Kinetics of Intracomplex Electron Transfer and of Reduction of the Components of Covalent and Noncovalent Complexes of Cytochrome *c* and Cytochrome *c* Peroxidase by Free Flavin Semiquinones†

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ABSTRACT: The kinetics of reduction of free flavin semiquinones of the individual components of 1:1 covalent and electrostatic complexes of yeast ferric and ferryl cytochrome *c* peroxidase and ferric horse cytochrome *c* have been studied. Covalent cross-linking between the peroxidase and cytochrome *c* at low ionic strength results in a complex that has kinetic properties both similar to and different from those of the electrostatic complex. Whereas the cytochrome *c* heme exposure to exogenous reductants is similar in both complexes, the apparent electrostatic environment near the cytochrome *c* heme edge is markedly different. In the electrostatic complex, a net positive charge is present, whereas in the covalent complex, an essentially neutral electrostatic charge is found. Intracomplex electron transfer within the two complexes is also different. For the covalent complex, electron transfer from ferrous cytochrome *c* to the ferryl peroxidase has a rate constant of 1560 s^{-1}, which is invariant with respect to changes in the ionic strength. The rate constant for intracomplex electron transfer within the electrostatic complex is highly ionic strength dependent. At μ = 8 mM a value of 750 s^{-1} has been obtained [Hazzard, J. T., Poulos, T. L., & Tollin, G. (1987) *Biochemistry 26*, 2836–2848], whereas at μ = 30 mM the value is 3300 s^{-1}. This ionic strength dependency for the electrostatic complex has been interpreted in terms of the rearrangement of the two proteins comprising the complex to a more favorable orientation for electron transfer. In the case of the covalent complex, such reorientation is apparently impeded. These kinetic results are discussed in terms of the hypothetical model for the complex proposed by Poulos and Kraut [Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 10322–10330] and the proposal of Waldmeyer and Bosshard [Waldmeyer, B., & Bosshard, H. R. (1985) *J. Biol. Chem. 260*, 5184–5190] regarding the position of sites of covalent cross-linking.

A great deal of interest has been shown in the structural nature of electrostatically stabilized 1:1 redox protein complexes in order to more fully understand the factors that govern intracomplex electron transfer kinetics. It has generally been accepted that attractive electrostatic interactions are responsible for bringing the two proteins together in such a manner that the prosthetic groups have a favorable distance and orientation for electron transfer from one protein to the other (Salemme, 1978). Several hypothetical structures for electron-transfer complexes have been proposed on the basis of computer graphics modeling (Salemme, 1976; Simondsen et al., 1982; Poulos & Kraut, 1980; Poulos & Mauk, 1983; Mauk et al., 1986). In all these cases, complementary electrostatic interactions which optimize the mutual orientation and distance between redox centers have been a predominant criterion in the model building procedure. If electrostatic forces are indeed a primary factor in optimally orienting the reaction partners, it is reasonable to expect that *both* the complex association constant and the first-order rate constant for the intracomplex processes associated with electron transfer (these would include any reorientations that influence redox center geometrical relations) should be inversely proportional to the ionic strength of the solution.

Equilibrium binding studies of cytochrome *c* to CcP[1] (Erman & Vitello, 1980), to cytochrome b_5 (Mauk et al., 1982), and to cytochrome *c* oxidase (Michel & Bosshard, 1984) all show the expected decrease in binding affinity with increasing ionic strength. Reports of ionic strength dependencies of intramolecular electron-transfer rates in protein complexes measured by direct transient-state techniques are rare. Those that have been reported show a variety of effects. Electron transfer in the flavocytochrome b_2–cytochrome *c* complex has a rate constant of 380 s^{-1} which is independent of ionic strength between 20 and 230 mM at pH 7 (Capeillere-Blandin, 1982). The electron-transfer rate constant in the flavodoxin–cytochrome *c* complex decreases slightly from 85 to 66 s^{-1} between μ = 48 and μ = 76 mM (Simondsen et al., 1982). Bhattacharyya et al. (1986) report on the electron transfer in the ferredoxin–ferrodoxin-$NADP^+$ reductase complex and find a substantial decrease in the rate constant, from 4000 to 1600 s^{-1}, for a change in ionic strength from 310 to 460 mM at pH 7. Such decreases in the apparent intracomplex electron-

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[1] Abbreviations: cyt *c*(III) and cyt *c*(II), ferric and ferrous forms of cytochrome *c*, respectively; CcP(III), ferric cytochrome *c* peroxidase; CcP(IV,$R^{\bullet+}$), peroxidase species oxidized by H_2O_2 to the Fe(IV) oxidation state containing (an) unidentified amino acid free radical(s), $R^{\bullet+}$ (i.e., compound I); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; $LfH^{\bullet}$, $FMNH^{\bullet}$, and 5-$DRfH^{\bullet}$, semiquinone species of lumiflavin, flavin mononucleotide, and 5-deazariboflavin, respectively.

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transfer rate constant with ionic strength have been attributed to formation of nonoptimal complexes that require geometrical rearrangement prior to electron transfer (Simondsen & Tollin, 1983; Hazzard et al., 1987b). Recent studies on the one-electron reduction of CcP(IV,R$^{\bullet+}$) by yeast iso-1 and iso-2 ferrocytochromes *c* show *increases* in the intracomplex electron-transfer rate constant with increasing ionic strength between 8 and 280 mM (Cusanovich et al., 1987). Thus, for the yeast cytochrome *c*–CcP complex, the formation of a strong electrostatic complex at low ionic strength is not coincident with the most efficient electron-transfer rate; rather, the fastest electron transfer occurs at ionic strengths at which the complex is considerably weakened.

In order to more thoroughly characterize the electrostatically stabilized cytochrome *c*–CcP complex, techniques for the covalent cross-linking of the two proteins have been developed (Waldmeyer et al., 1982; Waldmeyer & Bosshard, 1985; Moench et al., 1987), and the resultant covalent complex has been subjected to several biochemical and biophysical investigations (Waldmeyer et al., 1982; Waldmeyer & Bosshard, 1985; Bechtold & Bosshard, 1985; Erman et al., 1987; Moench et al., 1987). In this report we present a comparison of the kinetics of reduction of both the horse cytochrome *c* and the peroxidase components in the electrostatically stabilized and covalently cross-linked complexes by free flavin semiquinones, as well as the kinetics of intracomplex electron transfer for the two types of complex, at both low and intermediate ionic strengths. Our findings suggest that, although the overall geometry of the complexes at low μ are similar, there exist subtle, but significant, structural differences. The results also indicate that dynamic motions of the two proteins relative to one another are necessary in order to obtain an optimal electron-transfer complex, a process that is impeded by the formation of covalent cross-links.

Materials and Methods

Cytochrome *c* peroxidase was isolated, purified, and spectrally characterized as described previously (Yonetani & Ray, 1965; Erman & Vitello, 1980; Hazzard et al., 1987a). Horse heart cytochrome *c* (Sigma, type VI) was purified by ion-exchange chromatography on CM-cellulose. The covalently cross-linked complex between horse cyt *c* and CcP was formed and purified by a slight modification of the method of Waldmeyer and Bosshard (1985; Moench et al., 1987). CcP(IV,R$^{\bullet+}$) was prepared by the titration of CcP(III) with a buffered H_2O_2 solution immediately prior to kinetic experiments. 5-Deazariboflavin was the generous gift of Drs. William McIntire and Thomas P. Singer.

Three phosphate buffers containing 0.5 mM EDTA at pH 7 were employed for determination of ionic strength dependencies. Buffers which were 1, 3, and 15.4 mM in phosphate concentration were used for ionic strengths of 4, 8, and 30 mM, respectively. Lumiflavin concentration was 70 μM; 5-deazariboflavin concentration was 100 μM.

The laser flash photolysis apparatus and technique, as well as the preparation of free flavins, have been described previously (Simondsen & Tollin, 1983; Hazzard et al., 1987a). Under the experimental conditions employed in these studies, the concentration of free flavin semiquinone generated per laser pulse was <100 nM. For all kinetic experiments protein concentrations were $\geq$5 μM, and therefore these studies were performed under pseudo-first-order conditions. The reduction of cytochrome *c* in all complexes with CcP(III) was monitored at 575 nm. The reduction of cyt *c*(III) by LfH$^{\bullet}$ in the electrostatic complex with CcP(IV,R$^{\bullet+}$) at μ = 8 mM was monitored at 510 nm, a wavelength at which it was empirically determined that there is no net change in absorbance for both cyt *c* and CcP upon reduction by free flavin semiquinones. CcP(IV,R$^{\bullet+}$) reduction was monitored at 550 nm, a wavelength at which there is a significant bleach in the CcP(III,R) minus CcP(IV,R$^{\bullet+}$) redox difference spectrum (Hazzard et al., 1987a).

It has been previously shown that free flavin semiquinones do not directly react with CcP(IV,R$^{\bullet+}$) to any significant degree [cf. Figure 4 in Hazzard et al. (1987a)]. In the presence of cytochrome *c*, however, reduction of the ferryl peroxidase occurs, by a mechanism in which the free flavin semiquinone initially reduces the cytochrome *c*, which in turn transfers the electron, by an intracomplex mechanism, to the peroxidase. Reduction of CcP(IV,R$^{\bullet+}$) in the covalent complex was performed with lumiflavin semiquinone as the initial reductant. For reduction of CcP(IV,R$^{\bullet+}$) in the electrostatic complex, two techniques were utilized. At μ = 8 mM, equimolar concentrations of cytochrome *c* and CcP were reduced by using lumiflavin semiquinone (we have demonstrated previously that the rate constant for intracomplex electron transfer is independent of the flavin semiquinone species and that the second-order reduction of the complexed cytochrome *c* by the flavin is not rate-limiting at protein concentrations $\geq$20 μM). At μ = 30 mM, an ionic strength at which the complex should be considerably weakened, the cytochrome *c* concentration was held constant at 20 μM while the CcP(IV,R$^{\bullet+}$) concentration was varied. 5-Deazariboflavin semiquinone was used as a reductant in this experiment, since the second-order rate constant for reduction of free cytochrome *c* is 2×10^9 M^{-1} s^{-1}, and thus the initial reduction of cytochrome *c* is not rate-limiting even at low CcP concentrations. As a consequence of the small amount of free flavin semiquinone generated per flash (see above) under these conditions the reduction of CcP(IV,R$^{\bullet+}$) demonstrated pseudo-first-order kinetic properties at all CcP concentrations.

A minimum of four transient decay curves were averaged for each protein concentration. Pseudo-first-order rate constants k_{obsd} were calculated from semilogarithmic plots of Δ signal vs time, which were linear over four half-lives. For linear plots of k_{obsd} vs concentration, second-order rate constants were obtained directly from the slopes of these plots. For nonlinear plots of k_{obsd} vs concentration, computer modeling was performed based on the appropriate mechanism as given under Results and Discussion (Strickland et al., 1975; Simondsen et al., 1982). The estimated error of the rate constants reported below is $\pm$10%.

Computer graphics representations of the cyt *c*–CcP complex were displayed with the Evans and Sutherland PS300 system and the graphics program INSIGHT (Biosym Technologies, Inc.). Coordinates for the complex were generously donated by Dr. Thomas L. Poulos.

Results and Discussion

Reduction of the Cytochrome c Component in 1:1 Complexes with CcP(III). The steric accessibilities of the hemes of the cytochromes *c* of horse, tuna, and yeast iso-2 and both native and several site-directed mutants of yeast iso-1, within 1:1 electrostatic complexes with CcP, have been elucidated by using the kinetics of cytochrome reduction by exogenous neutral lumiflavin semiquinone (LfH$^{\bullet}$) as a probe (Hazzard et al., 1987a; Cusanovich et al., 1987). Figure 1a shows a plot of k_{obsd} vs [complex] for the reduction of horse cyt *c*(III) by LfH$^{\bullet}$ in the covalently cross-linked complex with CcP(III). The value for the second-order reduction rate constant obtained from this plot, $k = 2.8 \times 10^7$ M^{-1} s^{-1}, is given in Table I. Also given in Table I are the rate constants for reduction of free

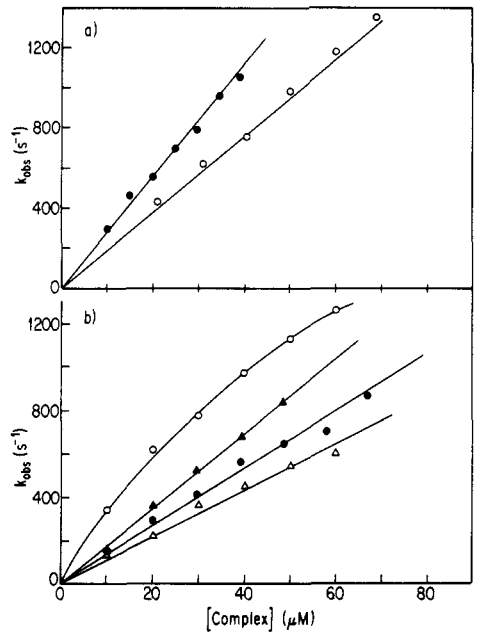

FIGURE 1: Plots of k_{obsd} vs concentration for reduction of horse cyt c(III) in 1:1 electrostatic and covalent complexes with CcP(III). (a) LfH• reduction of cyt c(III) at μ = 8 mM in the covalent (●) and electrostatic (○) complexes. (b) FMNH• reduction of cyt c(III) in the covalent complex at μ = 4 (●) and 8 (▲) mM; electrostatic complex at μ = 4 (○) and 8 (△) mM. Solutions were prepared by using 1 or 3 mM phosphate buffers containing 0.5 mM EDTA for the μ = 4 and 8 mM solutions, respectively, at pH 7. Flavin concentration was 70 μM.

Table I: Second-Order Rate Constants for Reduction of Free and CcP-Complexed Cyt c by LfH•[a]

cyt c species	$k \times 10^{-7}$ (M^{-1} s^{-1})	cyt c species	$k \times 10^{-7}$ (M^{-1} s^{-1})
free[b]	7.2	covalent complex	2.8
electrostatic complex[b]	1.9		

[a] Reactions were carried out at μ = 8 mM in phosphate buffer (3 mM) containing 0.5 mM EDTA and 70 μM Lf at pH 7. [b] Data for free and electrostatically complexed cyt c are from Hazzard et al. (1987a).

and electrostatically complexed horse cyt c (cf. also Figure 1a) under the same conditions. The cross-linked cyt c rate constant is 1.5 times larger than the value obtained for the electrostatic complex but is still significantly smaller than the value for free cyt c reduction. These results indicate that there is a slightly greater degree of accessibility of the cyt c heme edge to LfH• in the covalent complex compared with the electrostatic complex. We can conclude from this that, at least to the degree that the LfH• reduction kinetics are able to assess, the overall structural geometry of the covalent complex must be similar to, but not identical with, that of the electrostatic complex and that covalent cross-linking has probably not resulted in a gross reorientation of the two proteins relative to one another.

The sign and magnitude of the electrostatic charge near the exposed heme edge in the free cyt c and in either form of the complexed cyt c can be elucidated by employing the negatively charged semiquinone of FMN as a reductant at various ionic strengths (Meyer et al., 1984; Hazzard et al., 1987a). Figure 1b shows plots of k_{obsd} vs [complex] for reduction of cyt c in the cross-linked complex at μ = 4 and 8 mM; the second-order rate constants determined from these plots are given in Table II. For the covalently complexed cyt c, there is only a small change (within experimental error) in the value of k upon an increase in μ from 4 to 8 mM. These results differ from those

Table II: Second-Order Rate Constants for Reduction of Free and Complexed Cyt c by FMNH•[a]

cyt c species	μ (mM)	$k \times 10^{-7}$ (M^{-1} s^{-1})
free	4	19
	8	11
electrostatic complex	4	5.6
	8	1.1
covalent complex	4	1.4
	8	1.7

[a] For μ = 4 and 8 mM reactions, phosphate buffers of 1 and 3 mM, respectively, containing 0.5 mM EDTA and 70 μM FMN at pH 7 were used. [b] Data for free and electrostatically complexed cyt c are from Hazzard et al. (1987a).

obtained with the electrostatically complexed cytochrome. Reduction of horse cyt c by FMNH• in the electrostatic complex with CcP(III) was previously shown to give a rather large decrease (~80%) in the rate constant with an increase in ionic strength from 4 to 8 mM (cf. Figure 1b and Table II; Hazzard et al., 1987a), indicating that a net *positive* electrostatic charge existed at the FMNH• interaction site within the 1:1 electrostatic complex. Thus, the electrostatic potential that FMNH• experiences during heme reduction is *different* in the two forms of the complex; i.e., covalent cross-linking induces a neutralization of charge near the exposed cyt c heme edge, relative to the electrostatic complex. This implies that there may be sites of cross-linking (either inter- or intramolecular) in addition to those proposed by Waldmeyer and Bosshard (1985), a point to be addressed in more detail below.

A second major difference between the two forms of the horse cyt c complex lies in the fact that, for the cross-linked complex, plots of k_{obsd} vs [complex] remain linear over a wide concentration range, whereas at μ = 4 mM for the electrostatic complex, k_{obsd} deviates from linearity at relatively low protein concentrations (i.e., $\lesssim$30 μM). The approach of k_{obsd} to a limiting value for the flavodoxin–cyt c complex has previously been interpreted in terms of an isomerization between open and closed forms of the electrostatic complex, in which only the open form was capable of being reduced by FMNH• (Hazzard et al., 1987a).[2] The important feature in this mechanism is the frequency of collisions with the open form which at high protein concentrations becomes limited by the rate constant for the opening of the complex. Inasmuch as nonlinearity is not observed with the covalent complex, the results suggest that cross-linking significantly decreases dynamic motion within the 1:1 complex. The results are also consistent with a higher intrinsic reactivity of the covalently complexed cyt c toward FMNH• (compare k values at μ =

[2] An alternative mechanism that can explain the nonlinear concentration dependencies obtained with FMNH• is

$$\text{FMNH}^\bullet + \text{cyt } c\text{(III)–CcP} \rightleftharpoons \text{FMNH}^\bullet\text{–cyt } c\text{(III)–CcP}$$

$$\text{FMNH}^\bullet\text{–cyt } c\text{(III)–CcP} \rightarrow \text{FMN} + \text{cyt } c\text{(II)–CcP}$$

in which the rate-limiting process is the second reaction involving electron transfer within a ternary complex (Strickland et al., 1975). Previous studies of reduction of cyt c(III) within a 1:1 complex with flavodoxin indicated that the nature of the reductant had no effect on the rate-limiting step, and thus the results could not be interpreted in terms of this mechanism (Hazzard et al., 1986). Since we have not done comparable studies in the present system, we cannot exclude this possibility. However, in order to account for the present results using this formulation, it would be necessary to assume that the reduction of cyt c(III) within the ternary complex with CcP is much faster for the covalent complex than for the electrostatic complex. This seems to be a less satisfying explanation than the one presented in the text.

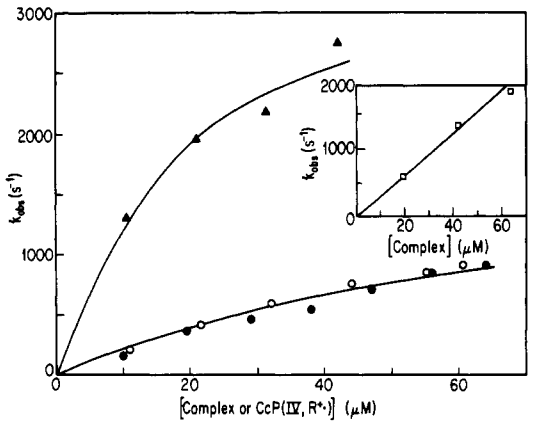

FIGURE 2: Plots of k_{obsd} vs concentration for reduction of CcP(IV,R$^{\bullet+}$) by cyt c(II) for the electrostatic and covalent complexes. (○) Reduction of CcP(IV,R$^{\bullet+}$) in the covalent complex at μ = 8 mM. (●) Reduction of CcP(IV,R$^{\bullet+}$) in the covalent complex at μ = 30 mM. For ○ and ●, LFH$^{\bullet}$ was used to reduce cyt c(III). (▲) Titration of cyt c(II) (≤0.1 μM) by CcP(IV,R$^{\bullet+}$) at μ = 30 mM; 5-DRfH$^{\bullet}$ was used for reduction of cyt c(III). CcP(IV,R$^{\bullet+}$) reduction was monitored at 550 nm. Insert: Reduction of horse cyt c(III) by LfH$^{\bullet}$ in the 1:1 electrostatic complex with CcP(IV,R$^{\bullet+}$) at μ = 8 mM. Cyt c(III) reduction was monitored at 510 nm. Reaction solutions were prepared by using 3 and 27 mM phosphate buffers containing 0.5 mM EDTA for the μ = 8 and 30 mM solutions, respectively. Flavin concentration was 70 μM for Lf and 100 μM for 5-DRf.

8 mM in Table II), which is in accord with the lumiflavin data which indicate a greater degree of heme accessibility.

Intracomplex Electron Transfer from Cyt c(II) to CcP(IV,R$^{\bullet+}$). Of special interest is the comparison of the rate of intracomplex electron transfer from cyt c(II) to CcP(IV,R$^{\bullet+}$) between the cross-linked and electrostatic complexes. We have previously shown (Hazzard et al., 1987a) that CcP(IV,R$^{\bullet+}$) is not readily reduced by free flavin semiquinones.[3] However, in the presence of cyt c at μ = 8 mM, a one-electron reduction of the ferryl species of CcP can be achieved by the mechanism:

$$\text{FH}^{\bullet} + \text{cyt } c\text{(III)–CcP(IV,R}^{\bullet+}) \xrightarrow{k_1} \text{F} + \text{cyt } c\text{(II)–CcP(IV,R}^{\bullet+}) \quad (1)$$

$$\text{cyt } c\text{(II)–CcP(IV,R}^{\bullet+}) \xrightarrow{k_2} \text{cyt } c\text{(III)–CcP(III,R}^{\bullet+}) \quad (2)$$

where FH$^{\bullet}$ and F represent the semiquinone and oxidized species of the free flavin, respectively. Previous studies with the electrostatic complex indicated that at complex concentrations ≥20 μM, the reaction given in eq 2 was the rate-limiting step in the electron-transfer mechanism (Hazzard et al., 1987a). Although an increase in ionic strength would be expected to lead to more complicated kinetics in the case of the electrostatic complex due to weakening of the intermolecular interaction (a point that will be addressed in more detail below), the covalent complex should inherently be less affected by ionic strength changes. The plots of k_{obsd} vs complex concentration, shown in Figure 2, for the reduction of covalently complexed CcP(IV,R$^{\bullet+}$) at μ = 8 and 30 mM in fact verify this proposal. Thus, the data obtained at both ionic strengths are essentially the same for the covalent complex. For this species the k_{obsd} vs concentration plot is nonlinear, suggesting that at low complex concentrations the second-order reduction of cyt c(III), i.e., eq 1, is rate-limiting. Table III lists values for the two rate constants obtained by computer modeling using the mechanism shown in eq 1 and 2. There was no significant ionic strength dependence for either of these rate constants. For the covalent complex the value for the intracomplex electron-transfer rate constant is ~1600 s^{-1}, which is slightly greater than the value of 750 s^{-1} obtained for the electrostatic complex at μ = 8 mM. One reason for this difference in rate constants may be that the conditions under which the covalent complex was formed (pH 6.0; 10 mM cacodylate; μ ≤10 mM) stabilized the two proteins in a more reactive state compared to the electrostatic complex under these experimental conditions. It has also been demonstrated that in the covalent complex CcP is more reactive toward H_2O_2 than native CcP (Erman et al., 1987).

The cyt c heme accessibility in the covalent complex with CcP(IV,R$^{\bullet+}$) is apparently the same as in the covalent complex with the ferric peroxidase, on the basis of the values for the second-order reduction rate constants given in Tables I and III (k and k_1, respectively).

The insert in Figure 2 shows a plot of k_{obsd} vs complex concentration for the reduction of cyt c by LfH$^{\bullet}$ in the electrostatic complex with CcP(IV,R$^{\bullet+}$) at μ = 8 mM. The rate constant obtained from this plot (3×10^7 M^{-1} s^{-1}) is given in Table III. There is a small increase (~50%) in the second-order reduction rate constant compared with that for the CcP(III) complex, which implies an increase in accessibility of the cyt c heme when the peroxidase is oxidized by hydrogen peroxide. In contrast, for the covalent complex there is essentially no difference in the reduction rate constants for the complexes containing the two different oxidized CcP species. Thus, the apparent increase in heme c accessibility due to oxidation of CcP(III) is inhibited by covalent cross-linking.

The results for both the electrostatic and covalent complexes using horse cyt c are markedly different from those obtained by using yeast iso-1 and iso-2 cytochromes c, in which the rate constants for reduction of the CcP(IV,R$^{\bullet+}$)-complexed cyt c were 5 times that for cyt c in the complex with CcP(III) (Cusanovich et al., 1987). It appears, therefore, that the change in accessibility of the cyt c heme upon oxidation of CcP by H_2O_2 is strongly dependent on the species of cytochrome used. It is important to point out, however, that the heme accessibility in both of the horse cyt c complexes with CcP(III) is much larger than that of the electrostatically complexed yeast cytochromes. Furthermore, the rate constants for all of the cytochromes in the CcP(IV,R$^{\bullet+}$) complexes are approximately the same (~3×10^7 M^{-1} s^{-1}). Thus, the major differences appear to be in the CcP(III)-complexed cytochromes. It should also be noted that any change in cyt c accessibility upon H_2O_2 oxidation of CcP(III) must ultimately reflect a change in either the peroxidase conformation or in the contribution of CcP to the overall dynamics of the complex. This suggestion is consistent with recent crystallographic data that indicates a change in the orientation of the antiparallel β sheet of CcP from residues 175 to 190 upon conversion to the ferryl species (Edwards et al., 1987). In the Poulos–Kraut model (Poulos & Kraut, 1980; Poulos & Finzel, 1984) this region lies adjacent to the exposed edge of the cyt c heme [specifically, the thioether bridge of Cys-17, which has been suggested to be involved in electron transfer into the cyt c heme (Tollin et al., 1986)]. The observed variations in heme accessibility due either to the cyt c species involved or to the formation of covalent cross-links provide further support for the view that the geometric relationships of the proteins within all of these CcP complexes are not identical (Hazzard et al., 1987a).

[3] We cannot absolutely exclude the possibility of direct reduction of CcP(IV,R$^{\bullet+}$) by lumiflavin semiquinone in the covalent complex. However, we have not observed such direct reduction in free or electrostatically complexed CcP by either 5-DRfH$^{\bullet}$ ($E_{m,7}$ = −630 mV) or LfH$^{\bullet}$ ($E_{m,7}$ = −230 mV) at ionic strengths of 8, 30, or 280 mM.

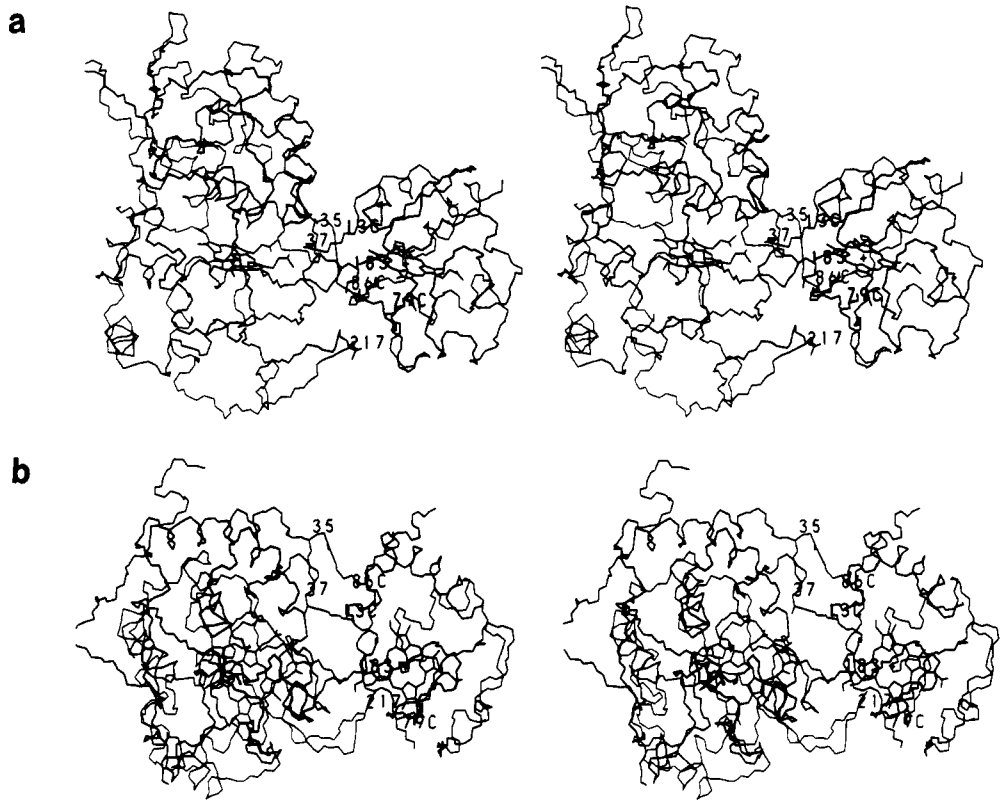

FIGURE 3: Computer graphic representations of the Poulos–Kraut model for the cyt *c*–CcP complex. Both CcP (left) and cyt *c* (right) are shown as C, CA, and N backbones with the heme groups included. Glu-35, Asp-37, and Asp-217 of CcP, Lys-183 of CcP, and lysines-13, -79, and -86 of cyt *c* are shown. Covalent cross-links between CcP and cyt *c* based on potential sites proposed by Waldmeyer and Bosshard (1985) are represented. (a) and (b) correspond to oblique and top views (relative to the heme planes), respectively, of the same object.

At higher ionic strengths the reaction mechanism for reduction of the electrostatic complex is complicated by dissociation of the complex. Under these conditions the following mechanism is operative:

$$FH^{\bullet} + \text{cyt } c(III) \xrightarrow{k_3} F + \text{cyt } c(II) \quad (3)$$

$$\text{cyt } c(II) + CcP(IV,R^{\bullet+}) \underset{}{\overset{k_4}{\rightleftharpoons}} \text{cyt } c(II)\text{–}CcP(IV,R^{\bullet+}) \quad (4)$$

$$\text{cyt } c(II)\text{–}CcP(IV,R^{\bullet+}) \xrightarrow{k_5} \text{cyt } c(III)\text{–}CcP(III,R^{\bullet+}) \quad (5)$$

where F and FH$^{\bullet}$ correspond to the oxidized and semiquinone forms of a free flavin. Reaction 4 corresponds to the formation of a transient complex prior to reduction of the CcP. Determination of the value of k_5 was accomplished as follows. To ensure that reduction of cyt *c*(III) (reaction 3) would not be rate-limiting in the overall mechanism, 5-deazariboflavin semiquinone was used as a reductant since $k_3 = 2 \times 10^9$ M^{-1} s^{-1} (Meyer et al., 1983), which is an order of magnitude larger than reported values for k_4 (Yonetani, 1976; see Table III). Also, since the amount of cyt *c*(II) generated per laser pulse is limited by the amount of 5-DRfH$^{\bullet}$ produced [typically $\leq$0.1 μM (Simondsen & Tollin, 1983)], for CcP concentrations $\geq$10 μM [cyt *c*(II)] $\ll$ [CcP(IV,R$^{\bullet+}$)]. At low concentrations of CcP(IV,R$^{\bullet+}$), k_{obsd} should be approximately equal to k_4[CcP(IV,R$^{\bullet+}$)], whereas $k_{obsd} = k_5$ when CcP(IV,R$^{\bullet+}$) is in such large excess that essentially all the cyt *c*(II) is complexed; i.e., reaction 5 becomes rate-limiting. One would thus expect that plots of k_{obsd} vs [CcP(IV,R$^{\bullet+}$)] would be hyperbolic. Figure 2 shows that, in fact, when cyt *c*(II) is titrated with CcP(IV,R$^{\bullet+}$) at μ = 30 mM, such hyperbolic behavior is observed. The solid line through these points corresponds to the best fit to the data from computer modeling based on the above mechanism (Strickland et al., 1975; Simondsen & Tollin, 1983). Rate constants obtained from the fitted data are given in Table III. It is apparent that, with an increase in ionic strength from 8 to 30 mM, there is a 4-fold increase in the intracomplex electron-transfer rate constant (from 750 s^{-1} to 3300 s^{-1}). The value of k_5 at μ = 30 mM is at least 2 times larger than the values obtained for the covalent complex at either ionic strength. Therefore, it appears that the more efficient electron-transfer complex is that which is formed from the individual components at higher ionic strengths, which implies that the electrostatic interactions that lead to a stable 1:1 complex at very low ionic strengths must be weakened in order to get high rates of electron transfer.[4] The absence of such an ionic strength dependent change in the intracomplex electron-transfer rate constant for the covalent complex is consistent with the view that the covalent linkages prevent the required reorientation of the two proteins within the complex. The fact that k_2 for the covalent complex is larger than the value of k_2 for the electrostatic complex at μ = 8 mM is a

[4] Another explanation that may be proposed for the increase in the intracomplex electron transfer with increasing ionic strength is that the formation of a strong electrostatic complex alters some thermodynamic property of the individual components such as the reduction potential. This would require that the difference in redox potentials be smaller in the electrostatic complex than when the proteins are free in solution or in a transient complex. While no data are available for the effect of either complexation or ionic strength on the reduction potential of CcP, electrostatic complex formation of cyt *c* with *C. pasteurianum* flavodoxin (Hazzard et al., 1986) or with liposomes, submitochondrial particles, and cytochrome *c* oxidase (Nicholls, 1974) results in small decreases of 30–40 mV in $E_{m,7}$. If a similar decrease occurs upon complexation with CcP(IV,R$^{\bullet+}$), then the intracomplex electron-transfer rate would increase. Thus, the kinetic data can only be explained if complexation with CcP results in a marked increase in $E_{m,7}$ for cyt *c*, relative to the free state, or a large decrease in the redox potential for CcP(IV,R$^{\bullet+}$). This seems unlikely, although further work needs to be done to explore the possible contribution of such effects.

Table III: Second-Order and Intracomplex Electron-Transfer Rate Constants for Reduction of the Cyt *c*–CcP(IV,R$^{\bullet+}$) Complexes[a]

complex species	μ (mM)	k_1[b] × 10^{-7} (M^{-1} s^{-1})	k_2 or k_5[b] (s^{-1})
electrostatic	8	3.0[c]	750[c]
	30	–[d]	3220
covalent	8	2.7	1560
	30	2.7	1560

[a] Reactions were performed in phosphate buffers at the given ionic strengths containing 0.5 mM EDTA and 70 μM Lf. [b] Rate constants for the covalent complex at μ = 8 and 30 mM and the electrostatic complex at μ = 8 mM were obtained by using LfH$^{\bullet}$. Data for the electrostatic complex at μ = 30 mM were obtained by using 5-DRfH$^{\bullet}$. [c] This value is from Hazzard et al. (1987a). [d] k_1 value is not obtainable from the data; however, $k_4 = 1.3 \times 10^8$ M^{-1} s^{-1}, which can be compared with the value of 5.6×10^8 M^{-1} s^{-1} reported from steady-state analysis by Yonetani (1976) under somewhat different conditions.

further indication that cross-linking alters the reactivity of the two proteins within the complex, due to either a change in relative orientations, a change in intrinsic reactivity of CcP, and/or changes in net electrostatic charge at the intermolecular interface due to EDC modification.

The present results concerning the effect of ionic strength on the rate constants for electron transfer within the noncovalent complex obtained with horse cyt *c* are in excellent agreement with results obtained previously with yeast iso-1, iso-2, and several site-specific mutants of iso-1 (Cusanovich et al., 1987). We feel confident in asserting, therefore, that the formation of the electrostatically stabilized complex at extremely low ionic strengths does not produce a species in which the intracomplex electron-transfer process is optimized. Rather, only by "loosening" the complex by increasing the ionic strength and diminishing the electrostatic interactions can the two proteins be allowed to reorient themselves to form a complex where rapid rates of electron transfer occur. In that respect, the covalent complex produced at pH 6.0 and at low ionic strength is more like the higher ionic strength electrostatic complex in terms of electron-transfer kinetics. An important question to be investigated is whether covalent cross-linking at higher ionic strengths produces complexes that have electron-transfer rate constants that are even more similar to the transient electrostatic complex.

The ionic strength dependency of k_5 for the electrostatic complexes is consistent with the earlier steady-state kinetic results of Kang et al. (1977) which showed that, for the oxidation of ferrous horse and yeast cytochromes *c*, the rates were highly dependent on ionic strength and that the optimal ionic strengths varied with the species of cytochrome used. It should be noted, however, that the maximum turnover number for horse cytochrome *c* in phosphate buffer reported by Kang et al., 350 s^{-1}, is significantly smaller than the 3300 s^{-1} value for k_5, suggesting that, in the steady-state experiment, dissociation of the first cyt *c* molecule which reduces CcP(IV,R$^{\bullet+}$) may be the rate-limiting step, as suggested by Kang and Erman (1982).

Correlation between the Kinetic Data, the Poulos–Kraut Hypothetical Model, and Proposed Sites of Covalent Cross-Linking. Panels a and b of Figure 3 show side and top views, respectively, of the Poulos–Kraut hypothetical complex (Poulos & Kraut, 1980) with two covalent cross-links, *cyt c Lys-13 to CcP Asp-37* and *cyt c Lys-86 to CcP Glu-35*, as suggested by Waldmeyer and Bosshard (1985) (Asp-37 and Glu-35 were not unambiguously identified by Waldmeyer and Bosshard to be the specific acidic residues involved in cross-linking). Note that the two covalent cross-links are located on the side of the cyt *c* heme which is less accessible to solvent. Thus, our results with LfH$^{\bullet}$ reduction of the covalent and electrostatic complexes are consistent with the location of these cross-links, inasmuch as they would not be expected to appreciably change heme accessibility within the complex. The kinetic experiments with FMNH$^{\bullet}$ indicate that covalent cross-linking results in net neutralization of charge near the cyt *c* heme edge. This suggestes that there may be additional sites of covalent modification, either intra- or intermolecular in nature. Two possibilities, which would satisfy this requirement, are *CcP Lys-183 to CcP Asp-217* or *cyt c Lys-79 to CcP Asp-217*. Cross-links at either location would not be expected to significantly change cyt *c* heme accessibility but would neutralize electrostatic charge. Furthermore, an additional interprotein cross-link at the bottom of the interface region might help to account for the fact that reduction of cyt *c* in the covalent complex with CcP(IV,R$^{\bullet+}$) shows no ionic strength dependence. In the absence of the additional interprotein cross-link, it would be expected that an increase in ionic strength would lead to destabilization of electrostatic interactions near the cyt *c* heme edge which could, in turn, result in an increase in steric accessibility of the cyt *c* heme. Further work is necessary to substantiate the possibility for additional covalent cross-links.

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Purification and Characterization of Two Types of NADH-Quinone Reductase from *Thermus thermophilus* HB-8†

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ABSTRACT: Two types of the NADH-quinone reductase were isolated from *Thermus thermophilus* HB-8 membranes, by use of the nonionic detergent, dodecyl β-maltoside, and NAD-agarose affinity, DEAE-cellulose, hydroxyapatite, and Superose 6 column chromatography. One of these (NADH dehydrogenase 1) is a complex composed of 10 unlike polypeptides, and the other (NADH dehydrogenase 2) exhibits a single band (M_r 53 000) upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The NADH-ubiquinone-1 reductase activity of the isolated NADH dehydrogenase 1 was about 14 times higher than that of the dodecyl β-maltoside extract and partially rotenone sensitive. The NADH-ubiquinone-1 reductase activity of the isolated NADH dehydrogenase 2 was about 30-fold as high as that of the dodecyl β-maltoside extract and rotenone insensitive. The purified NADH dehydrogenase 1 contained noncovalently bound FMN, non-heme iron, and acid-labile sulfide. The ratio of FMN to non-heme iron to acid-labile sulfide was 1:11-12:7-9. The high content of iron and labile sulfide is suggestive of the presence of several iron-sulfur clusters. The purified NADH dehydrogenase 2 contained noncovalently bound FAD and no non-heme iron or acid-labile sulfide. The activities of both NADH dehydrogenases were stable at temperatures of ≥80 °C. The occurrence of two distinct types of NADH dehydrogenase as a common feature in the membranes of various aerobic bacteria is discussed.

Among the enzyme complexes that comprise the mitochondrial oxidative phosphorylation system, the least studied is the NADH-ubiquinone oxidoreductase complex (complex I). The reason is perhaps associated with the fact that this enzyme has a highly complex structure. It is comprised of ≥25 unlike polypeptides (Heron et al., 1979; Hatefi, 1985) and eight iron-sulfur (FeS) clusters (Ohnishi et al., 1985; Hatefi, 1985). Three of the FeS clusters are EPR[1]-silent, and one has an apparent E_m of about −400 mV (Hatefi et al., 1985), leaving four clusters that can be reduced by NADH. The NADH-Q reductase segment of aerobically grown *Paracoccus denitrificans* membranes contains coupling site 1 (Stouthamer, 1980) and exhibits NADH-reducible EPR signals similar to those of the mitochondrial complex I (Albracht et al., 1980; Meinhardt et al., 1987). However, by comparison to mammalian complex I, the NADH dehydrogenase complex from *Paracoccus* membranes is composed of only 10 unlike polypeptides (Yagi, 1986), suggesting that the *Paracoccus* NADH dehydrogenase complex is simpler than its mammalian counterpart in terms of its polypeptide composition.

Thermus thermophilus HB-8, isolated from a hot spring in Japan, is a strictly aerobic and extremely thermophilic bacterium [Oshima & Imahori, 1971, 1974; also see Fee et al. (1986)]. The respiratory chain of *T. thermophilus* HB-8 contains the energy coupling site 1 (Ohnishi et al., 1987), and its NADH-Q reductase activity is sensitive to rotenone and piericidin A (Yagi, 1987; Ohnishi et al., 1987). In addition,

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[1] Abbreviations: EPR, electron paramagnetic resonance; E_m, oxidation-reduction potential; Q, quinone; NDH-1, NADH dehydrogenase 1; NDH-2, NADH dehydrogenase 2; EDTA, ethylenediaminetetraacetate; Q_1, ubiquinone 1; deamino-NAD, nicotinamide hypoxanthine dinucleotide; TCD buffer, buffer composed of 10 mM Tris-HCl (pH 7.5) and 0.1% dodecyl β-maltoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; M_r, molecular weight estimated from relative mobility in SDS-PAGE; TLC, thin-layer chromatography; R_f, relative mobility; NMR, nuclear magnetic resonance; DEAE-cellulose, (diethylaminoethyl)cellulose; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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