be expected to be relatively insensitive to complexation. However, the incoming free flavin must be properly oriented in order to be inserted into the peroxide hole. The lumiflavin ring is closely bounded (i.e., within van der Waals contact distance) from below by Tyr-187, on the right by Ser-185, and on the left by Asp-146. In the orientation shown, the lumiflavin ring and the CcP heme planes are essentially parallel. Furthermore, the 8-methyl group of the dimethylbenzene ring of the flavin is 4.3 Å from the methyl group of pyrrole ring C of CcP. The only freedom of movement for the flavin ring within this channel is either rotation about its long axis, resulting in a decrease in the coplanarity of the flavin and porphyrin rings, or translation toward Pro-80 above the flavin ring, which would increase the 8-methyl to pyrrole methyl distance to ~5 Å. Placement of a ribityl side chain at the N10 position of the isoalloxazine ring (i.e., the side next to Ser-185 in Figure 6a) would decrease the possibility of inserting the flavin ring as deeply in the access channel as is shown in Figure 6a. Therefore, we conclude that both the orientation requirement and the steric constraints imposed by the size of the flavin and the protein residues comprising the peroxide access channel must severely restrict the frequency of collisions that lead to electron transfer, as well as requiring a rather large average distance over which electron transfer from the free flavin to CcP(III) must occur. These considerations are consistent with the small rate constant observed for reduction of free CcP(III) by 5-DRFH• (see above). The 44% decrease in rate constant that was observed upon complexation with cytochrome *c* may reflect a change in either the protein structure or the flexibility of the peroxide channel. Both H_2O_2 and fluoride binding to CcP(III) are unaffected by complexation (Mochan & Nicholls, 1972; Hoth & Erman, 1984) with cytochrome *c*, suggesting that such changes must be relatively small. On the basis of the relatively tight fit of the flavin into the peroxide access channel, the structure of which was determined for uncomplexed peroxidase, such a suggestion is reasonable. Also, the hypothetical model for the cytochrome *c*–CcP complex cannot provide any insight into dynamic motions that may occur within the individual proteins or between the two proteins, since the model is based on time-averaged structures of both the cytochrome and the peroxidase.

A change in the structure or dynamics of the peroxide channel upon reaction of CcP(III) with H_2O_2 may also explain

our data involving reduction of free CcP(IV,P$^{\bullet+}$) and CcP(IV). On the basis of the CcP(III) reduction kinetics and the expected increase in $\Delta E_{m,7}$ due to the peroxide reaction (see above), we would have predicted a rapid reduction of both CcP(IV,R$^{\bullet+}$) and CcP(IV) by flavin semiquinones (Meyer et al., 1983; Tollin et al., 1986). On the contrary, we observed little or no rapid reduction of these ferryl CcP species in the absence of cytochrome *c*. Protein conformational changes related to redox state changes have also been observed in cytochrome *c* oxidase. Scholes and Malmstrom (1986) have shown that there is a marked increase in the rate of CN^- binding following two-electron reduction of the fully oxidized protein, which has been interpreted in terms of a two-state conformational model.

The requirement for the presence of cytochrome *c* in order to obtain rapid reduction of CcP(IV,R$^{\bullet+}$) and CcP(IV) supports the proposal made by Poulos and Finzel (1984) that complexation with cytochrome *c* may involve the establishment of an effective electron-transfer pathway from the molecular surface of CcP to its buried heme, which is not present in the free peroxidase. It can be conjectured that the cytochrome *c* dependency ensures that nonspecific reduction of the ferryl peroxidase by small physiological reductants would be less likely, thereby stabilizing what would otherwise be a highly reactive Fe(IV) heme. The facilitative role of cytochrome *c* has also been observed in the reduction of cytochrome *c* oxidase by exogenous reductants. Ahmad et al. (1982) have shown that, in the absence of cytochrome *c*, the oxidase is reduced very slowly by free flavin semiquinones. In the presence of the cytochrome *c*, a marked increase in the rate of reduction of the oxidase heme *a* was observed. As was the case for CcP(IV,R$^{\bullet+}$) reduction, electron transfer from flavin to oxidase occurred via cytochrome *c* rather than by direct interaction. In a similar study, Bickar et al. (1985) have suggested that cytochrome *c* facilitates an increase in the rate of reduction of the cytochrome a_3 component of cytochrome *c* oxidase using dithionite, NADH, or ascorbate. Therefore, it may be a common feature of oxygen-reducing enzymes, which have a high specificity for reduction by cytochromes, that upon oxidation of the enzyme a conformational change in the protein occurs such that nonspecific reduction becomes kinetically less feasible. In the presence of the cytochrome, an efficient electron-transfer conduit is established, perhaps by reorientation of a few surface amino acids on the enzyme, as has been proposed in the case of CcP (Poulos & Fenzel, 1984).

The species-dependent differences observed in the intracomplex electron-transfer rate constants, as well as the rate constants for reduction of the complexed cytochrome, illustrate that specific details of the hypothetical cytochrome *c*–CcP complex based on the tuna cytochrome structure are not applicable to complexes formed between CcP and other cytochromes *c*. These data are in accord with steady-state studies (Kang et al., 1977, 1978), which also indicate that there can be large differences between the kinetics involving yeast and either tuna or horse cytochrome *c*. For the hypothetical complex of tuna cytochrome *c* and yeast CcP, the distance from heme edge to heme edge is 17.8 Å (Poulos & Finzel, 1984). Using the equation of McLendon et al. (1985) relating electron-transfer rate constants with distance, $k_{et} = (kT/h)e^{-\alpha R}$, where $\alpha = 1.4$, we obtain values for R of 16.2 and 17.5 Å from the tuna and yeast cytochrome intracomplex electron-transfer rate constants (Table V), respectively. Therefore, a small (1.3 Å) change in heme to heme distance could, in principle, lead to a large (84%) decrease in the intracomplex electron-transfer rate constant.

Table VI: Differences in Amino Acid Composition at the Interface of the Hypothetical Electron-Transfer Complex for Tuna, Horse, and Yeast Cytochromes *c*

tuna residue[a]		amino acid substitution[b]	
no.	type	horse	yeast iso-2[c]
4	Ala	Glu	Lys
7	Lys		Ala
8	Lys		Thr
9	Thr	Ile	Leu
11	Val		Lys
12	Gln		Thr
13	Lys		Arg
15	Ala		Gln
28	Val	Thr	
81	Ile		Ala
85	Ile		Leu
88	Lys		Glu
89	Gly	Thr	Lys

[a] Alignment of primary sequences was based on the criteria of Meyer and Kamen (1982). [b] Absence of a substitution implies the presence of the same amino acid residue as found in tuna cytochrome *c*. [c] Yeast residue numbering is based on tuna amino acid sequence.

Our results also indicate that the steric accessibility of the cytochrome *c* heme changes with different cytochromes, presumably by differential masking of the cytochrome heme edges by CcP. Figure 6b shows a possible electron-transfer interaction of the lumiflavin molecule with the tuna cytochrome *c* heme. In this orientation, the flavin 8-methyl group is within 3.5 Å of the methyl group of pyrrole ring D and the 7-methyl group is within 3.8 Å of the thioether bridge sulfur of Cys-17 on pyrrole ring C. It has recently been shown (Tollin et al., 1986) that for free tuna cytochrome *c* this thioether bridge sulfur has a high degree of solvent exposure. In addition, appreciable delocalization of porphyrin π and Fe(III) d_π orbitals onto this sulfur atom indicated its importance in electron-transfer reactions (Tollin et al., 1986). In the complex model shown in Figure 6b, the heme edge comprised of pyrrole rings C and D remains relatively exposed. The presence of CcP does, however, decrease the accessibility of the Cys-17 sulfur relative to the free cytochrome. This could account for the decreases in the reduction rate constants for the complexed, compared to the free, cytochromes. The species-dependent differences in the magnitude of the decrease in rate constant may reflect changes in the degree of exposure of this sulfur in the various complexes.

Panels a and b of Figure 7 show the residues of tuna cytochrome *c* which are substituted in horse and yeast cytochromes, respectively. These changes are listed in Table VI. It should be noted that the number of residues occurring directly in the interprotein interface for yeast cytochrome is much greater than that for horse cytochrome. Furthermore, in the case of horse cytochrome only one substitution, Ala-4 to Glu-4, results in a change in electrostatic charge. The remaining changes involve uncharged residues, which are replaced by residues of similar steric bulk (except Gly-89 to Thr-89). Despite the close similarities in their amino acid composition as well as (presumably) in their three-dimensional structures, we obtained marked differences in reduction kinetics for the various cytochromes in the complex with CcP. We also observed that for the negatively charged flavin, FMNH$^\bullet$, the apparent electrostatic environments of the electron-transfer sites of tuna and horse cytochromes within the complex are quite different. Electrostatic potential calculations (data not shown) on the tuna cytochrome *c*–CcP structure indicated no predominance of either positive or negative charge at the protein interface. Rather, there were pockets of residual charges of both signs. This result is quite

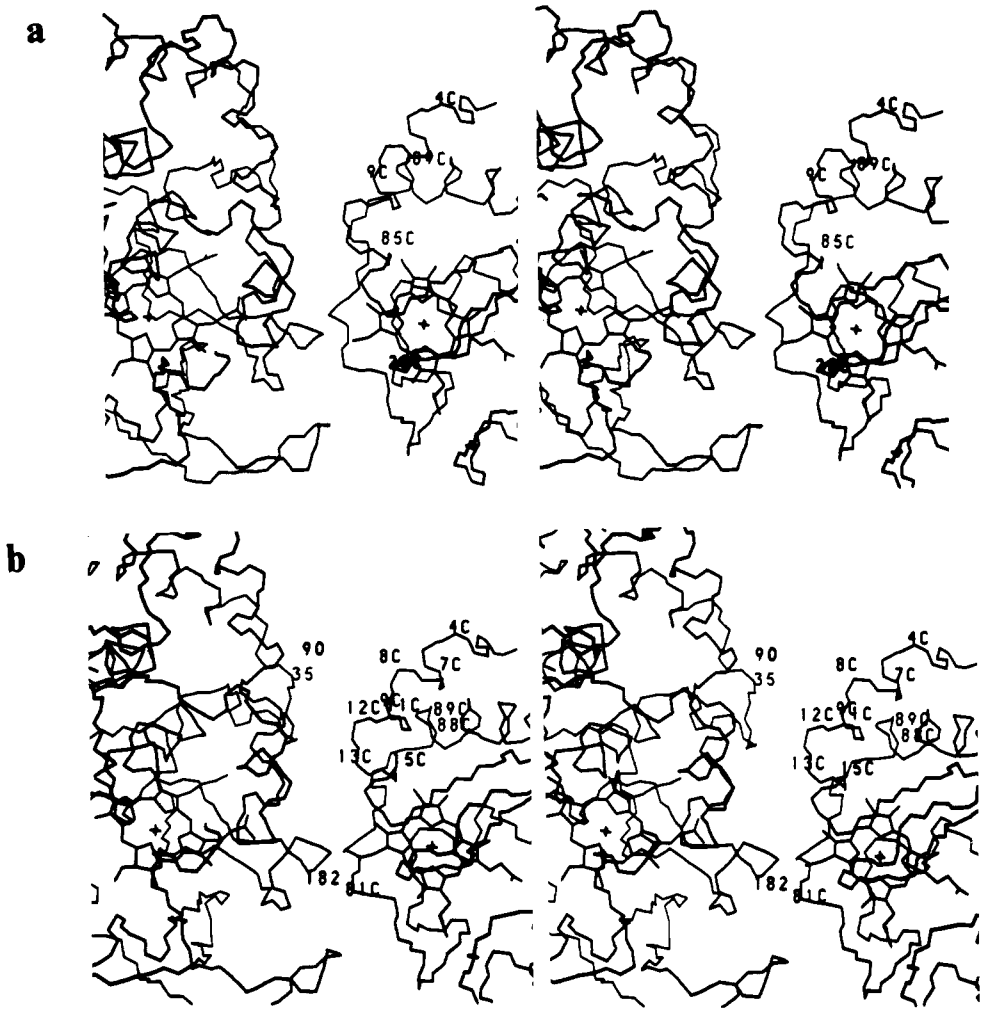

FIGURE 7: Orientations of amino acid substitutions of horse and yeast cytochromes *c*, relative to tuna cytochrome *c*, within the hypothetical cytochrome *c*–CcP complex. Both CcP (left) and cytochrome *c* (right) are shown as α-carbon, carbonyl carbon, and peptide nitrogen backbones. Specific substitutions for both cytochromes (denoted with the appended letter "C") are given in Table VI. (a) Amino acid substitutions of horse, relative to tuna, cytochrome *c*. (b) Amino acid substitutions of yeast iso-2, relative to tuna, cytochrome *c*.

different from that reported for the tuna cytochrome *c*–*Clostridum MP* flavodoxin complex (Matthew et al., 1983), in which a large net negative electrostatic potential was calculated to exist at the interprotein interface. In the CcP complex, therefore, it is possible that small changes in the orientation of the cytochromes within the complex can result in changes in the localized electrostatic environments surrounding the $FMNH^{\bullet}$–cytochrome *c* interaction site.

In the case of yeast cytochrome *c*, there are six substitutions involving changes in electrostatic charge, as well as several substitutions involving large changes in steric bulk. From the viewpoint of the orientation of the yeast cytochrome *c* within the complex with CcP, these substitutions may be significant. First, Val-11 in tuna is Lys in yeast, which would place Lys-11 in the yeast complex close to Lys-90 in CcP, possibly resulting in electrostatic repulsion in this region. Second, Lys-88 and Glu-89 in tuna are Glu and Lys, respectively, in yeast. This would alter the electrostatic environment in a key region of CcP involving the "carboxylate cluster" (residues 34, 35, and 37) and the cytochrome *c* "lysine cluster" centered on Lys-13 (Arg in yeast), which includes residues 87–89 in cytochrome *c* that have been shown by chemical modification and chemical cross-linking data to be involved in forming the complex (Bisson & Capaldi, 1981; Waldmeyer & Bosshard, 1985; Bechtold & Bosshard, 1985; Waldmeyer et al., 1982). Third, Ile-81 in tuna is Ala in yeast. Poulos and Finzel (1984) noted that Ile-81 in tuna cytochrome *c* is situated close to Leu-182 in CcP and provides a potential hydrophobic contact region. Indeed, this region presented problems in the molecular modeling since the approach of these two residues was always too close (T. L. Poulos, unpublished observations). Thus, a smaller side chain at this position might allow for a tighter fit between CcP and yeast cytochrome *c*. This could be especially important to heme accessibility since residue 81 in cytochrome *c* is below the thioether bridge of ring C. A tighter fit between the cytochrome and the peroxidase does not imply a priori a closer heme to heme distance, nor does it imply that the electron-transfer pathway from the yeast cytochrome to CcP is the same as that proposed for the complex involving tuna cytochrome. In fact, our intracomplex electron-transfer data would suggest that the distance between the two hemes may be greater in the case of the yeast cytochrome *c*. Thus, while the overall surfaces that interact within the complex may remain constant for all cytochromes *c*, a slight repositioning and tightening at the interface could alter both cytochrome *c* heme accessibility and the distance or the electron-transfer conduit between the two proteins.

To summarize, although our kinetic results are in overall agreement with the hypothetical cytochrome *c*–CcP complex proposed by Poulos and co-workers, the utilization of this model in the interpretation of kinetic data must be tempered by the realization that such a static model cannot take into account all aspects of the protein–protein interaction in solution. This is especially true of dynamic motions occurring

within the proteins themselves and between the two components (Hazzard et al., 1986). Furthermore, we have shown that relatively small variations in the structure of one of the partners can lead to marked changes in kinetic parameters, which of course is the essence of biological specificity. Finally, for any given electron-transfer complex, there is probably more than one orientation of the proteins comprising the complex that can permit efficient electron transfer, so that the observed kinetics reflect some average over those structures that are in rapid equilibrium with one another. Studies such as the one described herein may help to sort out the relative importance of these various factors in biological electron-transfer mechanisms.

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Conductance Routes for Protons across Membrane Barriers†

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ABSTRACT: Simple phospholipid bilayers show a high level of permeability to protons; in spite of this fact, large proton gradients existing across such bilayers may decay very slowly. In sealed systems, the free movement of protons across a membrane barrier is severely restricted by the coincident development of a proton diffusion potential. Using the fluorescent weak acid *N*-[5-(dimethylamino)naphth-1-ylsulfonyl]glycine (dansylglycine) to monitor the collapse of pH gradients across barrier membranes, it is revealed that in strongly buffered systems movement of the small number of protons giving rise to this electrical potential is insufficient to perturb the proton concentration gradient; significant flux of protons (and hence significant collapse of the concentration gradient) can only occur (a) if protons traverse the membrane as part of an electroneutral complex or (b) if there is a balancing flow of appropriate counterions. In both instances, proton flux is obligatorily coupled to the translocation of species other than protons. In weakly buffered systems, the small initial uncoupled electrogenic flux of protons may significantly alter the concentration gradient. This initial rapid gradient collapse caused by uncoupled electrogenic proton movements is then superimposed upon the residual collapse attributable to tightly coupled proton flux. The initial uncoupled electrogenic proton flux shows a temperature dependence very similar to that demonstrated for water permeation across simple lipid bilayers; upon cooling, there is a sharp decrease in flux at the temperature coinciding with the main gel–liquid-crystalline phase transition of the lipid. The coupled proton flux shows a markedly different temperature dependence with no dramatic change in rate at the phase transition temperature and strong similarity to the behavior previously seen with solutes known to be permeating as electrically neutral compounds. This temperature dependence profile for proton permeation supports the suggestion that this process has much more in common with water diffusion than with the translocation of other monovalent cations, such as Na^+, across membrane barriers.

The electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$, existing across the inner membrane of the mitochondrion and the cytoplasmic membrane of most bacterial cells, is believed to be the primary intermediate linking respiration to active ion transport and then to synthesis of ATP (Mitchell, 1979). Although $\Delta\tilde{\mu}_{H^+}$ is generally not accompanied by large chemical proton gradients, there are several examples, most notably that of the chloroplast thylakoid membrane, but also including the endosomal and lysosomal boundary membranes of most eukaryotic cells, of relatively substantial proton concentration gradients (ΔpH) being maintained across biological membranes (Hinkle & McCarty, 1978; Helenius et al., 1985). These large chemical gradients are generally estabished by the action of integral membrane proton pumps (Forgac et al., 1983) and are assumed to be stabilized by the combined effects of an intrinsically low proton permeability of the boundary membrane and the low concentration (<10 μM) of protons in the surrounding environment. The vital significance of $\Delta\tilde{\mu}_{H^+}$ and ΔpH to cellular metabolism, coupled with the fact that the principal obstacles to free permeation of polar solutes and ions across the endosomal, thylakoid, and mitochondrial matrix boundary membranes are created primarily by the impenetrable nature of the lipid matrix (Deamer & Bramhall, 1986), has prompted several attempts to quantify the proton permeability of lipid bilayers of defined composition. These measurements have established that protons permeate lipid bilayers several orders of magnitude faster than other small cations such as sodium or potassium and that, in fact, most membranes show a rel-

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