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Effects of Amino Acid Replacements in Yeast Iso-1 Cytochrome *c* on Heme Accessibility and Intracomplex Electron Transfer in Complexes with Cytochrome *c* Peroxidase†

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ABSTRACT: The kinetics of reduction of wild type and several site-specific mutants of yeast iso-1 cytochrome *c* (Arg-13 → Ile, Gln-16 → Ser, Gln-16 → Lys, Lys-27 → Gln, Lys-72 → Asp), both free and in 1:1 complexes with yeast cytochrome *c* peroxidase, by free flavin semiquinones have been studied. Intramolecular one-electron transfer from the ferrous cytochromes *c* to the H_2O_2-oxidized peroxidase at both low (8 mM) and high (275 mM) ionic strengths was also studied. The accessibility of the cytochrome *c* heme within the electrostatically stabilized complex and the rate constants for intramolecular electron transfer at both low and high ionic strength are highly dependent on the specific amino acids present at the protein–protein interface. Importantly, replacement by uncharged amino acids of Arg or Lys residues thought to be important in orientation and/or stabilization of the electron-transfer complex resulted in increased rates of electron transfer. In all cases, an increase in ionic strengths from 8 to 275 mM also produced increased intramolecular electron-transfer rate constants. The results suggest that the electrostatically stabilized 1:1 complex is not optimized for electron transfer and that by neutralization of key positively charged residues, or by an increase in the ionic strength thereby masking the ionic interactions, the two proteins can orient themselves to allow the formation of a more efficient electron-transfer complex.

Both biological energy transduction and much of intermediary metabolism ultimately depend on the control of simple one electron transfer reactions between proteins. Therefore, understanding the factors that govern the rate and specificity of protein to protein electron transfer is a matter of intense current research (Cusanovich et al., 1987a; Marcus & Sutin, 1985; McLendon et al., 1985; Scott et al., 1985).

One particularly useful paradigm for such studies is provided by the reduction of peroxide-oxidized cytochrome *c* peroxidase [commonly referred to as either ferryl peroxidase, compound I, "ES", or CcP(IV,R$^{\bullet+}$)] by ferrous cytochrome *c* [cyt*c*(II)].[1] This reaction is assumed to be involved in peroxide detoxification in yeast, utilizing cyt *c*(II) and CcP(IV,R$^{\bullet+}$) as natural redox partners. These two proteins are particularly well suited for detailed studies of protein to protein electron transfer for several reasons:

(1) The crystal structure of each protein is known at high resolution for various oxidation states (Poulos et al., 1980; Edwards et al., 1987; Swanson et al., 1976; Takano & Dickerson, 1981; Louie et al., 1988).

(2) Cyt *c* and CcP form a thermodynamically well characterized complex (Nicholls & Mochan, 1971; Mochan & Nicholls, 1971; Erman & Vitello, 1980), and a detailed hypothetical model has been proposed for the relative orientations of cyt *c* and CcP within this electron-transfer complex (Poulos & Kraut, 1980; Koppenol & Margoliash, 1982; Poulos & Finzel, 1984) although recent efforts to cocrystallize the two proteins have led to ambiguous results (Poulos et al., 1987). A number of specific ionic interactions within the complex have been suggested by chemical modification and/or covalent cross-linking measurements (Kang et al., 1977, 1978; Waldmeyer & Bosshard, 1985).

(3) Photochemical techniques have been developed which permit detailed single-turnover kinetic measurements of the reaction cyt *c*(II)·CcP(IV,R$^{\bullet+}$) → cyt *c*(III)·CcP(III,R$^{\bullet+}$) (Hazzard et al., 1987, 1988a,b).

(4) Numerous amino acid replacements of yeast cyt *c* (Hampsey et al., 1986; Pielak et al., 1985; Liang et al., 1987) as well as several site-specific mutants of CcP (Goodin et al., 1986; Fishel et al., 1987; Cusanovich et al., 1988; Mauro et

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[1] Abbreviations: cyt *c*(II) and cyt *c*(III), ferrous and ferric species of cytochrome *c*; CcP(III) and CcP(IV,R$^{\bullet+}$), ferric and H_2O_2-oxidized species of cytochrome *c* peroxidase; LfH$^{\bullet}$ and LfH^{-}, semiquinone and fully reduced lumiflavin species; 5-DRf and 5-DRfH$^{\bullet}$, oxidized and semiquinone species of 5-deazariboflavin; EDTA, ethylenediaminetetraacetic acid; Ser-16 etc., altered iso-1 cytochromes *c* with Ser-16 etc. replacement (see Table I).

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al., 1988) have been developed. Thus, detailed studies of the interaction between cyt *c* and CcP can be carried out with genetic techniques to modify either the interaction domain, residues involved in the formation of and/or stabilization of the free-radical species, $R^{\bullet+}$, or residues believed to be important in the electron-transfer pathway.

The ongoing development of the latter two areas in our laboratories has allowed us to investigate the effect of single amino acid replacements within cyt *c* on the reactivity of this protein with CcP. These studies show that single amino acid replacement of evolutionarily invariant residues, which are supposedly involved in binding of cyt *c*, can have significant effects on the processes controlling the rates of electron transfer between cyt *c* and CcP in either electrostatically stabilized or transiently formed 1:1 complexes.

Materials and Methods

Cytochrome *c* peroxidase was prepared from bakers' yeast (Red Star) and characterized as described in Hazzard et al. (1987). CcP(IV,$R^{\bullet+}$) was produced by titration of CcP(III) with a buffered 5 mM H_2O_2 solution. Cyt *c*(III) concentration was determined with $\epsilon_{412} = 118\ mM^{-1}\ cm^{-1}$. Preparation of lumiflavin was as previously described (Simondsen & Tollin, 1983). 5-DRf was the generous gift of Drs. Thomas P. Singer and William McIntire.

Yeast strains were grown, and the iso-1 cytochromes *c* were extracted and purified by the methods described by Sherman et al. (1968). The five altered proteins used in this study (Table I) were chosen from published (Hampsey et al., 1986) and unpublished collections of iso-1 cytochromes *c* having single amino acid replacements. The Ser-16 and Lys-16 replacements were previously determined by protein analysis (Sherman et al., 1974; J. W. Stewart and F. Sherman, unpublished results). The Ile-13 and Gln-27 replacements were uncovered among, respectively, *cyc1-75* and *cyc1-177* revertants. These Ile-13 and Gln-27 replacements, as well as others in the series, were determined by retrieving and sequencing the DNA regions encompassing the alterations, according to the procedures described by Hampsey et al. (1986). Total yeast DNA was isolated and digested with *Eco*RI and *Kpn*I restriction endonucleases, and the digested DNA was fractioned by electrophoresis on 2% low-melting agarose gels. The DNA fragments approximately 240 base pairs long were excised from the gel and inserted into the *Eco*RI and *Kpn*I sites of bacteriophage M13 mp19. The bacteriophages containing the *CYC1* inserts were detected by hybridization to the radiolabeled synthetic probe OL-9, and the region containing the alterations was subsequently sequenced by standard procedures.

The Asp-72 replacement was constructed by site-directed mutagenesis, with the bacteriophage M13-CYC1, and the two-primer method of Zollar and Smith (1984) and other methods described by Holzschu et al. (1987). The resulting *CYC1-785* gene, containing the Asp-72 replacement, was inserted into the yeast strain B-6748, with the plasmid pAB108 and according to the procedures described by Holzschu et al. (1987) (T. S. Cardilo and F. Sherman, unpublished results).

Reaction kinetics were measured under two ionic strength conditions. A 3 mM phosphate buffer at pH 7 containing 0.5 mM EDTA was used for low ionic strength studies (μ = 8 mM), and a 100 mM phosphate buffer containing 25 mM EDTA at pH 7 was used for high ionic strength conditions (μ = 275 mM).

Laser flash photolysis reduction of free flavins was carried out as described previously (Hazzard et al., 1987, 1988a). The free flavin concentration for the kinetic studies was 80 μM; under these experimental conditions, the concentration of flavin semiquinone generated by each laser flash was $\leq$0.1 μM (Simondsen et al., 1982). The oxidized protein concentration for concentration dependence studies was always $\geq$5 μM; thus, all experiments were carried out under pseudo-first-order conditions.

Table I: Altered Forms of Iso-1 Cytochrome *c*[a]

strain	gene symbol and amino acid and codon changes			replacement[b]	ref[c]
D-334	CYC1+	cyc1-2	CYC1-2-C	Ser-16	1
	Gln-21	End-21	Ser-21		
	CAA	UAA	UCA		
B-609	CYC1+	cyc1-2	CYC1-2-I	Lys-16	1
	Gln-21	End-21	Lys-21		
	CAA	UAA	AAA		
B-6591	CYC1+	cyc1-75	CYC1-75-F	Ile-13	2
	Arg-18	End-18	Ile-18		
	AGA	UGA	AUA		
B-1877	CYC1+	cyc1-177	CYC1-177-A	Gln-27	2
	Lys-32	End-32	Gln-32		
	AAG	UAG	CAG		
B-6842	CYC1+		CYC1-785	Asp-72	3
	Lys-77	site	Asp-77		
	AAG	directed	GAU		

[a] CYC1+ is the wild-type yeast iso-1 cytochrome *c*. [b] Residue numbers are based upon tuna cytochrome *c*. Yeast iso-1 cytochrome *c* has five additional residues at the N-terminus. [c] References: (1) Sherman and Stewart (1974); (2) this paper; (3) T. S. Cardillo and F. Sherman (unpublished results).

In the determination of rate constants for reduction of cyt *c* by flavin semiquinone in the complex with CcP(III), as well for intracomplex reduction of CcP(IV,$R^{\bullet+}$) at low ionic strength, a mole ratio of 1:1 between the two proteins was maintained throughout the entire concentration range. Experiments involving reduction of CCP(IV,$R^{\bullet+}$) at high ionic strength using 5-DRF were performed in the following manner. Cyt *c* was held constant at 30 μM while the CcP(IV,$R^{\bullet+}$) concentration was increased from an initial value of 10 μM. 5-DRf concentration was 100 μM. Free CcP(IV,$R^{\bullet+}$) is not reduced by 5-DRfH$^{\bullet}$ on a time scale relevant to the flash photolysis experiment (Hazzard et al., 1987). However, in the presence of oxidized cyt *c*, reduction of ferryl peroxidase by the flavin semiquinone occurs via the prior reduction of cyt *c* followed by reduction of the ferryl peroxidase by cyt *c*(II). Utilization of the highly reactive 5-DRfH$^{\bullet}$ as the reductant ensures that the initial reduction of cyt *c* is not rate limiting for the ultimate reduction of CcP(IV,$R^{\bullet+}$). The reduction kinetics of free and CcP(III)-complexed cyt *c* were monitored at 580 nm, whereas CcP(IV,$R^{\bullet+}$) reduction was followed at 550 nm (Hazzard et al., 1987).

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 docked complex were generously provided by Dr. Thomas L. Poulos.

Results

We have elected to investigate the five altered iso-1 cyt *c* species shown in Table I. Figure 1 shows the placement of these residues within the Poulos–Kraut hypothetical model, the importance of which shall be discussed below in more detail.[2] The Ile-13,[3] Gln-27, and Asp-72 proteins are re-

[2] The hypothetical complex has been generated with the coordinates from tuna cytochrome *c* and yeast CcP(III) (Poulos & Kraut, 1984; Poulos & Finzel, 1984). Comparisons between the absolute structures of the yeast and tuna cytochromes *c* must await the availability of high-resolution coordinates for the yeast cytochrome.

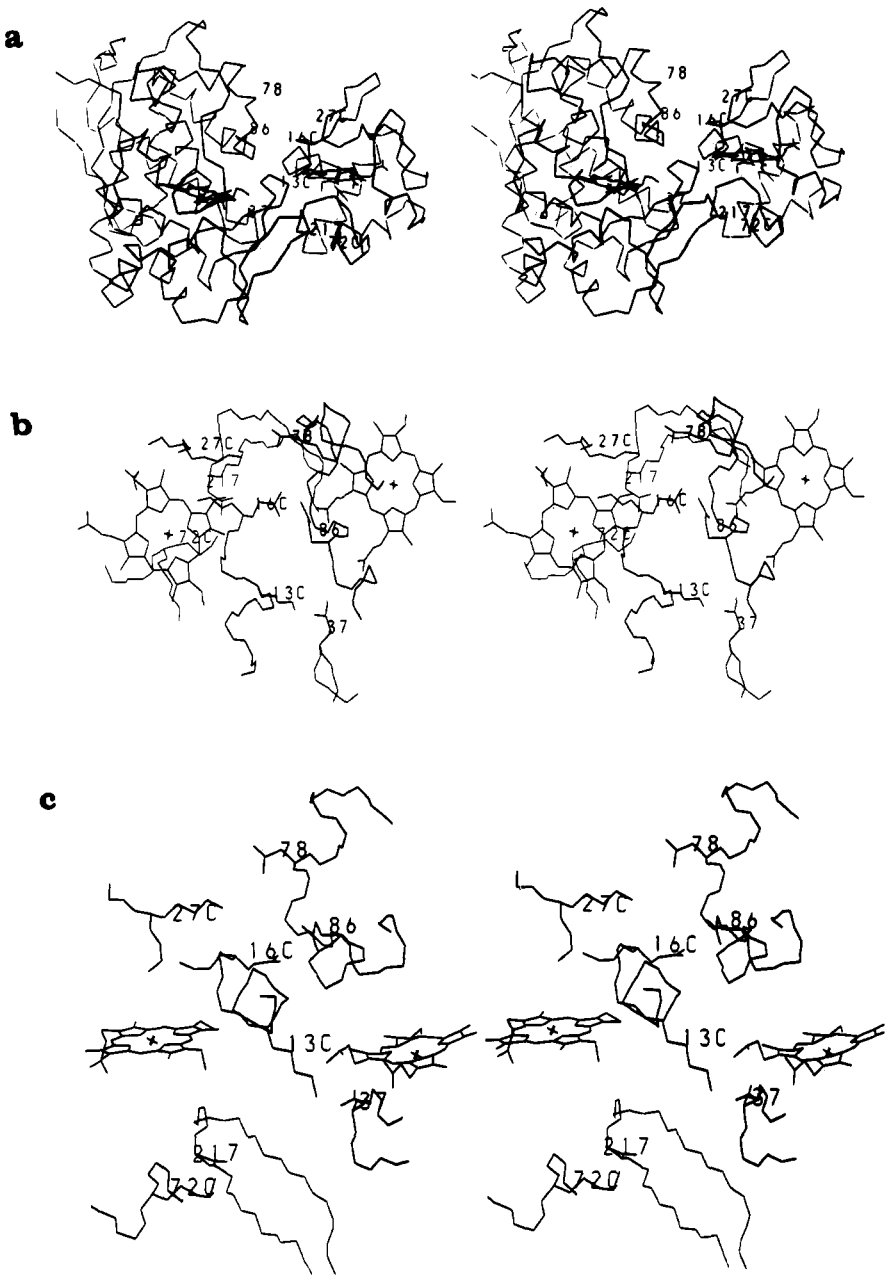

FIGURE 1: Computer graphic representations of the Poulos–Kraut model for the cyt *c*·CcP complex. Sites of specific amino acid replacement are indicated on cyt *c*. (a) Side view of the complex showing the orientation of cyt *c* (right) relative to CcP (left). (b) Closeup perspective of the interaction domain viewed from above. Note that (b) is related to (a) by a 180° rotation about a vertical axis between the two proteins. (c) Closeup oblique view of the interaction domain shown in (b).

placements of basic Lys and Arg residues that are supposedly important in the binding of cyt *c* to CcP. The Ser-16 and Lys-16 replacements occur at the surface of the cytochrome, presumably within the complex interaction domain, and residue 16 (Gln in wild type) has been suggested to affect electron transfer to cytochrome *c* from free flavin semiquinones by means of steric interactions (Meyer et al., 1984; Cusanovich et al., 1987a).

By utilization as a reductant of the small, electrostatically neutral lumiflavin semiquinone, $LfH^{\bullet}$, we can determine the relative accessibilities of the prosthetic groups of free and complexed redox proteins (Meyer et al., 1983; Hazzard et al., 1986, 1987, 1988a; Cusanovich et al., 1988). In cases where accessibility is very small, disproportionation of the flavin semiquinone to the fully reduced species, LfH^{-}, occurs. This can then react with the redox proteins, albeit with rate constants of significantly smaller magnitude than those obtained with the semiquinone species (Meyer et al., 1983). Second-order rate constants for the reduction of the wild types and of five mutant yeast cytochromes *c* in the free state by $LfH^{\bullet}$ are given in Table II. Free iso-2 cyt *c* is significantly more reactive toward $LfH^{\bullet}$ than is iso-1 cyt *c*, whereas all of the iso-1 mutants have essentially the same reactivity as does the wild type. Upon the formation of electrostatically stabilized 1:1 complexes with CcP(III) at low ionic strength (μ = 8 mM), large decreases in cyt *c* heme reactivity with $LfH^{\bullet}$ are observed [we have previously established (Hazzard et al., 1987) that free CcP(III) is not measurably reactive with $LfH^{\bullet}$]. The second-order reduction rate constants are presented in Table II. In the case of the complexed iso-1 cyt *c*, at the concen-

[3] The amino acid sequences of yeast iso-1 and iso-2 cytochromes *c* vary from those of horse or tuna cytochromes *c* by the presence of five or nine amino acids, respectively, at the N-terminus. For the sake of convenience, we have chosen the numbering system of the vertebrate cytochromes *c* in order to designate the locations of the mutation sites.

Table II: Rate Constants for Reduction of Free and Complexed Cytochromes by Reduced Lumiflavin Species[a]

	k_2 ($\times 10^{-7}$ M^{-1} s^{-1})			
		complexed		
		CcP(III)[b]		CcP(IV,R$^{\bullet+}$)[c]
cytochrome	LfH$^{\bullet}$	LfH$^{\bullet}$	LfH^{-}	LfH$^{\bullet}$
iso-1 (wild type)	3.5 ± 0.2	<0.5	0.1	2.4
iso-2	5.3[d]	0.5[d]	0.2	3.6
Ser-16	3.4 ± 0.1	<0.5	0.1	2.5
Lys-16	3.4 ± 0.3	<0.5	0.3	1.1
Ile-13	4.1 ± 0.2	1.4		4.1
Gln-27	4.1 ± 0.2	0.7		3.5
Asp-72	3.0 ± 0.2	<0.5		3.5

[a] Experiments were performed in a 3 mM phosphate buffer containing 0.5 mM EDTA and 50 μM lumiflavin (μ = 8 mM). [b] LfH$^{\bullet}$ values determined at a single protein concentration (30 μM); LfH^{-} values determined at several protein concentrations. [c] Rate constants for LfH$^{\bullet}$ reduction determined at a single protein concentration (30 μM). [d] Rate constants from Hazzard et al. (1987).

Table III: Intramolecular Rate Constants for Electron Transfer from Reduced Cytochromes *c* to CcP(IV, R$^{\bullet+}$)

cytochrome	k_2 (s^{-1}) (μ = 8 mM)[a]	k_5 (s^{-1}) (μ = 260 mM)[b]
iso-1 (wild type)	260[c] 230[d]	1460
iso-2	150[e]	1480
Ser-16	200	1350
Lys-16	100	600
Ile-13	1000	2240
Gln-27	260	780
Asp-72	440	

[a] Determined at a single protein concentration with lumiflavin semiquinone as the reductant. It has been shown that the intramolecular electron-transfer rate constant is not dependent on the flavin species utilized at low ionic strength. [b] Determined from nonlinear least-squares fits to the data based on the mechanism given by 3–5 represented as solid lines in Figure 2 (Hazzard et al., 1987b). [c] Measured at 30 μM in each component. [d] Measured at 50 μM in each component. [e] Value from Hazzard et al. (1987).

trations used, the reaction of LfH$^{\bullet}$ with the cytochrome is not able to compete with LfH$^{\bullet}$ disproportionation, and therefore the cyt *c* is not reduced by the semiquinone species. The complexed cyt *c* is, however, measurably reduced by the fully reduced flavin, LfH^{-} ($k = 1 \times 10^6$ M^{-1} s^{-1}). Under comparable conditions, CcP(III)-complexed iso-2 cyt *c* does react with LfH$^{\bullet}$, although the rate constant for this reaction is at the borderline of measurability for this flavin species with our techniques [$k \sim 5 \times 10^6$ M^{-1} s^{-1}; cf. Hazzard et al. (1987)]. Consistent with the greater reactivity of CcP(III)-complexed iso-2 cyt *c* is the fact that the rate constant for reduction by LfH^{-} is twice that of complexed iso-1. We conclude from these data that complexation of yeast iso-1 and iso-2 cyt *c* by CcP(III), at low ionic strength, markedly decreases the accessibility of reduced lumiflavin species to the exposed cyt *c* heme edges.

The mutant iso-1 cytochromes *c* with Ser-16, Lys-16, and Asp-72 substitutions are kinetically similar to the wild-type iso-1 and iso-2 cytochromes in terms of heme *c* accessibility within the CcP(III) complex (k < 10% of the uncomplexed rate constant). Thus, these substitutions have little effect on the change in heme accessibility. In contrast, the Ile-13 and Gln-27 mutant cytochromes are apparently more reactive than the wild type in the CcP(III) complex (15–30% of the uncomplexed rate constant for LfH$^{\bullet}$ reduction). For these two mutants, the increases in the reduction rate constants for the complexed cytochromes relative to the normal iso-1 cyt *c* value are significantly larger than the essentially negligible differences in the second-order rate constants for the free proteins. Thus, these mutations must result in a greater degree of exposure of the cyt *c* heme within the complex than is found for the complexed wild-type cytochrome. This suggests that there are significant differences in the steric environments near the lumiflavin interaction domain for the Ile-13 and Gln-27 mutants, presumably resulting from variations in the structure of the mutant cyt *c*·CcP(III) complex.

As is also shown in Table II when all of the yeast cytochromes are complexed to CcP(IV,R$^{\bullet+}$) at low μ, the accessibilities of the heme *c* edges to LfH$^{\bullet}$ are either equal to or somewhat less than those of the free cytochromes. Thus, the hemes of the normal iso-1 and iso-2 cytochromes as well as the Ser-16 mutant are ~70% as accessible to LfH$^{\bullet}$ as in the free state, the Ile-13, Gln-27, and Asp-72 mutants are ~100% as reactive, and the Lys-16 mutant is ~30% as reactive as the uncomplexed cytochromes. The above results emphasize two important features of the cyt *c*·CcP complex. First, alterations of single specific cyt *c* side chains can affect cyt *c* heme accessibility and by inference the geometric orientation of the two proteins within the complex. Second, upon peroxide oxidation of CcP(III) to the ferryl species, changes in the structure and/or conformation of the peroxidase must occur, which also alter the degree of cyt *c* heme accessibility within the complex.

We have previously demonstrated (Hazzard et al., 1987, 1988a,b) that it is possible with laser flash photolysis to directly measure the rate constant for intracomplex one-electron transfer between tuna, horse, and yeast iso-1 and iso-2 cyt *c*(II) and CcP(IV,R$^{\bullet+}$) at low and high ionic strengths. Depending upon the ionic strength, two mechanisms have been considered [this is discussed in more detail in Hazzard et al. (1988a)]. At low ionic strength (μ < 10 mM) the following sequence occurs:

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

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

where FH$^{\bullet}$ and F represent semiquinone and oxidized flavin, respectively, and cyt *c*·CcP represents an electrostatically stabilized complex. At higher ionic strength, the mechanism becomes

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

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

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

where reaction 4 represents the formation of a transient electron-transfer complex, which for reasons to be discussed below may not be the same as the electrostatic complex.

At μ = 8 mM, both iso-1 and iso-2 cytochromes *c* give similar intracomplex electron-transfer rate constants, $k_2 \sim 200$ s^{-1}, as shown in Table III. The Ser-16 and Gln-27 mutants yield approximately the same value for the rate constant as does the wild type, whereas the Lys-16 mutant is somewhat slower. The Ile-13 and Asp-72 mutants have rate constants which are appreciably greater than those obtained with the wild type. We have previously reported that the limiting first-order rate constants (k_5) for CcP(IV,R$^{\bullet+}$) reduction by iso-1 and iso-2 cyt *c*(II) (Hazzard et al., 1988b), as well as horse cyt *c*(II) (Hazzard et al., 1988a), at high ionic

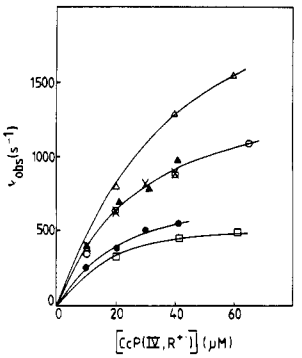

FIGURE 2: Plots of k_{obsd} vs CcP concentration for reduction of CcP(IV,R•+) by yeast cytochromes *c*(II) at high ionic strength. Symbols correspond to the various cytochrome species as follows: (△) Ile-13, (○) iso-1, (▲) iso-2, (×) Ser-16, (●) Gln-27, and (□) Lys-16. Ferric cytochrome *c* concentrations were 30 μM. Solid lines correspond to theoretical curves generated by nonlinear least-squares fits to the data (see text). CcP(IV,R•+) reduction was monitored at 550 nm. Reaction solutions were prepared in 100 mM phosphate buffer containing 25 mM EDTA at pH 7. 5-DRf concentration was 100 μM.

strengths were appreciably larger than the low μ values (k_2) for the intracomplex electron-transfer process. We have now carried out these same measurements with the iso-1 cyt *c* mutants, the results of which are shown in the plots of k_{obsd} vs CcP(IV,R•+) concentration in Figure 2, and the values for k_5 are given in Table III. In all cases, there is a significant increase in the limiting first-order rate constants with an increase in ionic strength. The Ser-16 mutant is identical with the wild type, whereas the Gln-27 and Lys-16 mutants have substantially smaller limiting rate constants, and the Ile-13 mutant has a much larger value. It is quite clear from these results that, without exception, the structure of the low ionic strength complex is less favorable for intracomplex electron transfer than is the case for the transient complex formed upon collision at high ionic strength. Furthermore, the Ile-13 mutant cyt *c* is a *better* electron-transfer reactant than is the wild type, at both low and high ionic strength.

Discussion

The present experiments have demonstrated that the various forms of yeast cyt *c* which we have studied yield a range of kinetic properties when they are complexed to CcP in terms of both cyt *c* heme accessibility and intramolecular electron transfer. Thus, it is of substantial interest to analyze these kinetic results in terms of known structural features of the individual components of the cyt *c*-CcP complex. One problem that arises is that no structural coordinates are yet available for yeast iso-2 cyt *c*. Although a hypothetical model for the cyt *c*-CcP complex, shown in Figure 1 with tuna cyt *c* and yeast CcP(III) coordinates has been proposed (Poulos & Kraut, 1980; Poulos & Finzel, 1984), it has been recently reported that cocrystallization of cyt *c* and CcP resulted in the inability to determine the orientation of cyt *c*, relative to CcP, presumably due to a high degree of disorder of cyt *c* within the crystal lattice (Poulos et al., 1987). Whereas this lack of definitive structural information introduces uncertainties into the analysis of the kinetic data, computer modeling does provide some guidance.

The present kinetic results provide four principal points to be addressed: (1) the differences in reactivity of free yeast iso-1 and iso-2 cytochromes toward lumiflavin semiquinone; (2) the change in accessibilities of the cytochrome hemes upon oxidation of CcP(III) by H_2O_2; (3) the effects of amino acid replacements on the heme accessibilities of the free and complexed cytochromes; (4) the differences in the intracomplex electron-transfer rate constants for the various mutant cytochromes with respect to iso-1 cyt *c*.

Accounting for the different reactivities of free iso-1 and iso-2 cytochromes with LfH• is difficult. The magnitude of the rate constant differences, although small, is well outside the experimental error and suggests that iso-1 and iso-2 cytochromes have significantly different heme accessibilities (i.e., steric constraints), since the redox potentials are identical and electrostatic interactions are not involved (LfH• is uncharged at pH 7) (Meyer et al., 1984). In the immediate region of the exposed heme edge, the amino acid sequences of the two yeast isocytochromes *c* are very similar (the principal regions of the sequence near the heme edge involve positions 8–17, 27–30, and 79–87, with position 72 somewhat more removed). The only differences are at positions 15 (Leu vs Gln) and 83 (Gly vs Ala) for iso-1 and iso-2, respectively. Moreover, the mutants studied provide no guidance, inasmuch as they all give rate constants with LfH• which are essentially the same as that of the iso-1 wild type. Thus, it must be concluded that some backbone relocation occurs in the iso-1 cytochrome relative to iso-2, which alters the steric constraints in the region of the exposed heme edge. A more definitive resolution of this question will have to await highly refined X-ray structural comparisons of iso-1 and iso-2 yeast cytochromes *c*.

As described above, the accessibilities of all of the cyt *c* hemes are quite different when they are complexed at low ionic strength with the CcP(III) and CcP(IV,R•+) redox states. The rate constants for reduction of CcP(IV,R•+)-complexed iso-1 and iso-2 cytochromes *c* increase by 5–10-fold, relative to those of the CcP(III)-complexed species. This suggests that formation of CcP(IV,R•+) causes a conformation change at the cyt *c*-CcP interface which makes the cyt *c* heme significantly more accessible. Recently, Edwards et al. (1987a) have reported the crystal structure of CcP(IV,R•+). A number of backbone structural changes occur when this peroxidase species is formed from CcP(III), including a twisting of a loop consisting of residues 175–190, which has the geometry of an antiparallel β-sheet. This loop contains Leu-182 which in the hypothetical model (Poulos & Kraut, 1980; Poulos & Finzel, 1984) interacts with position 81 of cyt *c*. Furthermore, in the model the loop is quite close to the sulfur atom in the thioether bridge formed between Cys-17 and the heme of cyt *c*, which has been suggested to be involved in electron transfer into the cyt *c* heme (Tollin et al., 1986). Thus, it is possible that on formation of CcP(IV,R•+) the 175–190 loop reorientation alters the interaction of CcP with cyt *c* in such a way that the cyt *c* heme becomes more accessible to LfH•.

In terms of the iso-1 cytochrome mutants studied, it is clear that the hemes of the Ile-13 and Gln-27 mutants are somewhat more accessible (>2-fold) than that of the wild type in the CcP(III) complex, suggesting that the loss of electrostatic interactions involving these residues results in a reorientation of cyt *c* within the complex. Interestingly, the chemical modification studies reported to date support an important electrostatic role for Lys-27 (Kang et al., 1978). An electrostatic interaction between Lys-13 of tuna cyt *c* (Arg in yeast) and Asp-37 of CcP has been proposed from the computer modeling. However, an electrostatic interaction involving Lys-27 is not apparent in the model, suggesting either a somewhat different complex structure in solution or a perturbation of the electrostatic field at the cyt *c*-CcP interaction domain resulting from replacement of Lys-27 by Gln which alters the complex structure.

In the case of the cyt *c*-CcP(IV,R•+) complexes, the situation with the mutants is clearer since the actual rate constants for

$LfH^{\bullet}$ reduction of cyt *c* could be measured in all cases (not just an upper limit). Substitution of Gln-16 with Ser has no effect, whereas substitution of the positively charged groups at positions 13, 27, and 72 all result in a somewhat increased accessibility of the cyt *c* heme, consistent with an alteration of the cyt *c* surface electrostatic potential leading to apparently similar complexes in terms of heme accessibility. In contrast, substitution of Gln-16 by Lys results in a decreased heme accessibility in the cyt *c*·CcP(IV,$R^{\bullet+}$) complex, suggesting that this extra positive charge results in a complex which is distinct from that of the wild type.

Intracomplex electron transfer is also quite sensitive to the precise interactions between cyt *c* and CcP. The iso-1 and iso-2 cytochromes and the Ser-16 mutant have essentially the same intracomplex electron-transfer rate constant at both ionic strengths studied, which is consistent with their similar cyt *c* heme accessibilities. The results obtained with the Ile-13 mutant cast serious doubt on the importance of Lys-13 (or Arg in yeast cytochrome) in the proposed electron-transfer pathway (Poulos & Kraut, 1980; Poulos & Finzel, 1984). In the hypothetical model, Lys-13 forms a salt bridge with Asp-37 of CcP. As a result of this ionic interaction, His-181 of CcP, which in the crystal structure of free CcP is hydrogen bonded to Asp-37, has increased freedom of motion and plays a key role as an electron-transfer conduit into the peroxidase. On the basis of this model, it would be reasonable to presume that converting this lysine (or arginine) to isoleucine would have a deleterious effect on the electron-transfer rate constant. To the contrary, the rate constant increases at low ionic strength with the Ile-13 substitution, suggesting that when the Lys-13 (or Arg)–Asp-37 electrostatic interaction is lost, the complex can more easily adopt a structure in which the heme-to-heme distance and/or the orientation is improved in terms of the kinetics of electron transfer. The rate constant for intracomplex electron transfer is also larger at high ionic strength for the Ile-13 substitution as compared to that of the wild-type, but the relative increase is smaller ($k_{Ile}/k_{Arg} \sim 1.6$ at $\mu = 260$ mM vs 4 at $\mu = 8$ mM). Kang et al. (1977) first noted that, for the steady-state turnover of CcP(IV,$R^{\bullet+}$) in the presence of horse and iso-1 cytochromes *c*, maximal turnover rates in phosphate buffer were obtained at intermediate ionic strengths of 50 and 200 mM. At lower ionic strengths, the decrease in the activity was attributed to the rate-limiting effect of the dissociation of the oxidized cyt *c*. Similarly, we have recently pointed out (Hazzard et al., 1988a,b) that the increase in the limiting rate constant for intracomplex electron transfer which occurs at high ionic strength for horse, iso-1, and iso-2 cytochromes apparently results from the masking of electrostatic interactions, which allows the complex to reach a more favorable conformation for electron transfer than at low ionic strength. Consistent with this idea is the finding that when horse cyt *c* was covalently cross-linked with the ferryl CcP, no increase in intramolecular electron transfer occurred with an increase in ionic strength from 8 to 30 mM, whereas in the noncovalent complex the rate constant increased 4.4-fold (Hazzard et al., 1988a). Thus, the increased rate constants obtained with the Ile-13 substitution suggest that Lys or Arg at this position impedes the conformational rearrangement associated with electron transfer. Although the Lys to Asp substitution at position 72 has only been studied at low ionic strength, it also leads to an increased rate constant for intracomplex electron transfer, consistent with formation of a more flexible complex, which readily rearranges to a more active form. On the other hand, at high ionic strength both the Lys-16 and Gln-27 substitutions result in a 2–3-fold decreased rate constant for intramolecular electron transfer. Thus, in these cases a less favorable interaction is obtained.

The results described above raise several interesting questions about the currently accepted criteria utilized in computer modeling of protein–protein electron-transfer complexes first developed by Salemme (1976). It has been generally accepted that electrostatic forces act to orient and to stabilize two proteins within an intermediate complex in a manner which is favorable to the electron-transfer event. Experimental results of ionic strength dependencies in at least three cases, flavodoxin–cyt *c* (Simondsen et al., 1982; Simondsen & Tollin, 1983), ferredoxin–ferredoxin $NADP^+$ reductase (Bhattacharyya et al., 1986), and *R. capsulata* cyt c_2–*R. rubrum* reaction centers (Rickle & Cusanovich, 1979), are consistent with this argument. In two other cases, *R. rubrum* cyt c_2–*R. rubrum* reaction centers and horse cyt *c*–*R. rubrum* reaction centers, the electron-transfer rate constant is independent of ionic strength (Rickle & Cusanovich, 1979). The present results obtained with yeast iso-1, Ile-13 and Asp-72 mutants, and the iso-2 cytochromes clearly demonstrate that the more efficient electron-transfer complex is not the electrostatically most stabilized one. In these cases, rapid electron transfer is approached at ionic strengths at which there must be considerable masking of specific charged residues on both proteins. Therefore, caution should be taken in ascribing a disproportionate importance to the quantitative role of electrostatic forces in the attainment of favorable electron-transfer complexes under physiological conditions. It is also clear from this and previous kinetic and spectroscopic data (Ho et al., 1985; Cheung et al., 1986; Hazzard et al., 1987; Moench et al., 1988) that the structures of the complexes formed between CcP and the cytochromes *c* from various species are certainly not the same. Furthermore, on the basis of the large changes in kinetic properties which occur upon the mutation of a single specific residue in cyt *c*, it is also possible that there exist several complexes for a given cyt *c* species, all of which can carry out efficient electron transfer. This conclusion is supported by the proposal of Poulos et al. (1987) that the inability to resolve cyt *c* in crystals with CcP could result from the presence of multiple cyt *c* orientations. The present results also emphasize the need for highly refined structures for all of the cytochromes studied, as well as a better understanding of the structures of the proteins, both free and within the complex before extrapolations from one cyt *c* to another can be made.

In summary, it is clear that both cyt *c* heme accessibility and the rate constant for intracomplex electron transfer are quite sensitive to amino acid substitutions which occur at the proposed cyt *c*–CcP interface. In at least two cases, complexes can be produced which are more effective in terms of one of the physiologically relevant electron-transfer processes than is the case with the wild-type cytochrome. On the basis of the kinetic results presented here, it appears that a variety of cyt *c*·CcP complex structures can exist, depending upon the side chains present at the interface, the ionic strength of the medium, and the redox state of CcP. Thus, the interfacial structure of the cyt *c*·CcP complex is evidently highly malleable, a conclusion which, if it can be generalized to other protein–protein complexes, must have important physiological consequences.

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