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Factors influencing intermolecular and intramolecular electron transfer in the cytochrome *c*: Cytochrome *c* peroxidase complex

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Factors Influencing Intermolecular and Intramolecular Electron Transfer in the Cytochrome c : Cytochrome c Peroxidase Complex.

by

James Taylor Hazzard

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by James Taylor Hazzard

entitled Factors Influencing Intermolecular and Intramolecular Electron Transfer in the Cytochrome c: Cytochrome c Peroxidase Complex.

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Gordon Tollin	December 13, 1989 Date

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Signed: James T. Hazard

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This dissertation is dedicated with love
to my wife

Jo

and to my son

Jamie

both of whom provided the foundation
upon which this work was built.

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LIST OF ABBREVIATIONS

CcP: native cytochrome c peroxidase isolated from baker's yeast as opposed to the cloned enzyme expressed in E. coli (see below).

CcP(II): ferrous or reduced cytochrome c peroxidase.

CcP(III): ferric or oxidized cytochrome c peroxidase.

CcP(IV,$R^{+}\cdot$): the peroxidase species oxidized by H_2O_2 to the Fe(IV) oxidation state containing an unidentified amino acid(s) free radical, $R^{+}\cdot$ (i.e., Compound I).

CcP(IV): the peroxidase species oxidized to the Fe(IV) state without R-group oxidation (i.e., Compound II).

Cyt c (II): ferrous or reduced cytochrome c.

Cyt c (III): ferric oxidized cytochrome c.

Cyt c(II)-CcP(IV,$R^{+}\cdot$): the transiently formed complex at high ionic strength.

5-DRF and 5-DRFH$\cdot$: the oxidized and neutral semiquinone species of 5-deazariboflavin, respectively.

EDC: 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride.

EDTA: ethylenediaminetetraacetic acid.

$E_{m,7}$: midpoint reduction potential measured at pH 7.

F_{ox} and FH$\cdot$: the oxidized and neutral semiquinone of a generic flavin.

LF and LFH$\cdot$: the oxidized and neutral semiquinone species of lumiflavin, respectively.

MI-CcP: the wild-type enzyme as expressed in E. coli which contains an additional Met-Ile sequence at the N-terminus.

μ: the symbol used to denote ionic strength of the buffer solution.

$NADP^{+}$: oxidized nicotinamide adenine dinucleotide phosphate.

RF and RFH·: the oxidized and neutral semiquinone species of riboflavin, respectively.

LIST OF EQUATIONS

I.1) $CcP(III) + H_2O_2 + H^+ \xrightarrow{k_{I.1}} CcP(IV,R^{+}\cdot) + H_2O$

I.2) $\text{cyt } \underline{c}(II) + CcP(IV,R^{+}\cdot) + H^+ \xrightarrow{k_{I.2}} \text{cyt } \underline{c}(III) + CcP(III,R^{+}\cdot) + H_2O$

I.3) $\text{cyt } \underline{c}(II) + CcP(III,R^{+}\cdot) \xrightarrow{k_{I.3}} \text{cyt } \underline{c}(III) + CcP(III,R)$

II.1) $FH\cdot + \text{cyt } \underline{c}(III)\{:CcP(III)\} \xrightarrow{k_{II.1}} F_{ox} + \text{cyt } \underline{c}(II)\{:CcP(III)\}$

II.2) $DRFH\cdot + CcP(III)\{:\text{cyt } \underline{c}(II)\} \xrightarrow{k_{II.2}} DRF + CcP(II)\{:\text{cyt } \underline{c}(II)\}$

II.3) $FH\cdot + \text{cyt } \underline{c}(III):CcP(IV,R^{+}\cdot) \xrightarrow{k_{II.3}} F_{ox} + \text{cyt c(II)}:CcP(IV,R^{+}\cdot)$

II.4) $\text{cyt c(II)}:CcP(IV,R^{+}\cdot) \xrightarrow{k_{II.4}} \text{cyt c(III)}:CcP(III,R^{+}\cdot)$

III.1) $FH\cdot + \text{cyt } \underline{c}(III) \xrightarrow{k_{III.1}} F_{ox} + \text{cyt } \underline{c}(II)$

III.2) $\text{cyt } \underline{c}(II) + CcP(IV,R^{+}\cdot) \underset{k_{-III.2}}{\overset{k_{III.2}}{\rightleftharpoons}} \text{cyt } \underline{c}(II)\text{-}CcP(IV,R^{+}\cdot)$

III.3) $\text{cyt } \underline{c}(II)\text{-}CcP(IV,R^{+}\cdot) \xrightarrow{k_{III.3}} \text{cyt } \underline{c}(III)\text{-}CcP(III,R+.)$

III.4) $$k_{obs} = \frac{k_{III.2}k_{III.3}[CcP(IV,R^{+}\cdot)]}{k_{III.2}[CcP(IV,R^{+}\cdot)] + k_{-III.2} + k_{III.3}}$$

III.5) $$k_{obs} = \frac{k_{III.2}k_{III.3}[CcP(IV,R^{+}\cdot)]}{k_{III.2}[CcP(IV,R^{+}\cdot)] + k_{-III.2}}$$

ABSTRACT

The kinetics of reduction by free flavin semiquinones of the individual components of 1:1 complexes of yeast cytochrome c peroxidase and the cytochrome c from horse, tuna, and yeast, including several site-specific mutants of either the cytochrome c or cytochrome c peroxidase, have been studied. The orientations of the various cytochromes c within electrostatically-stabilized complexes with the peroxidase are not equivalent. This is shown by differential decreases in the rate constants for cytochrome reduction by neutral flavin semiquinones upon complexation which are in the order: tuna >> horse > yeast iso-2 > yeast iso-1. We have also directly measured the physiologically-significant intracomplex one-electron transfer rate constants from the ferrous cytochromes c to the peroxide-oxidized species of the peroxidase at several ionic strengths. The rate constants at low ionic strength are highly species dependent, again consistent with the contention that the orientations of the various cytochromes within the complex with CcP are not the same. Increasing the ionic strength in all cases resulted in an increase in the rate constant for the first-order process which controls electron transfer from cytochrome c to the peroxidase Compound I species of the peroxidase. When the two proteins are immobilized by covalent cross-linking, no

such rate enhancement is observed, suggesting that the ionic strength effect is manifested by an increase in the number of geometric orientations between the two proteins which results in more rapid electron transfer. Similar rate enhancing effects are observed when positively charged residues on the surface of cytochrome c are converted to electrostatically neutral amino acids by site-specific mutagenesis. The effect of site-specific mutagenesis of two residues of cytochrome c peroxidase have also been studied. His-181, when converted to a glycine has little effect on the electron transfer rate constant, whereas when Trp-191 is converted to a phenylalanine no intracomplex electron transfer could be observed, indicating an obligatory role of this residue in the electron transfer process.

CHAPTER I

INTRODUCTION, OVERVIEW, AND EXPERIMENTAL MATERIALS AND METHODS

Introduction

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 which govern the rates and specificity of biological electron transfer processes. Such studies are of interest since both biological energy transduction and much of intermediary metabolism ultimately depend on the control of simple one electron transfer reactions between proteins. The overall topic of biological electron transfer has been extensively reviewed (Marcus and Sutin, 1985, McLendon et al., 1985; Scott et al., 1985; Mayo et al., 1986; Hoffman and Ratner, 1987; Kuki and Wolynes, 1987; Tollin et al., 1986a; Cusanovich et al., 1988a; and McLendon, 1988).

In order to better understand the structural relationships of proteins within 1:1 electron transfer complexes, workers have utilized computer graphics capabilities to model such complexes based on the x-ray crystallographically determined three dimensional structures for the individual proteins. Computer modeling has been necessitated due to the fact that to date no 1:1

electron transfer protein complex has been crystallized in a manner allowing the atomic coordinates for both proteins to be resolved. In doing such hypothetical modeling of docked proteins, it has generally been accepted that attractive electrostatic interactions are predominately responsible for proper long range orientation and attraction of the two proteins to generally reactive regions on the protein surfaces such that the prosthetic groups have a favorable distance and orientation for electron transfer from one protein to the other (Salemme, 1978; Koppenol and Margoliash, 1982; Matthew et al., 1983; Northrup et al., 1988). Several hypothetical structures for electron transfer complexes have recently been modeled (Salemme, 1976; Simondsen et al., 1982; Poulos and Kraut, 1980; Poulos and Mauk, 1983; Mauk et al., 1986; Poulos and Finzel, 1984; Stewart et al., 1988; Stayton et al., 1989). 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 <u>both</u> the complex association constant and the first-order rate constant for the intracomplex processes associated with electron transfer (these would

include any reorientations which influence redox center geometrical relations) should be inversely related to the ionic strength of the solution.

Equilibrium binding studies of cytochrome c to CcP (Erman and Vitello, 1980), to cytochrome b_5 (Mauk et al., 1982), and to cytochrome c oxidase (Michel and Bosshard, 1984; Michel and Bosshard, 1989; Michel et al., 1989) 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). Such decreases in the apparent intracomplex electron transfer rate constant with ionic strength have been attributed to formation of non-optimal complexes which require geometrical rearrangement prior to electron transfer (Simondsen and Tollin, 1983). Bhattacharyya et al.(1986;1987) have studied electron transfer in the

spinach ferredoxin: spinach ferredoxin-$NADP^+$ reductase complex and report a substantial decrease in the rate constant, from 4000 to 1600 s^{-1}, for a change in ionic strength from 310 mM to 460 mM at pH 7. However, more current research on this complex in our laboratory (Mark A. Walker, personal communication) indicates that this decrease is a phosphate ion induced effect rather than a general ionic strength effect and suggests that there is no change in the rate limiting first-order electron transfer process from 100 to 460 mM ionic strength. In contrast to this, in the case of Anabaena ferredoxin:Anabaena ferredoxin-$NADP^+$ reductase, a maximum first-order electron transfer rate constant was found to occur at μ = 110 mM, with values dropping off at ionic strengths less than and greater than 110 mM. Thus, the kinetic data on complexes which have been studied are not entirely consistent with a simple electrostatic model.

Recently, Northrup et al. (1988) have developed a dynamic docking simulation which incorporates electrostatic energy calculations as well as Brownian motion of the protein molecules. The picture which has emerged from such studies indicates that an ensemble of docked complexes can exist allowing the protein partners to experience a large number of geometric orientations many of which could conceivably lead to electron transfer. Based on such

simulations as well as the experimental data to be presented below, model builders are now deemphasizing the need for specific salt bridge formation and allowing for multiple docking geometries (see Stayton et al., 1989).

Consideration of inter-prosthetic group separation distance is of importance since the distance between closest contact can sometimes be as great as 18 - 20 Å, greatly outside the range of outer-sphere electron transfer. The rate constant for electron transfer should decrease exponentially with increasing distance (Marcus and Sutin, 1985; McLendon, 1988) for electron transfer processes not involving tunneling mechanisms. Furthermore, the prosthetic group of one of the redox proteins can often be buried within the matrix of the protein, such as in the case of cytochrome c peroxidase (Poulos et al., 1980). Thus, workers have been forced to propose specific residues which can act as electron transfer conduits.

One particularly useful paradigm for understanding the structure-function relationships of protein:protein electron transfer complexes is provided by cytochrome c and cytochrome c peroxidase. Cytochrome c peroxidase as isolated from the inner membrane space of yeast mitochondria exists in the ferric state. Addition of H_2O_2 converts CcP to a two electron oxidized species containing an oxy-ferryl Fe(IV) heme and an oxidized amino acid, $R^{+\cdot}$,

which has been recently suggested to be Trp-191 (Erman et al., 1989; Scholes et al., 1989; Sivaraja et al., 1989):

$$\text{I.1)}\quad \text{CcP(III)} + H_2O_2 + H^+ \xrightarrow{k_{I.1}} \text{CcP(IV,R}^+\cdot) + H_2O$$

The above reaction is quite rapid with a second order rate constant of ~108 M^{-1} s^{-1} (Yonetani, 1976). The peroxide-oxidized CcP species is commonly referred to as "ES", Compound I, or CcP(IV,$R^+\cdot$). The latter two designations will be used interchangeably throughout this work. This reaction is assumed to be involved in peroxide detoxification in yeast. Return of CcP(IV,R^+.) to the resting oxidation state involves two electron reduction. The classical mechanism (see Yonetani (1976) for a more detailed description) is given below:

$$\text{I.2)}\quad \text{cyt } \underline{c}\text{(II)} + \text{CcP(IV,R}^+\cdot) + H^+ \xrightarrow{k_{I.2}} \text{cyt } \underline{c}\text{(III)} + \text{CcP(III,R}^+\cdot) + H_2O$$

$$\text{I.3)}\quad \text{cyt } \underline{c}\text{(II)} + \text{CcP(III,R}^+\cdot) \xrightarrow{k_{I.3}} \text{cyt } \underline{c}\text{(III)} + \text{CcP(III,R)}$$

The CcP species produced in reaction I.2 is commonly referred to as Compound II. The oxidation state of the two redox components is questionable (Summers and Erman, 1988). In equation I.2, reduction of the Fe(IV) has been assumed

to occur. However, there is evidence to suggest that the thermodynamically more stable reduced species is that corresponding to CcP(IV,R). The evidence presented below, as well as stopped flow data of Summers and Erman (1988), indicate that the reduction of the oxidized amino acid, $R^{+\cdot}$, is a slow first-order process which occurs subsequent to the initial reduction of the heme Fe by ferrous cytochrome c. Summers and Erman (1988) have also suggested that the reduction of Compound II occurs by a disproportionation pathway instead of by the reaction shown in equation I.3. Since this latter point is outside the scope of this work we shall not consider this point further. The bulk of the work presented below deals with the one-electron reduction of Compound I by ferrous cytochrome c as depicted in equation I.2.

The two proteins in equation I.2 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 oxidization states and site-specific mutants (Poulos et al., 1980, Edwards et al., 1987, Swanson et al., 1977; Takano and Dickerson, 1981; Louie et al., 1988; Miller et al., 1988).
2) Cyt c and CcP form a thermodynamically well-

characterized complex (Nicholls and Mochan, 1971; Mochan and Nicholls, 1971; Erman and 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 and Kraut, 1980; Koppenol and Margoliash, 1982; Poulos and Finzel, 1984) although recent efforts to co-crystallize 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; Kang et al.,1978; Waldmeyer and Bosshard, 1985).

3) 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; Mauro et al., 1988; Miller et al.; 1988) have been developed. Thus, detailed studies of the interaction between cyt c and CcP can be carried out using genetic techniques to modify either the interaction domain, residues involved in the formation of

and/or stabilization of the free radical species, $R^{+\cdot}$, or residues believed to be important in the electron transfer pathway.

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 non-physiological 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 electrostatic environments of the protein prosthetic groups within the complexes, in addition to providing information concerning the intramolecular electron transfer process. (General principles of the use of free flavins as laser flash induced reductants has been reviewed in Tollin et al., 1987). Under optimal conditions, one is able to generate a free flavin reductant in situ which can specifically react with the lower potential protein redox partner, and then observe the intramolecular or intracomplex electron transfer to the higher potential protein partner. This methodology has

been applied to flavocytochromes c (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 ferredoxin-$NADP^+$ reductase complex (Pryziecki et al., 1985a), the spinach ferredoxin:spinach ferredoxin $NADP^+$ reductase complex (Bhattacharyya et al., 1986 and 1987), the horse cytochrome c:Clostridium pasteurianum flavodoxin complex (Hazzard et al., 1986), and the cytochrome c:cytochrome b_5 complex (Eltis et al., 1988). From such studies we can gain an understanding of the steric and electrostatic nature of the region near the prosthetic groups within these complexes, as well as the factors which influence the rates of electron transfer between redox proteins.

Overview of Thesis

The organization of this thesis is based primarily on the chronological order of the studies as they were performed. During the course of these investigations, it was this worker's privilege to collaborate with several investigators outside of the University of Arizona. However, all of the kinetic data which was obtained in these studies, as well as the interpretation of those data, was performed by this worker. In the brief description of each chapter which follows, recognition of the contribution

of these collaborators is given.

Chapter II deals with the general properties of the electrostatically-stabilized complexes between cytochromes c from tuna, horse and yeast with various oxidation states of CcP. It is also demonstrated in this chapter that we can utilize free flavin semiquinones to specifically reduce cytochrome c in the presence of Compound I, without the initial reduction of the CcP species, and subsequently follow the electron transfer from cytochrome c to Compound I. From these studies we demonstrated that despite a great deal of similarity in cytochrome c structures, there was a high degree of species specificity with respect to electron transfer. This work was done in collaboration with Dr. Thomas Poulos of the University of Maryland and the Center for Applied Research in Biophysics, who kindly provided coordinates for the hypothetical model of cytochrome c and CcP. This work has been published in Hazzard et al., 1987a.

Chapter III describes the differences between non-covalent and covalently cross-linked complexes of cytochrome c and CcP. This study provides the first evidence that the electrostatically-stabilized complex is not the most efficient electron transfer complex, and that covalently cross-linking the two proteins diminishes their ability to reorient themselves to find more optimal

geometric orientations. Dr. Susan Moench, working in the laboratory of Dr. James Satterlee at University of New Mexico, produced and characterized the covalent cross-linked complex. Dr. James Erman provided the CcP for Dr. Moench and made several valuable contributions during the course of the preparation of the manuscript. This work has been published in Hazzard et al., 1987a.

Chapter IV deals with the use of various single amino acid mutant species of yeast cytochrome c in which surface charged groups have been replaced by neutral or negatively charged amino acids. The results from this work clearly demonstrate that the complexes formed between cytochrome c and CcP at low ionic strength are less efficient electron transfer complexes than those formed at significantly higher ionic strengths. Furthermore, positively charged residues which have been suggested to be important to complex formation, are actually shown to inhibit the formation of the more efficient electron transfer complexes. This work was primarily done in collaboration with Dr. George McLendon at the University of Rochester. The mutants were isolated by Dr. Goutam Das working in Dr. Fred Sherman's laboratory at the University of Rochester. These results appeared in published form as Hazzard et al. (1988b and 1988c).

Chapter V describes kinetic studies involving two site-

directed mutants of CcP. The first mutation (His-181 to Gly) was produced by Dr. Mark Miller of the University of California, San Diego. The second mutant (Trp-191 to Phe) was engineered by Dr. Matthew Mauro of the same institution. Both work in the laboratory of Dr. Joseph Kraut. In this chapter we show that a residue which was postulated to be essential to electron transfer to CcP (Poulos and Finzel, 1984), His-181, is in fact not a prerequisite residue at all. Trp-191 is, however, quite essential, as shown by the observation that when it was converted to a phenylalanine residue no electron transfer could be observed. These results have been published in Mauro et al. (1988) and Miller et al. (1988).

In all chapters, the relevance of the kinetic data to the computer-derived hypothetical model is discussed and deficiencies in this model are pointed out.

Experimental Materials and Methods

Proteins

Horse (Type VI) and tuna cytochromes *c* were obtained from Sigma and were purified by ion exchange chromatography on CM-cellulose (Bio-Rad). Yeast iso-2 cytochrome *c*, isolated from Red Star baker's yeast, was the generous gift of Drs. Susan Moench and James Satterlee of the University of New Mexico. Wild-type yeast iso-1 cytochrome *c* as well as the single amino acid mutant cytochromes used in these studies were isolated and provided by Drs. George McLendon, Fred Sherman and Goutam Das of the University of Rochester. Details concerning the isolation of these proteins are given in Hazzard et al. (1988c).

Native yeast cytochrome *c* peroxidase used in all of the non-covalent complex studies was purified from commercial baker's 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 by 2x crystallization of the enzyme from glass distilled water. In order to check for enzymatic activity, 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 to a

concentration of 180 μM and the reaction was begun upon addition of 2 nM CcP. Reactions were performed in a 4 mM phosphate buffer containing 0.5 mM EDTA at pH 7.0. Initial velocity (v_o) 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_o/[cytochrome c] vs. v_o . Typically, a turnover number of 175 s^{-1} was obtained, which agrees well with the values reported by Yonetani (1965).

Cytochrome c peroxidase used for covalent cross-linking with cytochrome c was isolated, purified and spectrally characterized in the laboratory of Dr. James E. Erman at Northern Illinois University as described previously (Yonetani and Ray, 1965; Erman and Vitello, 1980).

The covalent cross-linking of horse cyt c to CcP using EDC, and purification of the 1:1 complex was performed in the laboratory of Dr. James D. Satterlee by Dr. Susan J. Moench using a slight modification of the method of Waldmeyer and Bosshard (1985) as described in Moench et al. (1987).

The site-directed mutants of yeast cytochrome c peroxidase were supplied by Drs. J. Matthew Mauro (W191F) and Mark A. Miller (H181G) of Prof. Joseph Kraut's laboratory at University of California, San Diego. Details concerning the production and isolation of these mutant

proteins from E. coli are given elsewhere (Fishel et al., 1987; Mauro et al., 1988; Miller et al., 1988).

Cytochrome c concentration was calculated using ϵ_{550}= 30.8 mM^{-1} cm^{-1} and 8.16 mM^{-1} cm^{-1} Hazzard (1982) for the ferrous and ferric states, respectively, or ϵ_{412} = 118 mM^{-1} cm^{-1} for the ferric species. CcP(III) concentration was determined using ϵ_{409}= 93 mM^{-1} cm^{-1} (Yonetani, 1967). The concentration of the ferric cytochrome c:CcP complex was determined using an extinction coefficient of 200 mM^{-1} cm^{-1} at 410 nm (Moench et al., 1987).

Generation of H_2O_2-Oxidized Species of CcP

Two forms of H_2O_2-oxidized CcP were produced. In order to generate CcP(IV,$R^{+}\cdot$), the ferric resting enzyme was titrated with a freshly prepared buffered 5 mM H_2O_2 solution, which had been bubbled with O_2-scrubbed Ar (see below) for ca. 45 minutes in the absence or presence of equimolar cytochrome c(III). The second oxidized species of the enzyme, CcP(IV), was obtained by steady-state photoreduction of CcP(III) to the ferrous species, using 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. In either case, the titration was monitored by obtaining a visible difference spectrum of oxidized sample minus ferric CcP. Kinetic experiments were performed immediately

following the production of both forms of the ferryl peroxidase.

Flavins and Other Reagents

Preparation of lumiflavin was 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. Determination of oxidized flavin concentration was made using extinction coefficients of 12.4 and 10 mM^{-1} cm^{-1} for lumiflavin (as well as riboflavin) at 440 nm and 5-deazariboflavin at 390 nm, respectively (Simondsen et al., 1982; Bhattacharryya et al., 1987).

Solutions of 5 mM H_2O_2 were prepared by addition of the appropriate volume of 3% stock peroxide (Baker), which was kept refrigerated to minimize decomposition, to the reaction buffer.

Laser Flash Photolysis Reduction Experimental Protocol

The laser flash photolysis apparatus and general principles of these reduction studies have been described previously (Ahmad et al., 1981 and 1982; Simondsen & Tollin, 1983). The basic buffer used throughout these experiments, unless otherwise stated, was a 3 mM phosphate buffer containing 0.5 mM EDTA at pH 7.0, which has an ionic strength of 8 mM. Generally, higher ionic strength buffers

were prepared by addition of the appropriate amount of KCl. In one set of experiments, the high ionic strength was obtained by using 100 mM phosphate and 25 mM EDTA, μ = 275 mM, at pH 7.0.

Solutions containing free flavins were prepared by addition of solid flavin to the appropriate buffer, followed by an approximately two hour mixing time. The solution was then filtered through a 0.45 micron filter in order to remove any undissolved flavin. Concentrations were determined using the above extinction coefficients. For reactions involving lumiflavin or riboflavin, concentrations of the flavin were 70 - 80 μM. For reactions using 5-deazariboflavin, concentrations were typically 100 μM.

Solutions were made essentially anaerobic by extensive bubbling of the reaction solution, minus proteins, for 30 minutes with Ar which was passed through a 3 cm x 1 m column packed with the O_2 removing catalyst R3-11 (Chemical Dynamics Corp.) and hydrated by bubbling through H_2O. To minimize O_2 contamination, vacuum tubing and glass fittings only were used on the gas train. All ground-glass joints were sealed with Apiezon Type M grease. The reaction cuvette was sealed with a rubber septum (Thomas Scientific). Simultaneously, the 5 mM solution of peroxide was degassed in a similar fashion. Prior to addition of

the proteins, the degree of anaerobiosis was determined by monitoring the intensity of the flavin semiquinone signal at 510 nm for 5-deazariboflavin and 570 nm for all other flavins generated in the laser flash apparatus. All reactions were performed at room temperature, 24 ^{o}C. Under these experimental conditions, the concentration of flavin semiquinone generated by each laser flash was determined to be 0.07 μM, based on the amount of ferrous cytochrome c generated per laser flash, which agrees well with the value given by Simondsen et al.(1982).

Following the determination of the anaerobic state of the flavin solution, protein(s) was added and an additional 5 minutes of de-oxygenation was performed by allowing a stream of the Ar to pass over the top of the reaction solution with strict care being taken to not allow any bubbling to occur. For experiments involving peroxide-oxidized CcP, titration of the protein with 5 mM H_2O_2 (see above) was performed immediately prior to the flash experiment. Due to the small volumes of peroxide introduced, typically < 2 μl, no further degassing was necessary.

For studies on the reduction of cytochrome c by flavin semiquinones in an electrostatically-stabilized complex at low ionic strengths with either CcP(III) or CcP(IV,$R^{+\cdot}$), as well for intracomplex reduction of CcP(IV,$R^{+\cdot}$) at low ionic

strength, a mole ratio of 1:1 between the two proteins was maintained throughout the entire concentration range. Experiments investigating the reduction of CcP(IV,$R^{+}\cdot$) at higher ionic strengths were performed in the following manner. Cyt c was held constant at 30 μM while the peroxidase concentration was increased from an initial value of either 5 or 10 μM. Reduction reactions were performed using 5-DRF, typically at 100 μM. As is shown below, free CcP(IV,$R^{+}\cdot$) is not directly reduced by 5-DRFH$\cdot$ on a time scale relevant to the flash photolysis experiment. 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(Fe(II)). Thus, with respect to the amount of ferrous cytochrome c generated per laser flash, the concentration of ferryl peroxidase was always in large excess, hence pseudo-first-order conditions were obtained.

The reduction of cytochrome c in all complexes with CcP(III) was monitored at 575 nm. The reduction of cyt c(III) by LFH$\cdot$ in the electrostatic complex with CcP(IV,$R^{+}\cdot$) at μ = 8 mM was also 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. Therefore, at this wavelength we are able

to monitor the oxidation of free flavin semiquinone due to its reduction of cytochrome c. CcP(Fe(IV),$R^{+\cdot}$) reduction was monitored at 550 nm, a wavelength at which there is a significant bleach in the CcP(III,R) minus CcP(IV,$R^{+\cdot}$) redox difference spectrum (see below). A minimum of four transient decay curves were averaged for each protein concentration, and typically for each protein concentration decay curves were obtained on two different time scales.

Kinetic Analysis

Pseudo-first-order rate constants were determined from the slopes of semi-log plots of the kinetic data (delta signal vs. time) which were linear over at least four half-lives. (During the course of these investigations, a commercial kinetic data analysis software package, KFIT (OLIS, Inc.), was utilized for determining rate constants from kinetic traces which had been stored in a computer memory. For traces which had good signal-to-noise ratios ($\geq$ 100:1), no difference between hand-fits and computer generated fits could be discerned).

Where appropriate, a second-order rate constant was calculated from the slopes of linear plots of k_{obs} vs. protein concentration. For non-linear plots of k_{obs} vs. concentration, computer modelling was performed based on the appropriate mechanism, details of which will be given below. The estimated error of the rate constants reported

below is ± 10%.

Molecular Graphics

Computer graphic representations of the cytochrome c-CcP complex were displayed using an Evans and Sutherland PS390 system interfaced with a Vax 1170 computer. Displays of the molecular structures was obtained using the graphics display program, INSIGHT (Biosym Technologies, Inc.). Coordinates for the hypothetical cyt c:CcP complex, which were kindly provided by Dr. Thomas L. Poulos, were obtained using coordinates for tuna cytochrome c and yeast cytochrome c peroxidase. The rational for the docking geometry has been described in Poulos and Finzel (1984). The coordinates for the lumiflavin molecule were obtained by deletion of the ribityl side chain from the FMN structure of Clostridium MP flavodoxin (Smith et al., 1977) deposited in the Brookhaven Protein Data Bank. For purposes of docking lumiflavin with CcP or cytochrome c, van der Waals surfaces were utilized to insure proper positioning of the molecules.

CHAPTER II

REDUCTION OF THE COMPONENTS OF ELECTROSTATICALLY-STABILIZED 1:1 COMPLEXES OF HORSE, TUNA, OR YEAST ISO-2 CYTOCHROMES C AND NATIVE YEAST CYTOCHROME C PEROXIDASE.

Introduction

The following chapter describes the interaction of mitochondrial cytochromes c from tuna, horse, and yeast with native yeast cytochrome c peroxidase in the ferric, oxy-ferryl, and Compound I oxidation states. Utilizing the native cytochromes c from the various species permits us to investigate the effect of cytochrome c primary structure on the nature of the 1:1 electrostatic complexes formed with CcP in terms of steric properties, as well as any differences in the rates of electron transfer from cytochrome c to Compound I. The effect of complexation on the reduction kinetics of ferric CcP by free flavin semiquinones is also investigated, the results of which suggest that there is a change in the dynamic properties of the peroxidase when complexed to cytochrome c, relative to the free protein.

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 correlation between the second-order reduction rate constants and the difference in midpoint reduction potentials between the reactants based upon Marcus theory (see Meyer et al., 1983 and Cusanovich et al., 1988). In order to facilitate an understanding of the protocol employed in the present studies, we have listed the midpoint reduction potentials of the relevant free flavins and proteins in Table II-1. The reduction potential for the ferryl species of CcP has not been directly determined by potentiometric titration. The indicated value, + 1 V, is a value for the midpoint potential of what is assumed to be the Fe(IV)/Fe(III) couple of the peroxidase Compound II species calculated from the kinetics of reduction by a variety of Fe(II) reductants at pH 5.6 (Purcell and Erman, 1976). The value is, however, quite close to that measured for the Fe(IV)/Fe(III) couple of Compound I of horseradish peroxidase (Yamazaki, 1974). In the low ionic strength experiments described in this chapter, the higher potential flavin semiquinones (e.g., LFH$^{\cdot}$ and RFH$^{\cdot}$) have been primarily used to study the reduction of free and

Table II-1

Reduction Potentials for the Free Flavins and Proteins

Flavin/Protein	Redox Couple	$E_{m,7}$(mV)	References
Lumiflavin	oxidized/semiquinone	-231	1
Riboflavin	oxidized/semiquinone	-231	2
5-DRF	oxidized/semiquinone	-630	3
Horse cyt c	Fe(III)/Fe(II)	+260	4
Tuna cyt c	Fe(III)/Fe(II)	+260	4
Yeast iso-2 cyt c	Fe(III)/Fe(II)	+260	5
Yeast CcP	Fe(III)/Fe(II)	-190	6
	Fe(IV)/Fe(III)	+1000	7

References

1. Draper and Ingraham (1968a).
2. Draper and Ingraham (1968b).
3. Blankenhorn (1976).
4. Tollin et al.(1984).
5. Henderson and Rawlinson (1961).
6. Conroy et al. (1978).
7. The $E_{m,7}$ has not been determined experimentally for Compound I of CcP. The indicated value is assumed for the midpoint potential of the CcP Fe(IV)/Fe(III) couple calculated from the kinetics of CcP Compound II reduction by Fe(II) reductants at pH 5.26 (Purcell and Erman, 1976). This value is quite similar to the $E_{m,7}$ for horse radish peroxidase (Yamazaki, 1974).

CcP-complexed cytochrome c (see below), whereas the lower potential 5-deazariboflavin semiquinone (5-DRFH·) has been employed for reduction of the ferric form of CcP(III), both free and in the complex with ferrous cytochrome c.

Figure II-1 shows the steady-state redox difference spectra (reduced minus oxidized) of horse cytochrome c and yeast 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 II-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, based on an association constant of $\sim 10^6$ 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, by the higher potential lumiflavin reductant, which is to be expected based on the midpoint redox potentials for cytochrome c (+270 mV, Meyer et al., 1983) and CcP(III)

Figure II-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,$R^{+}\cdot$) 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,$R^{+}\cdot$), 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.

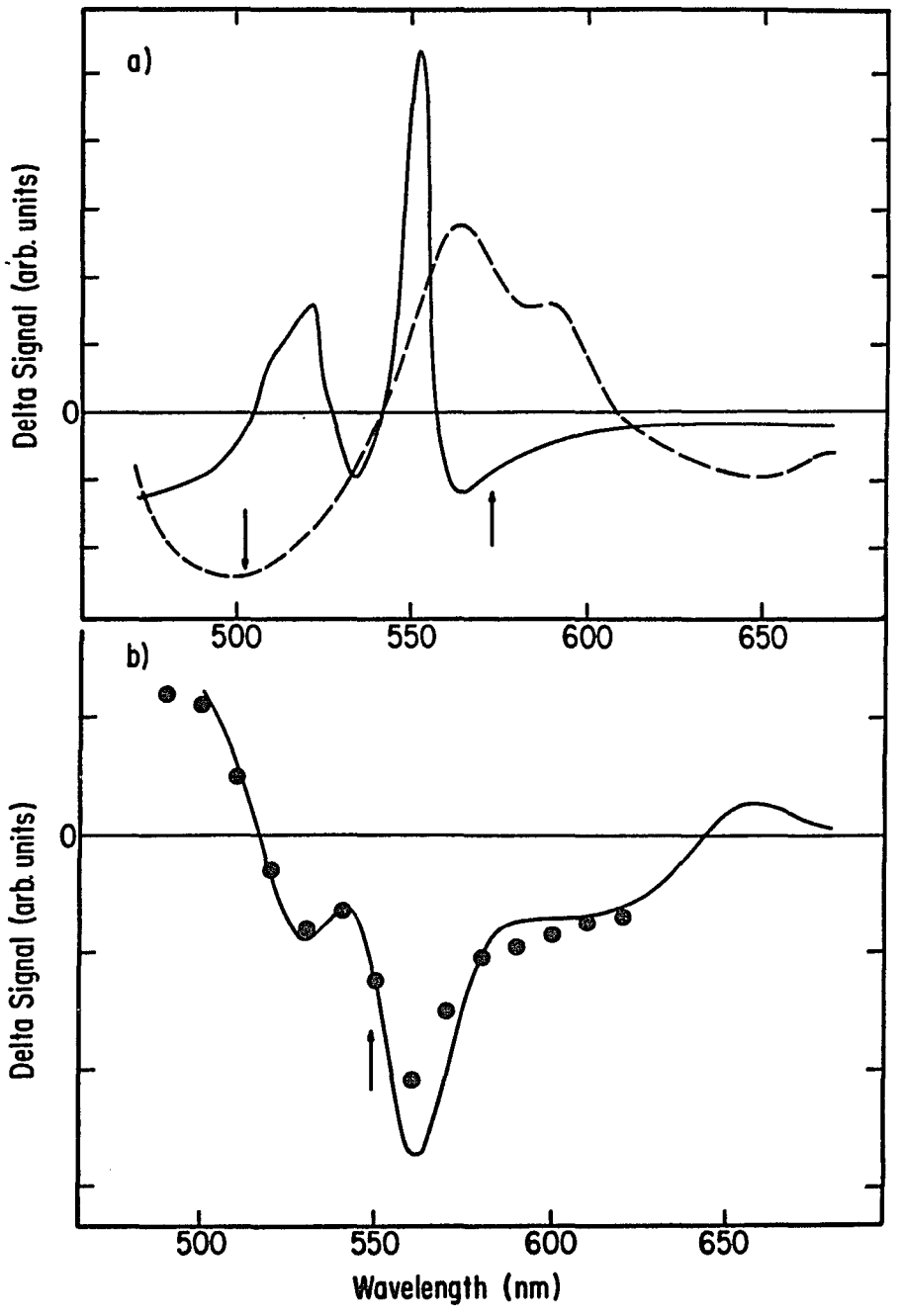


Figure II-1. Redox difference spectra (reduced minus oxidized) of cytochrome c and CcP.

(-190 mV, Conroy et al., 1978). Also shown in Figure II-1a is the steady-state redox difference spectrum obtained by photoreduction with white light of a 1:1 mixture of ferrous cytochrome c and ferric CcP at μ = 8 mM using the lower potential 5-DRFH· as a reductant. 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 peroxidase (Wittenberg et al., 1968; Ho et al., 1983). These results demonstrate that, under the appropriate experimental conditions, it is possible to separately photoreduce the individual components of electrostatically-stabilized complexes of ferric cytochrome c and CcP.

Figure II-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) in the absence of cytochrome c. (For reasons to be discussed in more detail below, reduction of Compound I occurs by the fully-reduced species of lumiflavin rather than by the semiquinone). The data points correspond to a time resolved (t = 30 ms) flash difference spectrum of a 1:1 mixture of CcP(IV,R^{+}·) and horse cytochrome c(III) at low ionic strength (μ = 8 mM) using LFH· as a reductant. Within the limits of the experimental error, the two spectra agree well; the

attenuation of the large absorption band at ~564 nm in the transient spectrum is the result of the greater slit-width of the monitoring beam monochromator (1 mm) for the flash apparatus as opposed to that of the spectrophotometer. Thus, the species which was reduced at t = 30 ms after the flash was CcP(IV,$R^{+}\cdot$). In the absence of cytochrome c(III) and on the time scale used to obtain the transient spectrum of Figure II-1b, CcP(IV,$R^{+}\cdot$) was reduced to a much smaller extent, with kinetics which were consistent with reduction primarily by fully-reduced lumiflavin (produced by disproportionation of LFH$\cdot$), and to a much lesser degree by LFH$\cdot$ (see below). These results demonstrate that the reduction of CcP(IV,$R^{+}\cdot$) 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 II-4).

Reduction Kinetics of Free and Complexed Cytochrome c(III)

Based on the spectral properties observed above, reduction of cytochrome c in the presence of ferric CcP results in the reduction of cytochrome c alone. Plots of k_{obs} vs. concentration for flash-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$\cdot$ are shown in Figure II-2a. Over the concentration range studied, the plots remained linear with no evidence

Figure II-2. Plots of k_{obs} vs. concentration for reduction of free and CcP(III)-complexed horse, tuna, and yeast iso-2 cytochromes c(III) by LFH· and ^{R}FH·.

(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.

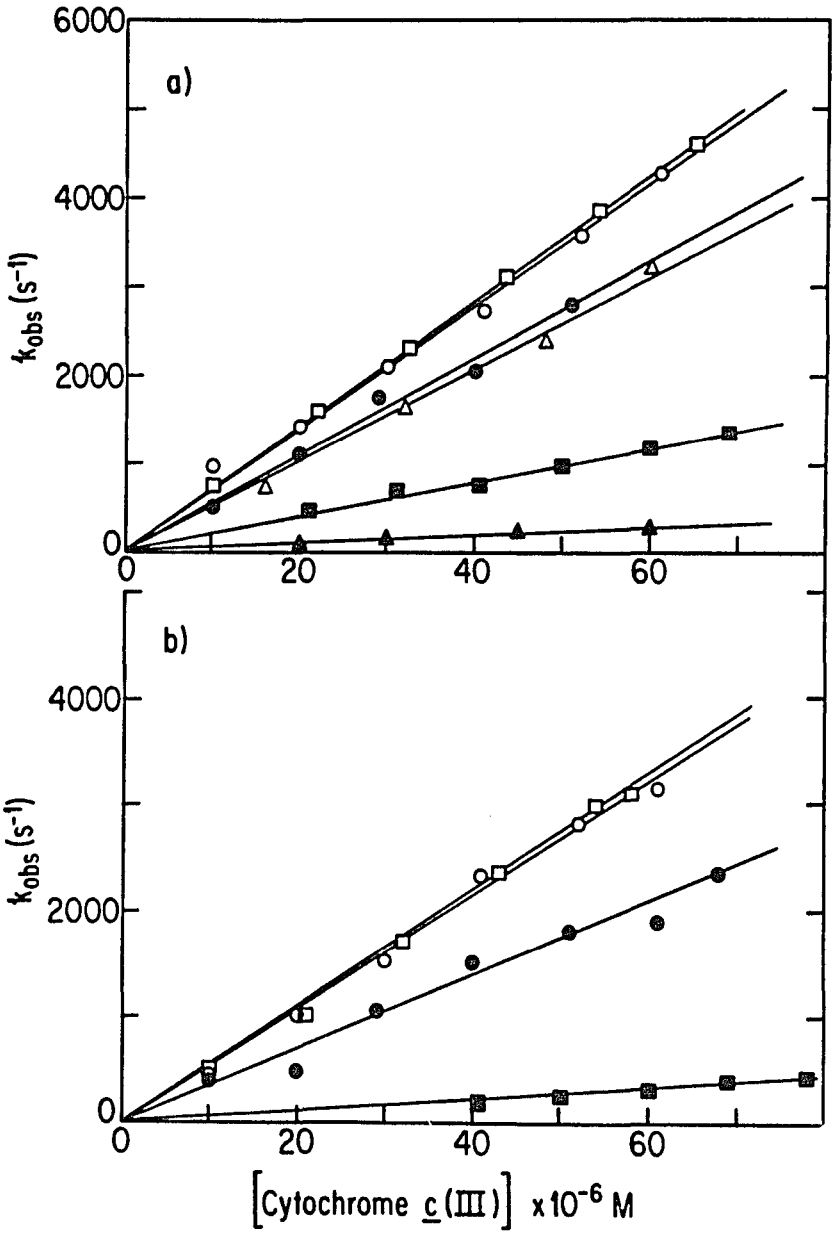


Figure II-2. Plots of k_{obs} vs. concentration for reduction of free and CcP(III)-complexed horse, tuna, and yeast iso-2 cytochromes c(III) by LFH· and RFH·.

of an approach to a rate limiting process. Thus the reaction between the free flavin reductant and complexed cytochrome c can be written as a simple bimolecular reaction:

$$\text{II.1)}\quad FH^{\cdot} + \text{cyt c(III)\{:CcP(III)\}} \xrightarrow{k_{II.1}} F_{ox} + \text{cyt c(II)\{:CcP(III)\}}$$

where the brackets around CcP refer to reactions performed in the absence and presence of CcP while $FH^{\cdot}$ and F_{ox} refer to the semiquinone and oxidized flavin species, respectively. The second-order rate constants for direct reduction of either the free or complexed cytochromes c, $k_{II.1}$, obtained from the slopes of these plots, are listed in Table II-2. The rate constants for the free horse and tuna cytochromes are in good agreement with values previously reported (Meyer et al., 1983; Meyer et al., 1984; Hazzard et al., 1986). In the case of free yeast iso-2 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., 1986b).

Table II-2

Second-order Rate Constants for Reduction of Free and CcP(III)-Complexed Cytochromes c(III) by LFH· and RFH·

	$k_{II.1} \times 10^{-7}$, 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 Figures II-2a and b.

Complex formation with CcP(III) decreased the second-order reduction rate constant of the cytochrome heme to varying degrees dependent on the cytochrome species. The magnitude of the decrease in $k_{II.1}$ was of the order: tuna < horse < yeast iso-2, with decreases 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 interfaces 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 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 site of reduction 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_{obs} 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 II-2b. Again the plots remained linear over the range of concentrations investigated. The rate constants derived from these plots are given in Table II-2. For the free cytochromes, decreases in $k_{II.1}$ of 24% relative to the values obtained for LFH$^{\cdot}$ reduction were observed, in good agreement with the previous findings. For tuna cytochrome c, a decrease in $k_{II.1}$ 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 $k_{II.1}$ for LFH$^{\cdot}$ reduction of yeast cytochrome within the complex, determination of the rate constant for RFH$^{\cdot}$ reduction was not experimentally feasible, since the time scale of the reaction was so slow that riboflavin semiquinone disproportionation to the fully reduced species occurred before the semiquinone could react with the complexed yeast iso-2 cytochrome over the concentration range employed). These effects of complexation were in the same direction as those obtained with LFH$^{\cdot}$, 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$^{\cdot}$ with the complexed cytochromes, which were not found for the free cytochromes. On the basis of the 91%

decrease in $k_{II.1}$ 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 with the free flavin reductants due to complexation, suggesting that in the complex this cytochrome heme is the most exposed.

Reduction Kinetics of Free and Complexed CcP(III)

Plots of k_{obs} 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 II-3. Again, based on the linear relationship between k_{obs} and concentration a simple bimolecular reaction may be used to describe the reaction:

$$\text{II.2)}\quad \text{DRFH}\cdot + \text{CcP(III)}\{:\text{cyt c(II)}\} \xrightarrow{k_{II.2}} \text{DRF} + \text{CcP(II)}\{:\text{cyt c(II)}\}$$

where the brackets signify reactions in the absence or presence of cytochrome c(II). The second-order rate constants, $k_{II.2}$, obtained from these plots are listed in Table II-4. For free CcP(III), a value of 9×10^6 M^{-1} s^{-1} was obtained. This rate constant is quite small when

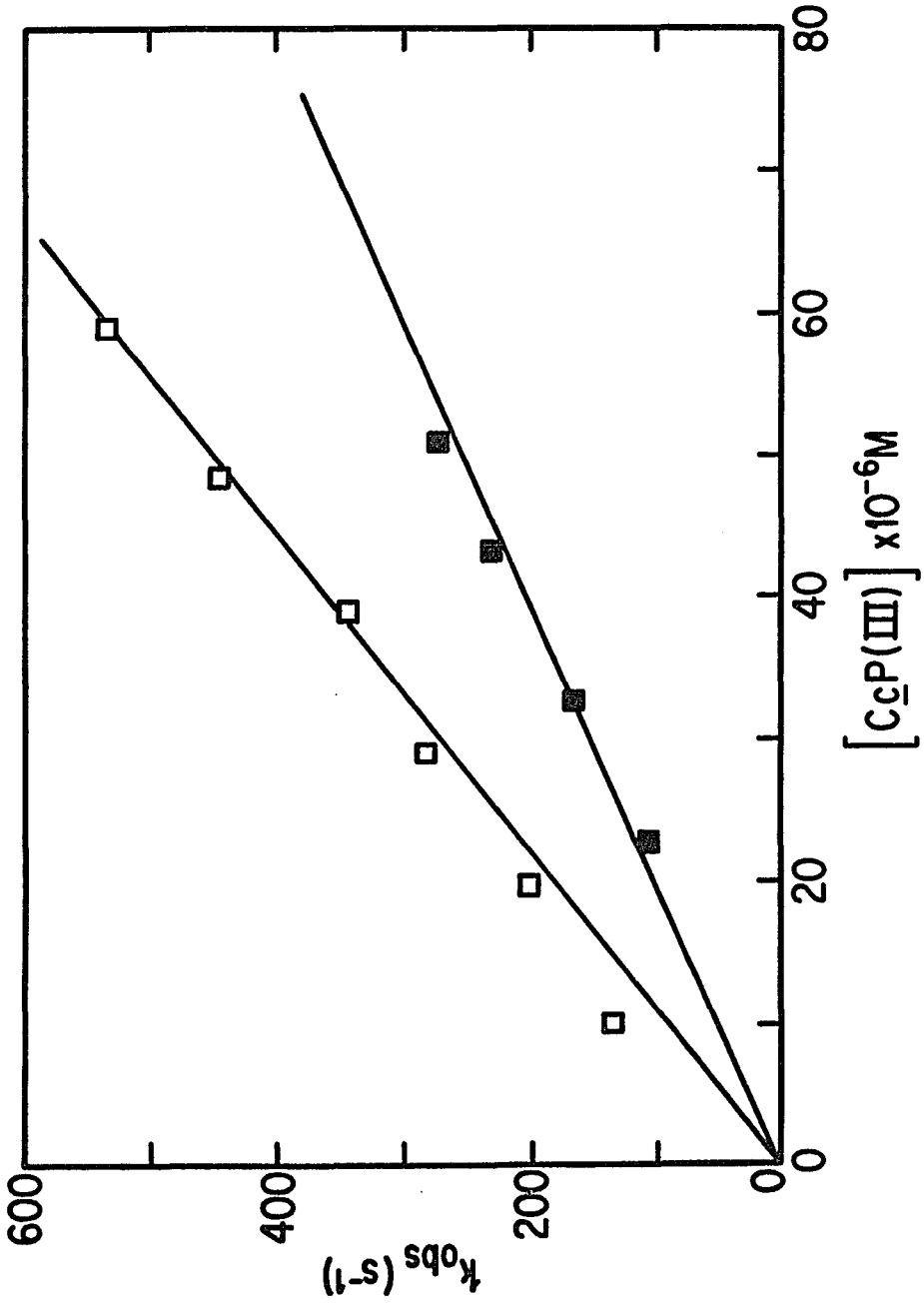


Figure II-3. Plots of k_{obs} vs. concentration for reduction of free (□) and horse cytochrome c(II)-complexed CcP(III) (■) by 5-DRFH· at μ = 8 mM.

Table II-3

Second-order Rate Constants for Reduction of Free and Cytochrome c(II)-Complexed CcP(III) and Cytochrome c_3 by 5-DRFH·

	$k_{II.2} \times 10^{-6}$, M^{-1} s^{-1}
CcP(III)	9[a]
CcP(III):Horse cyt c(II)	5[a]
Cyt c_3(Fe(III)	2400[b]

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

compared with the value of ~2 x 10^9 M^{-1} s^{-1} obtained by Meyer et al. (1983) for the tetraheme cytochrome $\underline{c}_3$, which has an average reduction potential between - 350 and -250 mV (Postgate, 1956; Hori & Kamen, 1961; Fan et al., 1989), which is somewhat less than that of CcP(III) (-190 mV). Based on redox potential alone, CcP(III) should have been more reactive than the cytochrome $\underline{c}_3$. Although the rate constant for cytochrome $\underline{c}_3$ 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 three important structural differences between the heme environments in cytochrome $\underline{c}_3$ and in CcP. First, all four of the cytochrome $\underline{c}_3$ hemes 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 $\underline{c}_3$ 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. Thirdly, because $\underline{c}_3$ is a small protein with four exposed hemes, one can expect electron transfer to occur over a greater proportion of the proteins surface area than would be the case with CcP where the heme is unsymmetrically buried within the protein matrix. As a result of the difference in the reactive surface area differences, there would be a greater likelihood of productive collisions between the reductant and the protein in the case of cytochrome $\underline{c}_3$. Therefore, the ~500-fold difference in the reduction rate constants for CcP(III) and cytochrome $\underline{c}_3$ probably reflects both the relative accessibility of the hemes to incoming reductants (Tollin et al., 1986a) and any structural changes which may occur in the peroxidase heme ligation sphere upon reduction and the differing reactive surface areas.

In the complex with horse cytochrome $\underline{c}$(II), the second-order rate constant for reduction of CcP(III) had a value of 5×10^6 M^{-1} s^{-1} (Table II-3), 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 $\underline{c}$ reduction by RFH· (89%), which has steric properties equivalent to 5-DRFH·. Thus, we conclude that access to the peroxidase heme is much less

affected by complex formation than is access to the horse and yeast cytochrome hemes; this will be addressed in more detail below. This is as expected based on the relative distances of the hemes from the surface in the free proteins.

Reduction Kinetics of Free and Complexed CcP(IV,$R^{+}\cdot$) 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 been oxidized (Peisach et al., 1968; Wittenberg et al., 1968; Yonetani, 1976; Hoffman et al., 1981; Ho et al., 1983; Horio & Yonetani, 1985; Myers & Palmer, 1985; Goodin et al., 1986). Although the reduction potential of the Fe(IV)/Fe(III) couple has not been directly measured, it has been approximated to be +1 V from calculations made from the kinetics of reduction of the peroxidase Compound II species, which is believed to contain an Fe(IV) and a reduced amino acid residue, R, by a variety of Fe(II) reductants at pH 5.2 (Purcell and Erman, 1976). The value is also close to that of horseradish peroxidase Compound I, ~1 V (Yamazaki, 1974), which contains an Fe(IV) and a porphyrin radical cation. Based on these determinations, the midpoint potential for the H_2O_2-oxidized species of CcP is appreciably higher than that of

cytochrome c (+260 mV) or CcP(III)(-190 mV). Therefore, we would expect to observe a rapid reduction of CcP(IV,$R^{+}\cdot$) by both LFH$\cdot$ and 5-DRFH$\cdot$.

Figure II-4a shows the transient decay curve obtained upon laser flash photolysis of a lumiflavin solution containing EDTA in the absence of either cytochrome c or CcP which is monitored at 550 nm. A rapid increase in signal, due to the formation of LFH$\cdot$, is followed by a non-exponential (second-order) decay back to the baseline prior to the laser flash due to semiquinone disproportionation. In the presence of CcP(IV,$R^{+}\cdot$), as shown in Figure II-4b, there is a small change in the shape of the decay curve which suggests that there is a slow reduction of CcP(IV,$R^{+}\cdot$). Based on 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,$R^{+}\cdot$) by LFH$\cdot$ of $\sim 10^{6}$ M^{-1} s^{-1}. (The kinetics of this reaction will be addressed in more detail in a subsequent chapter). Addition of horse cytochrome c(III) dramatically changes the characteristics of the transient decay curve, as illustrated in Figure II-4c. Within 3 ms after the laser flash, the decay curve goes markedly below the pre-flash baseline, indicating a much

Figure II-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 uM 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).

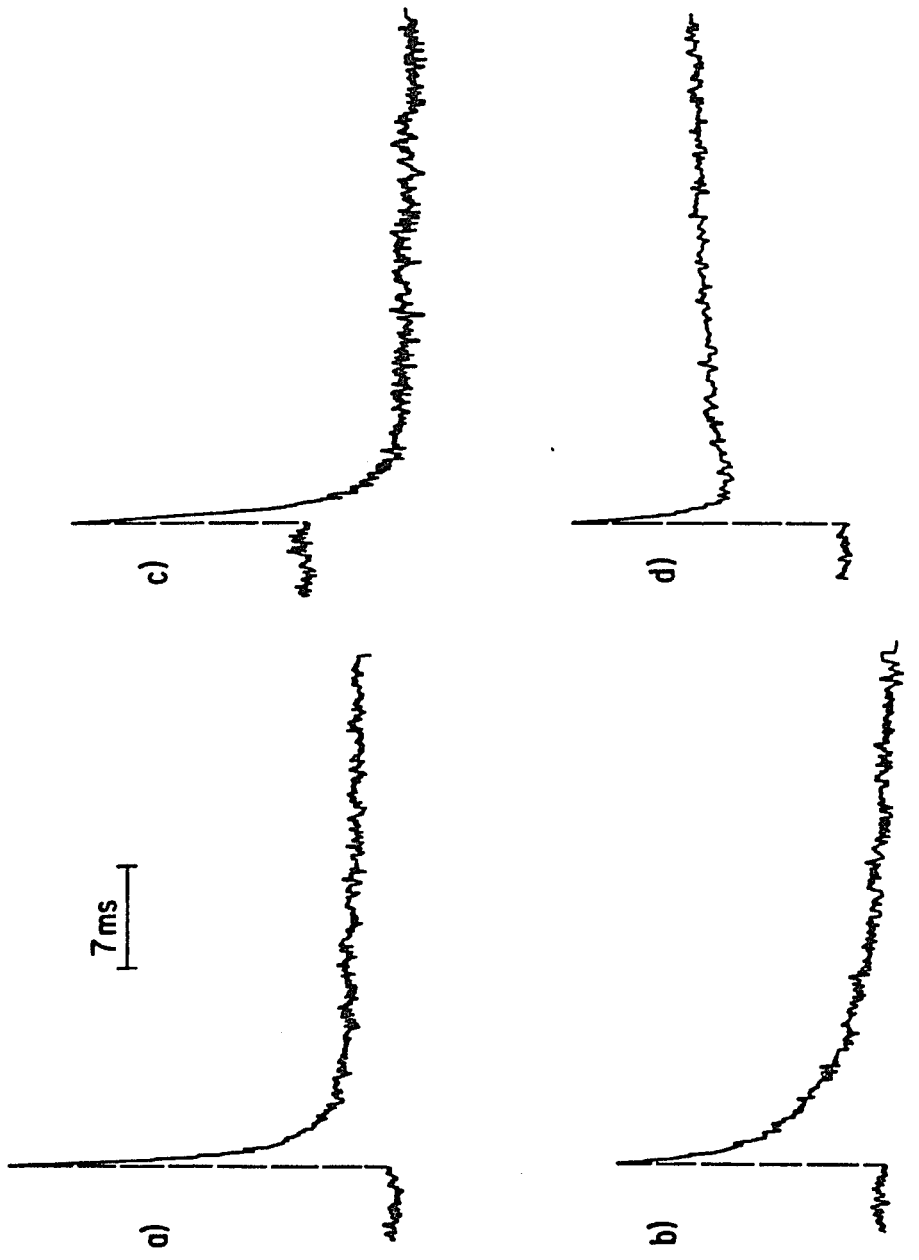


Figure II-4. Laser flash transient decay curves for LFH· disproportionation and cytochrome c and CcP(IV,R^{+}·) reduction monitored at 550 nm.

greater extent of reduction than observed with CcP(IV,$R^{+}\cdot$) alone. A net decrease in signal below the pre-flash baseline at 550 nm is consistent with the redox difference spectrum of CcP(III) minus CcP(IV,$R^{+}\cdot$) (Figure II-1b). Note also that the reaction in the case of the cytochrome c:CcP(IV,$R^{+}\cdot$) mixture is essentially complete within 10 ms, whereas the reaction with CcP(IV,$R^{+}\cdot$) 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, whereas in the reduction of CcP there is considerable contribution of the fully-reduced lumiflavin (Simondsen et al., 1982). For comparison, Figure II-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 pre-flash baseline and to the transient curve obtained with lumiflavin alone, consistent with the cytochrome c reduction difference spectrum shown in Figure II-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). Based on Figures II-4c and II-4d, we can conclude that the reduction of CcP(IV,$R^{+}\cdot$) by LFH$\cdot$ is greatly facilitated by the presence of cytochrome c. (Similar results utilizing 5-DRFH$\cdot$ have been obtained and

will be addressed in more detail in subsequent chapters.)

Two mechanisms for peroxidase reduction at low ionic strength in the presence of cytochrome c can be proposed. First, direct electron transfer from the flavin semiquinone to CcP(IV,$R^{+}\cdot$) could occur via a pathway which is induced by the complexation with cytochrome c(III), without any reduction of the cytochrome c. Second, an initial rapid reduction of cytochrome c by the flavin semiquinone could occur, which is followed by a rapid intracomplex one-electron transfer to CcP(IV,$R^{+}\cdot$):

$$\text{II.3)}\quad FH\cdot + \text{cyt } c(III):CcP(IV,R^{+}\cdot) \xrightarrow{k_{II.3}} F_{ox} + \text{cyt } c(II):CcP(IV,R^{+}\cdot)$$

$$\text{II.4)}\quad \text{cyt } c(II):CcP(IV,R^{+}\cdot) \xrightarrow{k_{II.4}} \text{cyt } c(III):CcP(III,R^{+}\cdot)$$

In either mechanism, no net reduction of cytochrome c would be observed, however, these two mechanisms would have other quite different kinetic characteristics. In the absence of any rate determining isomerization of the complex, the direct reduction mechanism should obey simple bimolecular kinetics, whereas the latter might show limiting first-order kinetics under conditions in which the rate constant for intracomplex electron transfer, $k_{II.4}$, was smaller than the (pseudo-first order) rate constant for

cytochrome c reduction by the flavin semiquinone. Furthermore, if there is little decrease in the reactivity of the cytochrome c heme due to complex formation, the limiting reaction in the second mechanism should not show any kinetic dependency on the particular flavin used as the reductant provided sufficiently high concentrations are reached. (It will be shown in subsequent chapters, that for reactions employing yeast cytochromes c, where there is a large effect of complexation on $k_{II.3}$, the choice of the flavin is critical to ensure that the pseudo-first order rate constant for reaction II.3 is > $k_{II.4}$).

In order to test these mechanisms, we measured the concentration dependencies for reduction of CcP(IV,$R^{+\cdot}$) in a 1:1 complex with horse cytochrome c by 5-DRFH· and LFH·. The kinetic plots from these experiments are shown in Figure II-5. For both flavin species, the value of k_{obs} at low ionic strength (Fig. II-5a) did not change over a wide range of protein concentrations. Furthermore, the value of k_{obs} was independent of the chemical nature of the flavin semiquinone. These results provide strong evidence that one-electron reduction of CcP(IV,$R^{+\cdot}$) at low μ 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. Similar concentration independent first-order kinetics were

Figure II-5. Plots of k_{obs} vs. concentration for reduction of CcP(IV,$R^{+}\cdot$) 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,$R^{+}\cdot$) in the complex with horse cytochrome c(III). LFH· reduction of CcP(IV,$R^{+}\cdot$) in 1:1 complexes with tuna (O) and yeast iso-2 (◧) cytochromes. LFH· reduction of CcP(IV) (Δ) in 1:1 complex with horse cytochrome c(III).

(b) Reduction of CcP(IV,$R^{+}\cdot$) 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.

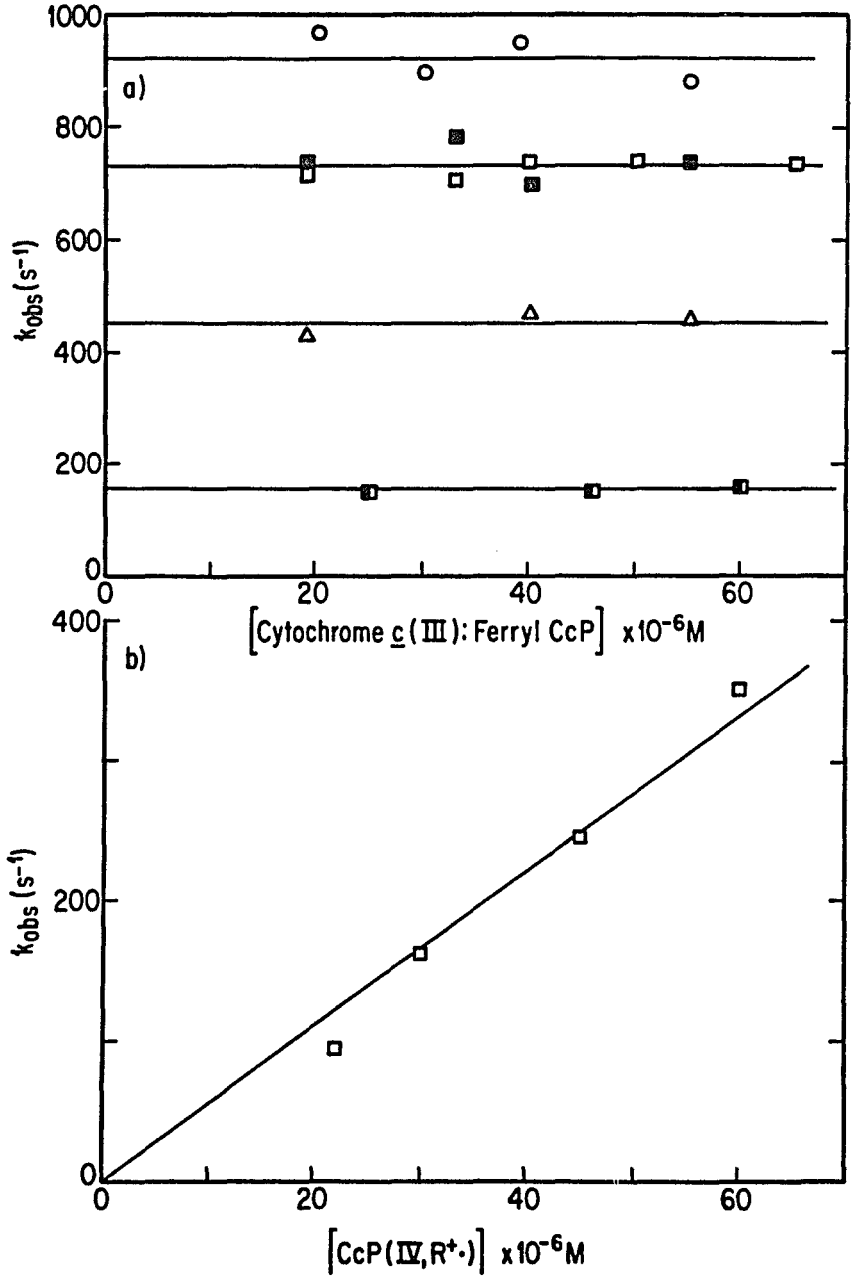


Figure II-5. Plots of k_{obs} vs. concentration for reduction of CcP(IV,R^{+}·) and CcP(IV) in 1:1 complexes with cytochromes c(III) by 5-DRFH· and LFH·.

Table II-4

First-order Rate Constants for Intracomplex Electron Transfer from Cytochrome c(III) to CcP(IV,$R^{+}\cdot$) and CcP(IV).

	$k_{II.4}$ (s^{-1})[a]	
	LFH·	5-DRFH·
CcP(IV,$R^{+}\cdot$):Horse cyt c(III)	730	730
CcP(IV,$R^{+}\cdot$):Tuna cyt c(III)	920	---
CcP(IV,$R^{+}\cdot$):Iso-2 cyt c(III)	150	---
CcP(IV):Horse cyt c(III)	450	---

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

observed for complexes prepared with tuna and yeast iso-2 cytochromes, and are also shown in Figure II-5a The rate constants obtained from these data are given in Table II-5. 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 versus 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,$R^{+}\cdot$) 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 reactions, which are in essence reverse electron transfer reactions relative to the physiological reaction and the reactions described in these studies, the rate constants for yeast cytochrome c reduction are approximately ten

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,$R^{+}\cdot$) 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 significant reduction of CcP(IV,$R^{+}\cdot$) could again be achieved only in the presence of cytochrome c. As shown in Figure II-5b, the linear increase in k_{obs} 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,$R^{+}\cdot$) 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, using LFH$\cdot$ as the reducing species 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 (Fig. II-5b). The ability to resolve the first-

order intracomplex electron transfer reaction at higher ionic strengths was achieved using the more powerful reductant 5-DRFH·, the subject of which will be addressed in more detail in subsequent chapters.

Another aspect of the peroxide-oxidized CcP species which 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) to an oxy-ferryl species occurs, without oxidation of any amino acid group(s) based upon the absence of a diagnostic g = 2 EPR signal. Based on these spectral properties, it was suggested by these workers that this form of CcP corresponded to what is commonly referred to as Compound II, that is the one electron reduction product of Compound I. As shown in Figure II-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 two-fold decrease in the intracomplex rate constant

for CcP(IV) relative to Ccp(IV,$R^{+}\cdot$) was found, the oxidized R-group(s) is apparently not tightly coupled to the peroxidase heme and does not have a dominating effect 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 which undergoes oxidation, as suggested by Hoffman and coworkers (1979; 1981) based on 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,$R^{+}\cdot$). Furthermore, very little change in the visible or EPR spectrum of CcP(IV,$R^{+}\cdot$) 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 the present 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 illustrates the necessity to take into account such factors as protein dynamics and species-dependent changes in cytochrome structure which 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, 1984).

Figure II-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 channel. 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 towards 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 II-6a) would

Figure II-6. Stereo-views 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-Me group of LFH· to the thioether bridging sulfur of Cys-17(C) is 3.8 Å.

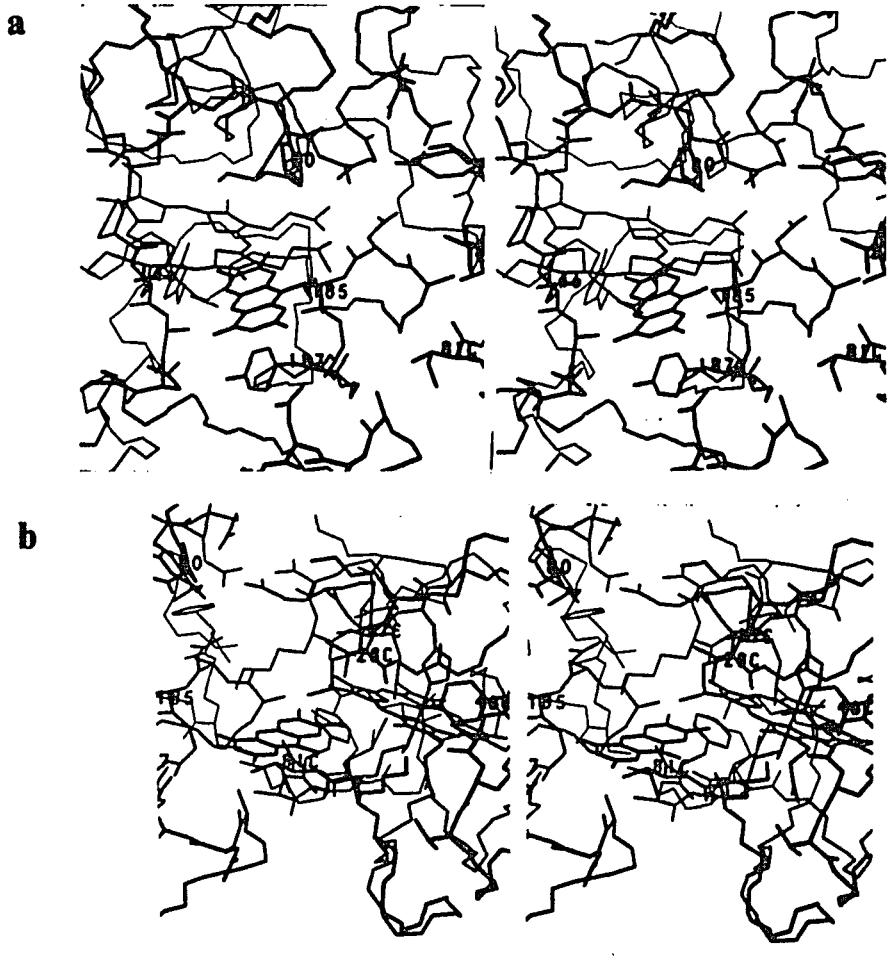


Figure II-6. Stereo-views of the interaction of LFH· with the hypothetical tuna cytochrome c:yeast CcP complex.

decrease the possibility of inserting the flavin ring as deeply in the access channel as is shown in Figure II-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 which lead to rapid 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 which was observed upon complexation with cytochrome c may reflect a change in either the protein structure or in 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. Based on 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 which 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 in crystals.

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,$R^{+\cdot}$) and CcP (IV). Based on the CcP(III) reduction kinetics and the expected increase in $*E_{m,7}$ due to the peroxide reaction (see above), we would have predicted a rapid reduction of both CcP(IV,$R^{+\cdot}$)and CcP (IV) by flavin semiquinones (Meyer et al., 1983; Tollin et al., 1986a). 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,$R^{+\cdot}$) 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 non-specific 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,$R^{+\cdot}$) reduction, electron transfer from flavin to oxidase occurred via cytochrome c rather than by direct interaction. In similar studies, Bickar et al. (1985) have suggested that cytochrome c facilitates an increase in the rate of reduction of the cytochrome a component of cytochrome c oxidase using dithionite, NADH, or ascorbate as well as an increase of the electron transfer from the cytochrome a to the cytochrome a_3 component of cytochrome c oxidase. 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 non-specific 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 & Finzel, 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 cytochromes 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 II-4), 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.

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 II-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., 1986b) 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., 1986b). In the complex model shown in Figure II-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.

Figures II-7a and II-7b show the residues of tuna cytochrome c which are substituted in horse and yeast cytochromes, respectively. These changes are listed in Table II-5. It should be noted that the number of residues occurring directly in the interprotein interface for yeast cytochrome is much greater than 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.

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

Figure II-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 II-6.

(a) Amino acid substitutions of horse, relative to tuna, cytochrome c.

(b) Amino acid substitutions of yeast iso-2, relative to tuna, cytochrome c.

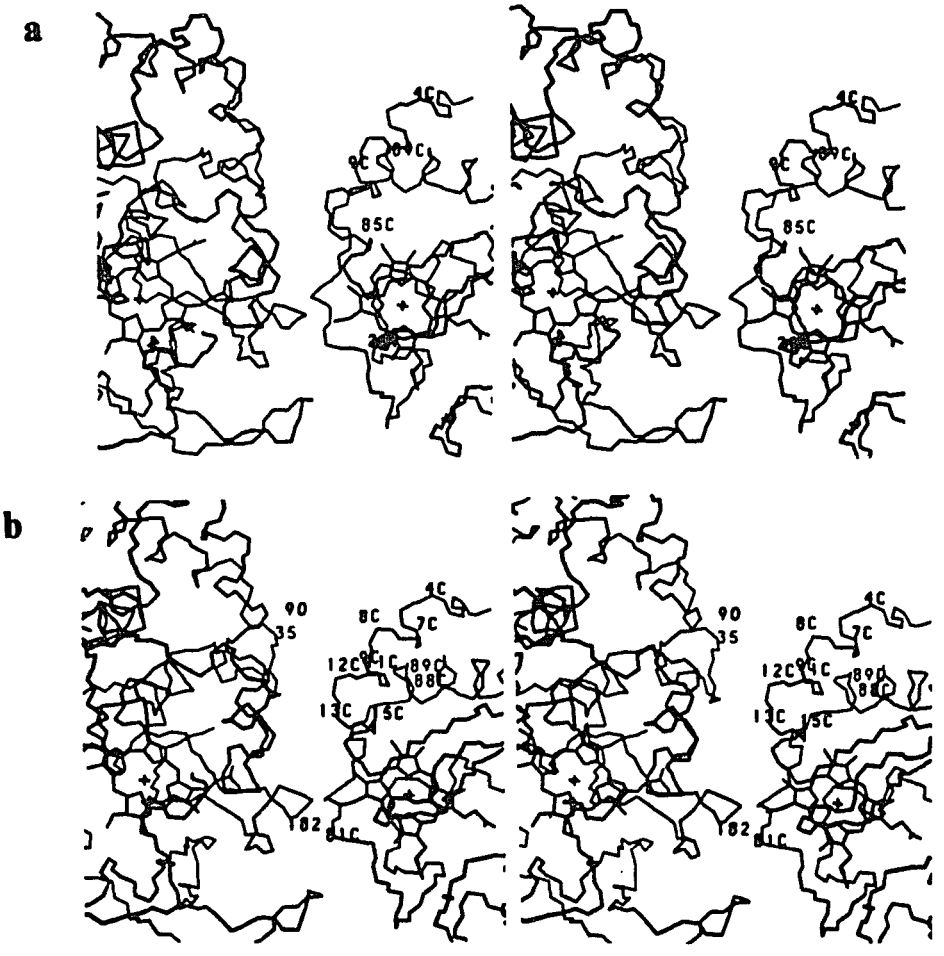


Figure II-7. Orientations of amino-acid substitutions of horse and yeast cytochromes c, relative to tuna cytochrome c, within the hypothetical cytochrome c:CcP complex.

Table II-5

Differences in Amino Acid Composition at the Interface of the Hypothetical Electron Transfer Complex for Tuna, Horse, and Yeast Cytcohromes c.

Tuna Residue No. and Type[a]	Amino Acid Substitution[b] 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	Glu

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

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 which 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 (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 as well as the distance or the electron transfer conduit between the two proteins.

To summarize, although these kinetic results are in general 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 which

are in rapid equilibrium with one another. The following chapters will describe experiments designed to directly test this latter point.

CHAPTER III

KINETICS OF INTRACOMPLEX ELECTRON TRANSFER AND OF REDUCTION OF THE COMPONENTS OF COVALENT AND NON-COVALENT COMPLEXES OF CYTOCHROME C AND CYTOCHROME C PEROXIDASE BY FREE FLAVIN SEMIQUINONES.

Introduction

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 using the water soluble carbodiimide EDC (Waldmeyer et al., 1982; Waldmeyer and 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 and Bosshard, 1985; Bechtold and Bosshard, 1985; Erman et al., 1987; Moench et al., 1987). In this chapter a comparison is made 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 which is impeded by the formation of covalent cross-links.

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 within 1:1 electrostatic complexes with CcP was investigated using the kinetics of cytochrome reduction by the exogenous neutral lumiflavin semiquinone (LFH·) as a probe. Figure III-1a shows a plot of k_{obs} vs. [complex] for the reduction of horse cytochrome c(III) by LFH· in a covalently cross-linked complex with CcP (III). The value for the second-order reduction rate constant obtained from this plot, $k_{II.1} = 2.8 \times 10^7\ M^{-1}\ s^{-1}$, is given in Table III-1. Also given in Table III-1 are the rate constants for reduction of free and electrostatically-complexed horse cytochrome c (cf. also Fig. III-1a) under the same conditions derived from the data of Figure II-1. The rate constant for reduction of the cross-linked cytochrome c is 1.5 times larger than the value obtained for the electrostatic complex, but is still significantly smaller than the value for free cytochrome c reduction. These results indicate that there is a slightly greater degree of accessibility of the cytochrome 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 LFH· reduction

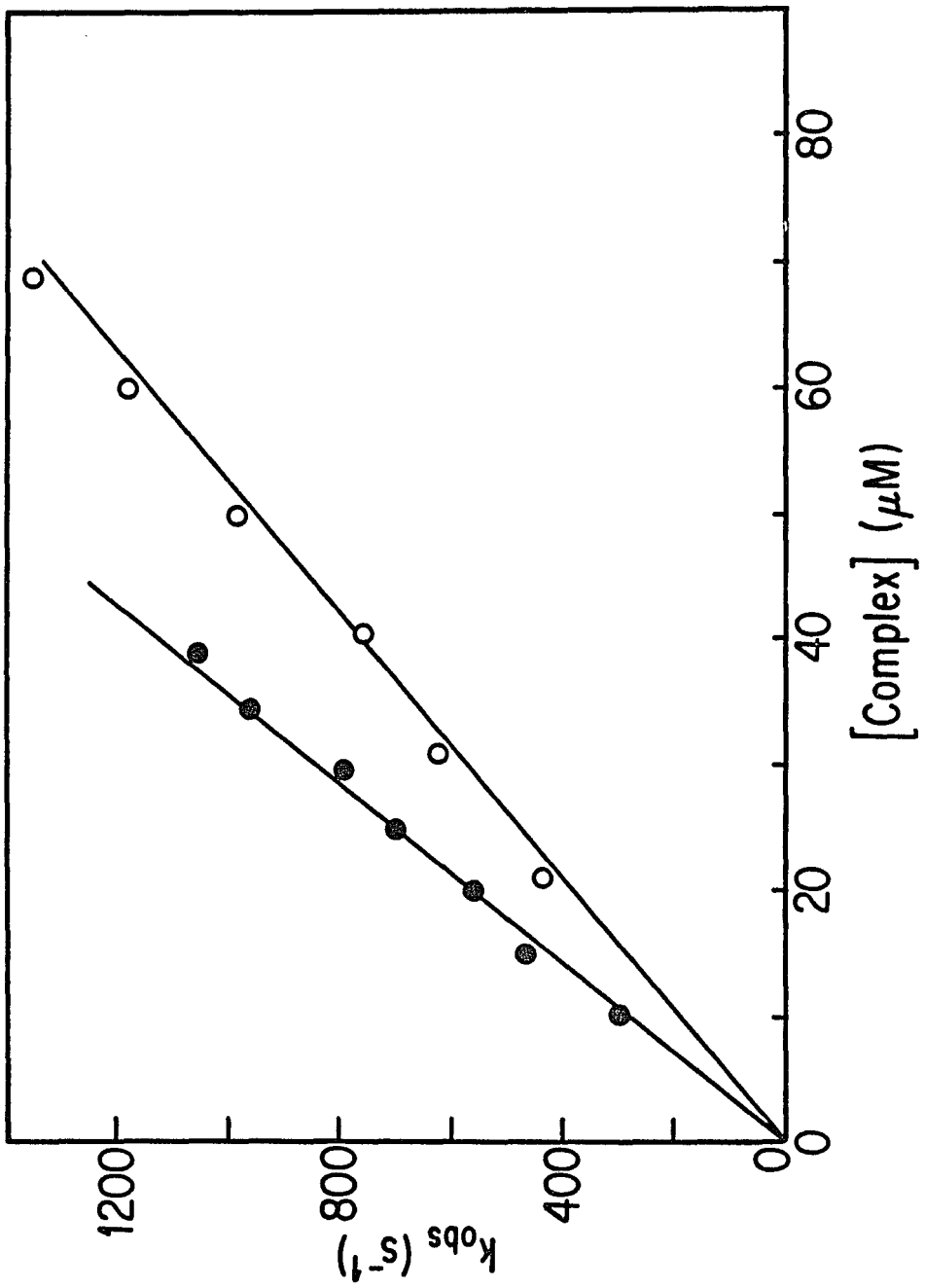


Figure III-1. Plots of k_{obs} vs. concentration for reduction of horse cytochrome c(III) in 1:1 electrostatic (O) and covalent (●) complexes with CcP(III) by LFH· at μ = 8 mM.

Table III-1

Second-order Rate Constants for Reduction of Free and CcP-Complexed Cytochrome c by LFH·

cyt c species	$k_{II.1} \times 10^{-7}$ (M^{-1} s^{-1})
free	7.2
electrostatic complex	1.9
covalent complex	2.8

[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 cytochrome c from Chapter II.

kinetics is able to assess, the overall structural geometry of the covalent complex must be similar, but not identical to, 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.

Intracomplex Electron Transfer from Cytochrome c(II) to CcP(Fe(IV),$R^{+\cdot}$).

Of special interest is the comparison of the rate of intracomplex electron transfer from cytochrome c(II) to CcP(Fe(IV),$R^{+\cdot}$) between the cross-linked and electrostatic complexes. It has been shown in Chapter II that CcP(Fe(IV),$R^{+\cdot}$) is not readily reduced by free flavin semiquinones. However, to reiterate, in the presence of cytochrome c at μ = 8 mM, i.e. an ionic strength that favors the formation of an electrostatically-stabilized complex, a one-electron reduction of the ferryl species of CcP can be achieved by the mechanism given in equations II.3 and II.4. The studies with the electrostatic complex described in Chapter II indicated that at complex concentrations $\geq$ 20 μM, the rate-limiting step was the electron transfer from ferrous cytochrome c to Compound II. 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 which will be addressed

in more detail below), the covalent complex should inherently be less affected by ionic strength changes. The plots of k_{obs} vs. complex concentration, shown in Figure III-2, for the reduction of covalently-complexed CcP(IV,$R^{+}\cdot$) at μ = 8 and 30 mM in fact verify this proposal. The data obtained at both ionic strengths are essentially the same for the covalent complex. For this species the k_{obs} vs. concentration plot is nonlinear, suggesting that at low complex concentrations the bimolecular reduction of complexed cytochrome c(III) by flavin semiquinone, i.e. equation II.3, is rate-limiting. Table III-2 lists values for the two rate constants obtained by computer modelling using the mechanism given in equations II.3 and II.4 (see below for a more detailed description of the computer modeling). 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 two-fold 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; $\mu \leq$ 10 mM) stabilized the two proteins in a more reactive state compared to the electrostatic complex under these experimental conditions.

Figure III-2. Plots of kobs vs. concentration for reduction of CcP(IV,$R^{+}\cdot$) by cytochrome c(II) for the electrostatic and covalent complexes.

(O) reduction of CcP(IV,$R^{+}\cdot$) in the covalent complex at μ = 8 mM. (●) reduction of CcP(IV,$R^{+}\cdot$) in the covalent complex at μ = 30 mM. For (O , ●) LfH$\cdot$ was used to reduce cytochrome c(III). (▲) titration of cytochrome c(II) ($\leq$ 0.1 μM) by CcP(IV,$R^{+}\cdot$) at μ = 30 mM; 5-DRfH$\cdot$ was used for reduction of cytochrome c(III). CcP(IV,$R^{+}\cdot$) reduction was monitored at 550 nm.

Insert: Reduction of horse cytochrome c(III) by LfH$\cdot$ in the 1:1 electrostatic complex with CcP(IV,$R^{+}\cdot$) at μ = 8 mM. Cytochrome c(III) reduction was monitored at 510 nm. Reaction solutions were prepared using 3 mM 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.

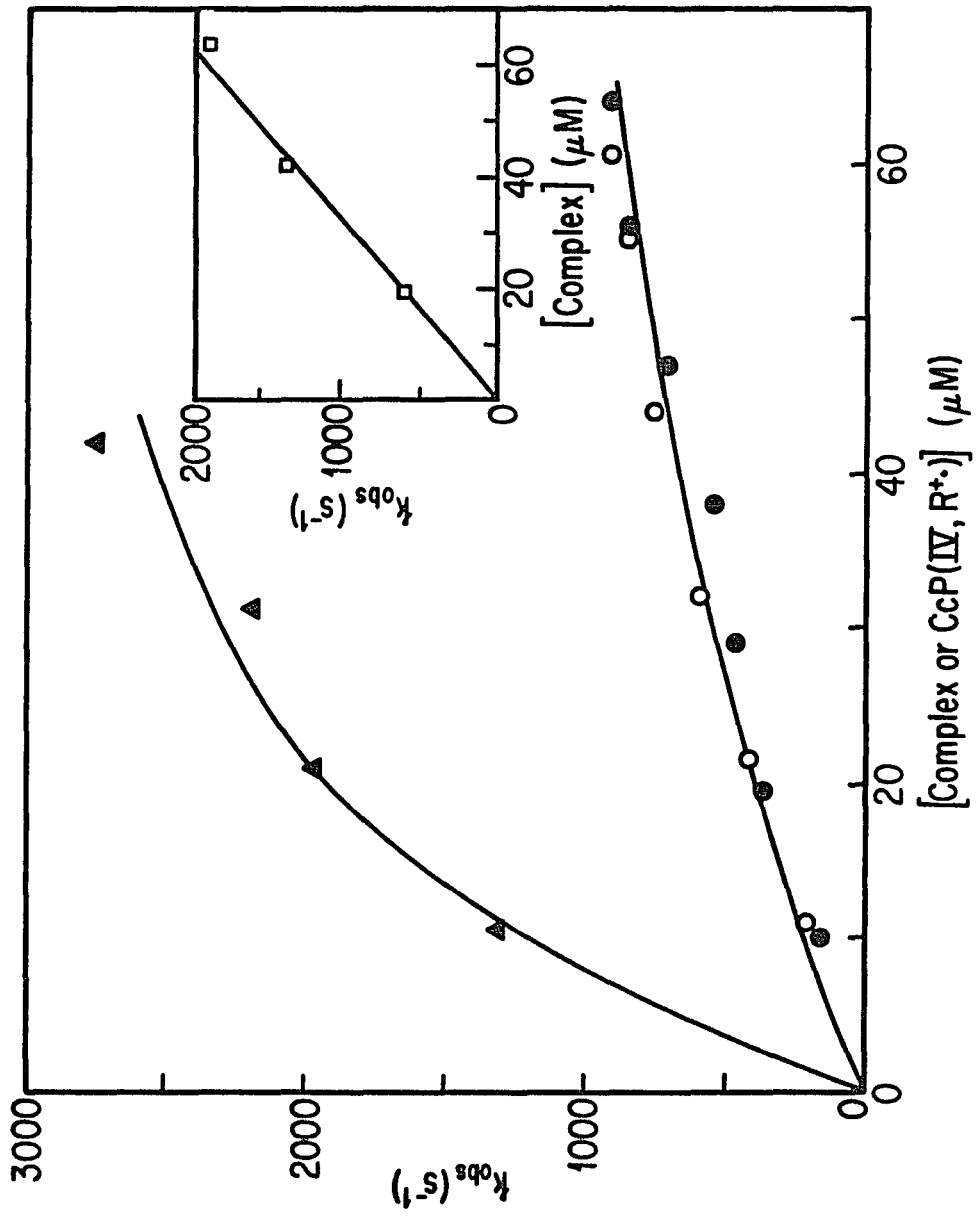


Figure III-2. Plots of kobs vs. concentration for reduction of CcP(IV,$R^{+\cdot}$) by cytochrome c(II) for the electrostatic and covalent complexes.

Table III-2

Second-order and Intracomplex Electron Transfer Rate Constants for Reduction of the Cytochrome c:CcP(IV,$R^{+}\cdot$) Complexes[a]

Complex species	μ (mM)	$k_{II.3}$[b] x 10^{-7} (M^{-1} s^{-1})	$k_{II.4}$ or $k_{III.3}$[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 using LFH·. Data for the electrostatic complex at μ = 30 mM were obtained using 5-DRFH·.
[c] This value is from Chapter II.
[d] $k_{II.3}$ value is not obtainable from the data; however, $k_{III.2}$ = 1.3 x 10^{8} M^{-1} s^{-1}, which can be compared with the value of $k_{association}$ = 5.6 x 10^{8} M^{-1} s^{-1} reported from steady state analysis by Yonetani (1976) under somewhat different conditions.

We can not absolutely exclude the possibility of direct reduction of CcP(IV,$R^{+}\cdot$) 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$\cdot$ ($E_{m,7}$ = -630 mV) or LFH$\cdot$ ($E_{m,7}$ = -230 mV) at ionic strengths of 8, 30 or 280 mM. On the other hand, it has been demonstrated that in the covalent complex CcP is more reactive towards H_2O_2 than native CcP (Erman et al., 1987).

The cytochrome c heme accessibility in the covalent complex with CcP(IV,$R^{+}\cdot$) is apparently the same as in the covalent complex with the ferric peroxidase, based on the values for the second-order reduction rate constants given in Tables III-1 and III-2 ($k_{II.1}$ and $k_{II.3}$, respectively). The insert in Figure III-2 shows a plot of k_{obs} vs [complex] concentration for the reduction of cytochrome c by LFH$\cdot$ in the electrostatic complex with CcP(IV,$R^{+}\cdot$) at μ = 8 mM. The rate constant obtained from this plot (3 x 10^7 M^{-1} s^{-1}) is given in Table III-2. There is a small increase (~50%) in the second-order reduction rate constant compared with that for the CcP(III) complex (Table III-1), which implies an increase in accessibility of the cytochrome 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, 2.8 x 10^7 M^{-1} s^{-1} for the CcP(III) complex vs. 2.7 x 10^7 M^{-1} s^{-1} for the CcP(IV,$R^{+}\cdot$) complex. 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 cytochrome c are markedly different from those obtained using yeast iso-1 and iso-2 cytochromes c, in which the rate constants for reduction of the CcP(IV,$R^{+}\cdot$)-complexed cytochrome c were five times that for cytochrome c in the complex with CcP(III) (see Cusanovich et al., 1988b and Chapter IV below). It appears, therefore, that the change in accessibility of the cytochrome 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 cytochrome 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,$R^{+}\cdot$) complexes are approximately the same (~3 x 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 cytochrome c accessibility upon

H_2O_2 peroxidation 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 which indicate a change in the orientation of the anti-parallel beta sheet of CcP from residues 175 to 190 upon conversion to the ferryl species (Edwards et al., 1987). In the Poulos-Kraut model (Poulos and Kraut, 1980; Poulos and Finzel, 1984) this region lies adjacent to the exposed edge of the cytochrome c heme (specifically the thioether bridge of Cys-17 which has been suggested to be involved in electron transfer into the cytochrome c heme (Tollin et al., 1986b)). The observed variations in heme accessibility due either to the cytochrome 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 as suggested in Chapter II.

Figure III-2 also shows that for the electrostatic complex formed between horse cytochrome c and Compound I at higher ionic strength, μ = 30 mM, there is a non-linear relationship between k_{obs} and CcP(IV,$R^{+}\cdot$) concentration. Thus, as Compound I concentration increases, k_{obs} approaches a limiting value. The simplest interpretation of the data presented for the electrostatic complex is the

following mechanism:

III.1) $FH^{\cdot} + \text{cyt } \underline{c}(III) \xrightarrow{k_{III.1}} F_{ox} + \text{cyt } \underline{c}(II)$

III.2) $\text{cyt } \underline{c}(II) + CcP(IV,R^{+\cdot}) \underset{k_{-III.2}}{\overset{k_{III.2}}{\rightleftharpoons}} \text{cyt } \underline{c}(II)\text{-}CcP(IV,R^{+\cdot})$

III.3) $\text{cyt } \underline{c}(II)\text{-}CcP(IV,R^{+\cdot}) \xrightarrow{k_{III.3}} \text{cyt } \underline{c}(III)\text{-}CcP(III,R+.)$

where F and $FH^{\cdot}$ correspond to the oxidized and semiquinone forms of a free flavin. Reaction III.1 is the same reaction as given in equation II.1, rewritten here for clarification. Reactions III.2 and III.3 correspond to the formation of and electron transfer within a transient complex. Based on the large reduction potential differences, the reverse reaction of equation III.3 can be neglected. Also, to ensure that reduction of cyt $\underline{c}$(III) (reaction III-1) would not be rate-limiting in the overall mechanism, 5-deazariboflavin semiquinone was used as a reductant since $k_{III.1} = 2 \times 10^9\ M^{-1}\ s^{-1}$ (Meyer et al., 1983), which is an order of magnitude larger than reported values for $k_{III.2}$ (Yonetani, 1976; see Table III-2 below). Finally, since the amount of cytochrome $\underline{c}$(II) generated per laser pulse is limited by the amount of 5-DRFH$^{\cdot}$ produced (typically $\leq 0.1\ \mu M$ (Simondsen and Tollin, 1983)), for CcP concentrations $\geq 10\ \mu M$ [cyt $\underline{c}$(II)] << [$CcP(IV,R^{+}.)$].

When the steady-state approximation is applied to the above mechanism, the following relationship can be written for k_{obs} (Strickland et al., 1975):

$$\text{III.4)}\quad k_{obs} = \frac{k_{III.2}k_{III.3}[CcP(IV,R^{+\cdot})]}{k_{III.2}[CcP(IV,R^{+\cdot})] + k_{-III.2} + k_{III.3}}$$

If one assumes that the reaction represents a rapid equilibrium formation of the electron transfer complex followed by electron transfer (i.e., $k_{III.3} << k_{III.2}[CcP(IV,R^{+\cdot})] + k_{-III.2}$), then saturation behavior will be observed and under these conditions equation III.4 reduces to:

$$\text{III.5)}\quad k_{obs} = \frac{k_{III.2}k_{III.3}[CcP(IV,R^{+\cdot})]}{k_{III.2}[CcP(IV,R^{+\cdot})] + k_{-III.2}}$$

At low concentrations of $CcP(IV,R^{+\cdot})$, k_{obs} should be approximately equal to $K_{III.2}k_{III.3}[CcP(IV,R^{+\cdot})]$, whereas $k_{obs} = k_{III.3}$ when $CcP(IV,R^{+\cdot})$ is in such large excess that essentially all the cytochrome c(II) is complexed, i.e. reaction III.3 becomes rate-limiting.

The data for k_{obs} as a function of Compound I concentration were modeled by treating the mechanism as two consecutive first-order reactions (since all reactions were

conducted under pseudo-first-order conditions) and programming an exact solution for the rate equations (Frost and Pearson, 1961). A constraint that any possible solution was > 90% monophasic was imposed on the data fitting process. The program used a steepest descent procedure to obtain a minimum least-squares error in fitting the experimental data. Values for $K_{III.2}$ (= $k_{III.2}/k_{-III.2}$) and $k_{III.3}$ can be determined directly, whereas only a minimum value can be specified for $k_{III.2}$. Any value larger than the reported $k_{III.2}$ gives identical fits, within experimental error, when $K_{III.2}$ is held constant. The values for $k_{III.3}$ derived from this procedure are also reported in Table III-2.

The solid line through the data points in Figure III-2 corresponds to the best fit to the data from computer modeling. 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 $k_{III.3}$ at μ = 30 mM is at least two 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 which lead to a stable 1:1

complex at very low ionic strengths must be weakened in order to get higher rates of electron transfer. Another explanation which may be proposed for the increase in the intracomplex electron transfer rate constant 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, cytochrome c electrostatic complex formation 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,$R^{+}\cdot$), 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 cytochrome c, relative to the free state, or a large decrease in the redox potential for CcP(IV,$R^{+}\cdot$). This seems unlikely, although further work needs to be done to explore the possible contribution of such effects).

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 $k_{II.4}$ for the covalent complex is larger than the value of $k_{II.4}$ for the electrostatic complex at μ = 8 mM is a further indication that cross-linking alters the reactivity of the two proteins within the complex, either due to a change in relative orientations, intrinsic reactivity of CcP, and/or changes in net electrostatic charge at the intermolecular interface due to EDC modification.

These results concerning the different effects of ionic strength on the rate constants for electron transfer within the non-covalent and covalent complexes of horse cytochrome c and native CcP suggest 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 apparently is

"frozen" in an orientation which is somewhere between the low and high ionic strength non-covalent complexes.

The ionic strength dependency of $k_{III.3}$ 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 steady-state 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 $k_{III.3}$, suggesting that in the steady-state experiment, dissociation of the first cytochrome c molecule which reduces CcP(IV,$R^{+}\cdot$) 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.

Figures III-3a and III-3b show top and side views, respectively, of the Poulos-Kraut hypothetical complex (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

Figure III-3. Computer graphic representations of the Poulos-Kraut model for the cytochrome c:CcP complex.

Both CcP (left) and cytochrome 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 cytochrome c are shown. Covalent cross-links between CcP and cytochrome 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.

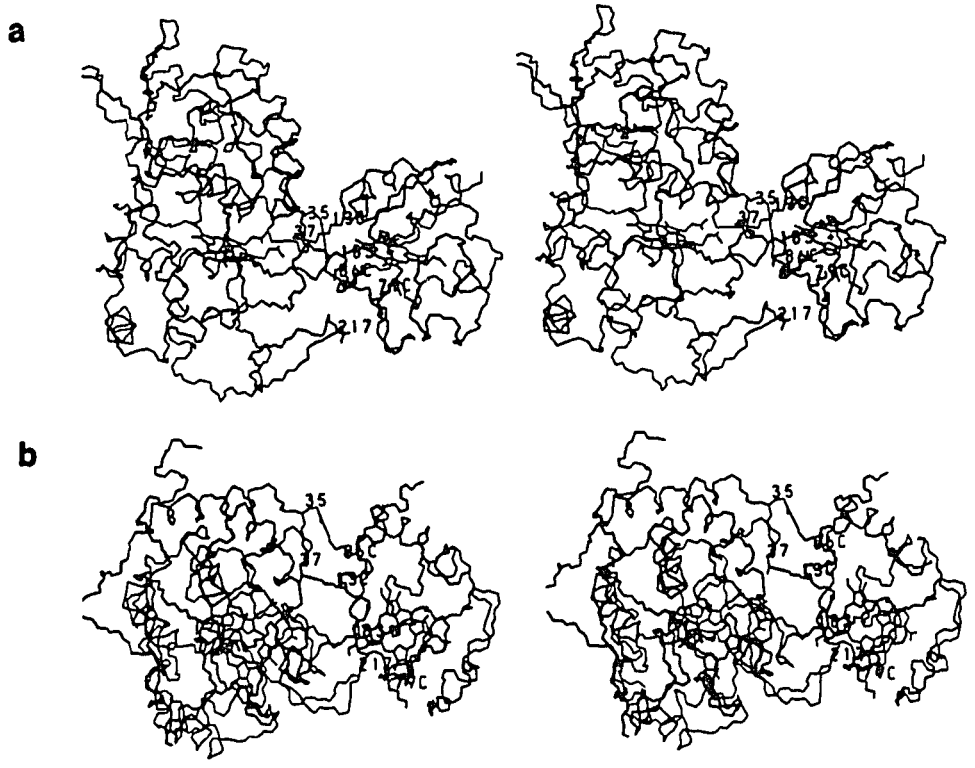


Figure III-3. Computer graphic representations of the Poulos-Kraut model for the cytochrome c:CcP complex.

the specific acidic residues involved in cross-linking). Note that the two covalent cross-links are located on the side of the cytochrome c heme which is less accessible to solvent. Thus, our results with LFH· 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.

CHAPTER IV

EFFECTS OF AMINO ACID REPLACEMENTS IN YEAST ISO-1-CYTOCHROME C ON HEME ACCESSIBILITY AND INTRACOMPLEX ELECTRON TRANSFER IN COMPLEXES WITH NATIVE CYTOCHROME C PEROXIDASE.

Introduction

In Chapter II differences in the properties of electrostatically-stabilized complexes of tuna, horse, and yeast iso-2 cytochromes c and CcP were observed. Most importantly, large differences between the rate constants for electron transfer from the ferrous cytochromes c and Compound I of CcP were observed at low ionic strength, with yeast iso-2 cytochrome c having the smallest value. It was also suggested that there was a significant difference in the accessibilities of the cytochrome hemes in the complexes with CcP. Furthermore, data was presented in Chapter III which suggested that the electrostatically-stabilized complex which is formed at low μ is not a complex is not optimal for electron transfer. This was supported by the fact that when cytochrome c and Compound I are covalently cross-linked there is an apparent decrease in the ability of the two proteins to rearrange and find more favorable orientations at higher ionic strength. In order to clarify these unexpected results, further studies were carried out using the physiological redox partner of CcP, yeast iso-1 cytochrome c_1 expressed by a variety of yeast of randomly generated revertant mutants in which single amino acid replacements are found, or as modified by site-directed mutagenesis. The cytochrome c species chosen for these investigations were those which have

substitutions in residues which have been suggested to be important in the formation of the electron transfer complex with CcP. In the chapter which follows, data are presented which support the hypothesis given in Chapter III, that the criteria for construction of the Poulos-Kraut hypothetical model might lead to an oversimplification of the actual variables involved in the *in vivo* electron transfer complex.

Results

A listing of the five iso-1-cyt c species, produced either in the randomly generated revertant mutants or by site-specific mutagenesis, investigated in this chapter are shown in Table IV-1. Figure IV-1 shows the placement of these residues within the Poulos-Kraut hypothetical model, the importance of which shall be discussed below in more detail. It should be noted that 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 5 and 9 amino acids, respectively, at the N-terminus. We have chosen the numbering system of the vertebrate cytochromes c in order to designate the locations of the mutation sites. We have also chosen to designate the mutant proteins according to the convention explained in Footnote c of Table IV-1. The R13I, K27Q and K72D proteins are replacements of basic Lys and Arg residues that are supposedly important in the binding of cytochrome c to CcP. The Q16S and Q16K 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., 1988a).

As in the previous chapters, the small electrostatically

Table IV-1

Amino Acid Replacements of iso-1-cytochrome c

Strain #	Gene symbol[a], amino acid[b], and codon		Designation[c]	Ref.[d]
	Wild Type	Mutant		
D-334	CYC1±	CYC1-2-c[e]	Q16S	1
	Gln16	Ser16		
	CAA	UCA		
B-609	CYC1±	CYC1-2-I[e]	Q16K	1
	Gln16	Lys16		
	CAA	AAA		
B-6591	CYC1±	CYC1-75-F[e]	R13I	2
	Arg13	Ile13		
	AGA	AUA		
B-1877	CYC1±	CYC1-177-A[e]	K27Q	2
	Lys27	Gln27		
	AAG	CAG		
B-6842	CYC1±	CYC1-785[f]	K72D	3
	Lys72	Asp72		
	AAG	GAU		

[a] CYC1± is the wild type yeast iso-1-cytochrome c.
[b] Residue numbers are based upon tuna cytochrome c, using the cysteine residues which form the thioether linkage with the heme as a common frame of reference. Yeast iso-1 cytochrome c has five additional residues at the N-terminus.
[c] The designation used specifies the one letter code for the wild-type amino acid residue, the residue number, and the residue in the mutant cytochrome. Hence, Q16S, represents the conversion of Gln-16 to Ser in the mutant protein.
[d] References: 1 Sherman & Stewart (1974); 2 Hazzard et al., 1988b; 3 T.S. Cardillo & F. Sherman, unpublished results.
[e] These are randomly generated revertant mutants.
[f] This is a site-directed mutant species.

Figure IV-1. Computer graphic representations of the Poulos-Kraut model for the cytochrome c:CcP complex. Sites of specific amino acid replacement are indicated on cytochrome c.

(a) Side view of the complex showing the orientation of cytochrome c (right) relative to CcP (left).

(b) Close-up perspective of the interaction domain viewed from above. Note that (b) is related to (a) by a 180^{o} rotation about a vertical axis between the two proteins.

(c) Close-up oblique view of the interaction domain shown in (b).

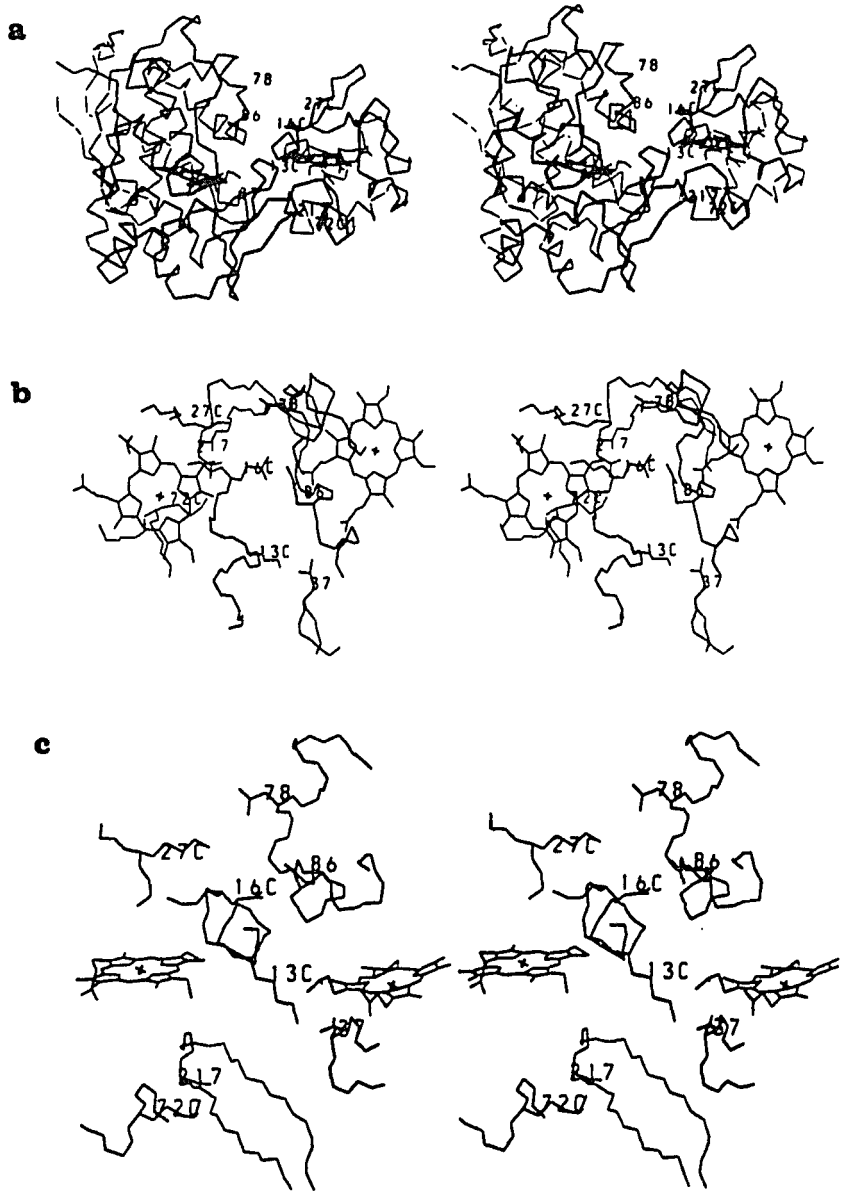


Figure IV-1. Computer graphic representations of the Poulos-Kraut model for the cytochrome c:CcP complex. Sites of specific amino acid replacement are indicated on cytochrome c.

neutral lumiflavin semiquinone, LFH·, has been used as a reductant in order to determine the relative accessibilities of the prosthetic groups of free and CcP-complexed yeast iso-1-cytochrome c. To reiterate, in cases where accessibility is very small, disproportionation of the flavin semiquinone yields a fully-reduced species, LFH^-, which can, in turn, react with the redox protein, albeit with rate constants of significantly smaller magnitude than those obtained with the semiquinone species (Meyer et al., 1983). Second-order rate constants for reduction of the wild-type iso-1 and the five mutant cytochromes c in the free state by LFH· are given in Table IV-2. For comparative purposes, the rate constant for free iso-2-cytochrome c is also included in Table IV-2. Iso-2-cytochrome c is significantly more reactive towards LFH· than is iso-1-cytochrome 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 cytochrome c heme reactivity with LFH· are observed. The second-order reduction rate constants are presented in Table IV-2. In the case of the complexed iso-1-cytochrome c, at the concentrations used, the reaction of LFH· with the cytochrome is not able to compete with LFH·

Table IV-2

Rate constants for reduction of free and complexed cytochromes by reduced lumiflavin species

	$k \times 10^{-7}$ (M^{-1} s^{-1})[a]			
	Free	Complexed		
		CcP(III)[b]		CcP(IV,$R^{+\cdot}$)[c]
Cytochrome	LFH$\cdot$	LFH$\cdot$	LFH^-	LFH$\cdot$
iso-1 (w.t.)	3.5 ± 0.2	< 0.5	0.1	2.4
iso-2	5.3[d]	0.5[d]	0.2	3.6
Q16S	3.4 ± 0.1	< 0.5	0.1	2.5
Q16K	3.4 ± 0.3	< 0.5	0.3	1.1
R13I	4.1 ± 0.2	1.4	---	4.1
K27Q	4.1 ± 0.2	0.7	---	3.5
K72D	3.0 ± 0.2	< 0.5	---	3.5

Experiments were performed in a 3 mM phosphate buffer containing 0.5 mM EDTA and 50 μM lumiflavin (μ = 8 mM).
a- The rate constants for free and CcP(III) complexed cytochromes *c* correspond to $k_{II.1}$; for the complex with CcP(IV,$R^{+\cdot}$) the rate constant is $k_{II.3}$.
b- LFH$\cdot$ values determined at a single protein concentration (30 μM); LFH^- values determined at several protein concentrations.
c- rate constants for LFH$\cdot$ reduction determined at a single protein concentration (30 μM).
d- rate constants from Chapter II.

disproportionation, and therefore the cytochrome c is not reduced by the semiquinone species. The complexed cytochrome c is, however, measurably reduced by the fully-reduced flavin, LFH^- ($k = 1 \times 10^6\ M^{-1}\ s^{-1}$). Even though LFH^- can act as a two electron reductant, cytochrome c can accept only one electron. Under comparable conditions, CcP(III)-complexed iso-2-cytochrome c does react with $LFH^{\cdot}$, although the rate constant for this reaction is at the borderline of measurability for this flavin species with our techniques because of the high absorption of laser light at the concentrations of protein required to obtain the data ($k_{II.1} \sim 5 \times 10^6\ M^{-1}\ s^{-1}$, cf. Chapter II). Consistent with the greater reactivity of CcP(III)-complexed iso-2-cytochrome 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, as was the case with yeast iso-2 cytochrome c, complexation of yeast iso-1 by CcP (III), at low ionic strength, markedly decreases the accessibility of reduced lumiflavin species to the exposed cytochrome c heme edge.

The mutant iso-1-cytochromes c with Q16S, Q16K and K72D 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 R13I and K27Q mutant cytochromes are apparently more reactive than the wild-type in the CcP(III) complex (15-30% of the uncomplexed rate constant for LFH· reduction). For these two mutants, the increases in the reduction rate constants for the complexed cytochromes relative to the normal iso-1-cytochrome 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 R13I and K27Q mutants, presumably resulting from variations in the structure of the mutant cytochrome c:CcP(III) complex.

As is also shown in Table IV-2, when all of the yeast cytochromes are complexed to CcP(IV,R^{+}·) at low μ, the accessibilities of the heme c edges to LFH· 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 Q16S mutant are ~70% as accessible to LFH· as in the free state, the R13I, K27Q and K72D mutants are ~100% as reactive and the Q16K mutant is ~30% as reactive as are the uncomplexed cytochromes. The

above results emphasize two important features of the cytochrome c:CcP complex. First, alterations of single specific cytochrome c side chains can affect cytochrome 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 cytochrome c heme accessibility within the complex.

We have demonstrated in Chapter II that it is possible using laser flash photolysis to directly measure the rate constant for intracomplex one-electron transfer between tuna, horse and yeast iso-2-cytochrome c(II) and CcP(IV,$R^{+}\cdot$) at low ionic strength. The reaction mechanism at low ionic strength (μ < 10 mM) is given by equations II.3 and II.4. At higher ionic strength, electron transfer from cytochrome c to Compound I can also occur, via the formation of a transiently formed complex as described in equations III.1 - III.3. Furthermore, it was suggested in Chapter II that the transient electron transfer complex may not be the same as the electrostatic complex.

At μ = 8 mM, both iso-1 and iso-2 cytochromes c give similar first-order intracomplex electron transfer rate constants, $k_{II.4} \sim 200\ s^{-1}$, as shown in Table III-3. The Q16S and K27Q mutants yield approximately the same value

Table IV-3

Intramolecular rate constants for electron transfer from reduced cytochromes c to CcP(IV,$R^{+}\cdot$)

cytochrome	$k_{II.4}$[a] (s^{-1}) μ = 8 mM	$k_{III.3}$[b] (s^{-1}) μ = 260 mM
iso-1 (wild type)	260[c]	1460
	230[d]	
iso-2	150[e]	1480
Q16S	200	1350
Q16K	100	600
R13I	1000	2240
K27Q	260	780
K72D	440	---

a- determined at a single protein concentration using 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 non-linear least squares fits to the data based on the mechanism given by equations III.1, III.2 and III.3 represented as solid lines in Figure IV-2.
c- measured at 30 μM in each component.
d- measured at 50 μM in each component.
e- value from Chapter II.

Figure IV-2. Plots of k_{obs} vs. CcP concentration for reduction of CcP(IV,$R^{+}\cdot$) by yeast cytochromes c(II) at high ionic strength.

Symbols correspond to the various cytochrome species as follows: (Δ) R13I, (O) iso-1, (▲) iso-2, (✕) Q16S, (●) K27Q, and (□) Q16K. Ferric cytochrome c concentrations were 30 μM. Solid lines correspond to theoretical curves generated by non-linear least squares fits to the data (see text). CcP(IV,$R^{+}\cdot$) reduction was monitored at 550 nm. Reaction solutions were prepared using 100 mM phosphate buffer containing 25 mM EDTA at pH 7. 5-DRf concentration was 100 μM.

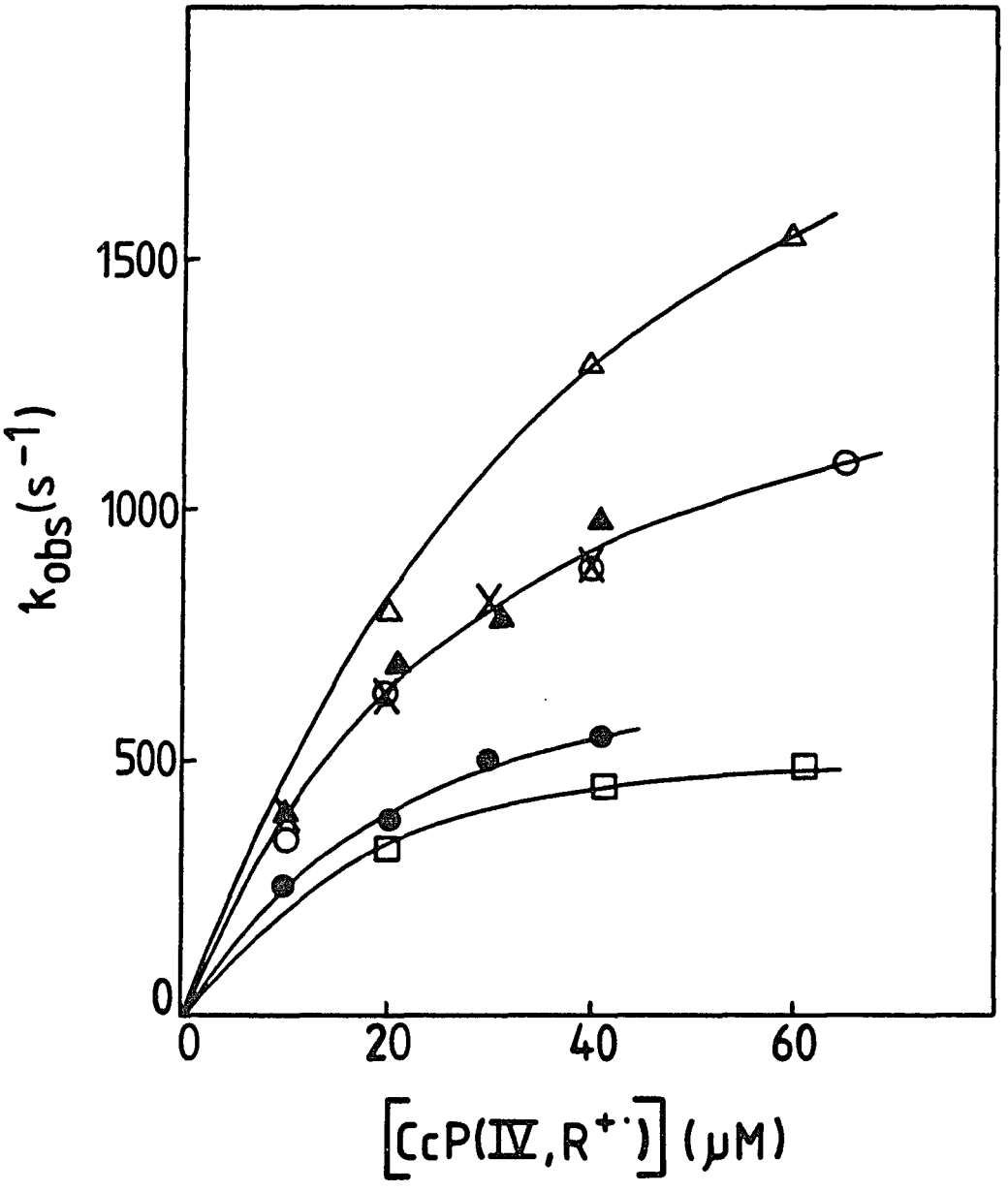


Figure IV-2. Plots of k_{obs} vs. CcP concentration for reduction of CcP(IV,$R^{+\cdot}$) by yeast cytochromes c(II) at high ionic strength.

for the rate constant as does the wild type, whereas the Q16K mutant is somewhat slower. The R13I and K72D mutants have rate constants which are appreciably greater than those obtained with the wild-type.

Figure IV-2 shows plots of k_{obs} vs [CcP(IV,$R^{+}\cdot$)] for yeast iso-1, iso-2, and the iso-1 mutant cytochromes c for the reduction of Compound I at high ionic strength (μ = 260 mM). In all cases, both wild types and mutants, there is a significant increase in the limiting first-order rate constants with an increase in ionic strength. The Q16S mutant is identical with the wild type, whereas the K27Q and Q16K mutants have substantially smaller limiting rate constants, and the R13I 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 R13I mutant cytochrome 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 cytochrome c which we have studied yield a range of kinetic properties when they are complexed to CcP in terms of both cytochrome c heme accessibility and

intramolecular electron transfer. The kinetic results presented in this chapter provide four principal points to be addressed: 1) the differences in reactivity of free yeast iso-1 and iso-2-cytochromes towards 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; and 4) the differences in the intracomplex electron transfer rate constants for the various mutant cytochromes with respect to iso-1-cytochrome c.

Accounting for the different reactivities of free iso-1 and iso-2-cytochromes with LFH· is difficult. The magnitude of the rate constant difference, 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 iso-cytochromes c are very similar (the principal regions of the sequence near the heme edge involve positions 8-17, 27-30, 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 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 the availability of the highly-refined x-ray structural coordinates for iso-1 cytochrome c and determination of the iso-2 cytochrome c structure.

As described above, the accessibilities of all of the cytochrome c hemes are quite different when they are complexed at low ionic strength with the (III) and (IV,R^{+}·) redox states of CcP. The rate constants for reduction of CcP(IV,R^{+}·)-complexed iso-1 and iso-2 cytochromes c increase by 5-10 fold, relative to the CcP(III)-complexed species. This suggests that formation of CcP (IV,R^{+}·) causes a conformation change at the cytochrome c:CcP interface which makes the cytochrome c heme significantly more accessible. Recently, Edwards et al. (1987) 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 anti-parallel beta-sheet. This loop contains Leu-182 which in the hypothetical model (Poulos and Kraut, 1980; Poulos and Finzel, 1984) interacts with position 81 of cytochrome 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 cytochrome c, which has been suggested to be involved in electron transfer into the cytochrome c heme (Tollin et al., 1986b). Thus, it is possible that on formation of CcP(IV,$R^{+}\cdot$) the 175-190 loop reorientation alters the interaction of CcP with cytochrome c in such a way that the cytochrome c heme becomes more accessible to LFH$\cdot$.

In terms of the iso-1 cytochrome mutants studied, it is clear that the hemes of the R13I and K27Q mutants are somewhat more accessible (>2-fold) than the wild type in the CcP(III) complex, suggesting that the loss of electrostatic interactions involving these residues results in a favorable reorientation of cytochrome c within the complex. An electrostatic interaction between Lys-13 of tuna cytochrome c (Arg in yeast) and Asp-37 of CcP has been proposed in the Poulos-Kraut computer model. Interestingly, the chemical modification studies reported to date support an important electrostatic role for Lys-27 (Kang et al., 1978). 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 cytochrome c:CcP interaction domain resulting from replacement of Lys-27 by Gln which alters the complex structure.

In the case of the cytochrome c:CcP(IV,$R^{+}\cdot$) complexes, the situation with the mutants is clearer since the actual rate constants for LFH· reduction of cytochrome 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 cytochrome c heme, consistent with an alteration of the cytochrome 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 cytochrome c:CcP(IV, $R^{+}\cdot$) 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 cytochrome c and CcP. The iso-1 and iso-2 cytochromes and the Q16S mutant have essentially the same intracomplex electron transfer rate constant at both ionic strengths studied, which is

consistent with their similar cytochrome c heme accessibilities. The results obtained with the R13I mutant cast serious doubt on the importance of Lys-13 (or Arg in yeast cytochrome) in the proposed electron transfer pathway (Poulos and Kraut, 1980; Poulos and 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 an increased freedom of motion and is suggested to play a key role as an electron transfer conduit into the peroxidase. (As will be shown in the next chapter, the role of the His-181 of CcP is not essential to electron transfer). Based on the Poulos-Kraut 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 R13I substitution, suggesting that when the proposed Lys(or Arg)-13: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 R13I substitution as compared to the wild-type, but the relative increase is

smaller (k_{ile}/k_{arg} ~1.6 at μ = 260 mM vs. 4 at μ = 8 mM). Kang et al. (1977) first noted that, for the steady-state turnover of CcP(IV,$R^{+}\cdot$) in the presence of horse and iso-1 cytochromes c, maximal turnover rates 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 cytochrome c. Similarly, we have suggested in the previous chapter that the increase in the limiting rate constant for intracomplex electron transfer which occurs at high ionic strength apparently results from the masking of electrostatic interactions which allow 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 cytochrome 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 non-covalent complex, the rate constant increased 4.4-fold. Thus, the increased rate constants obtained with the R13I substitution suggests 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 Q16K and K27Q 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 present results obtained with yeast iso-1, the R13I and K72D 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, contrary to the predominant criteria utilized to build hypothetical protein:protein complexes via computer modelling (Salemme, 1976). 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, based on the large changes in kinetic properties which

occur upon the mutation of a single specific residue in cytochrome c, it is also possible that there exist several complexes for a given cytochrome c species, all of which can carry out efficient electron transfer. This conclusion is supported by the fact that when cytochrome c is co-crystallized with CcP (Poulos et al., 1987) the atomic coordinates for cytochrome c could not be resolved due to a large degree of disorder, supposedly resulting from the presence of multiple cytochrome 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 cytochrome c to another can be made.

CHAPTER V.

THE EFFECTS OF HIS-181 ⟶ GLY AND TRP-191 ⟶ PHE MUTATIONS IN YEAST CYTOCHROME C PEROXIDASE ON THE REDUCTION KINETICS OF COMPOUND I BY CYTOCHROME C AND FREE FLAVINS.

Introduction

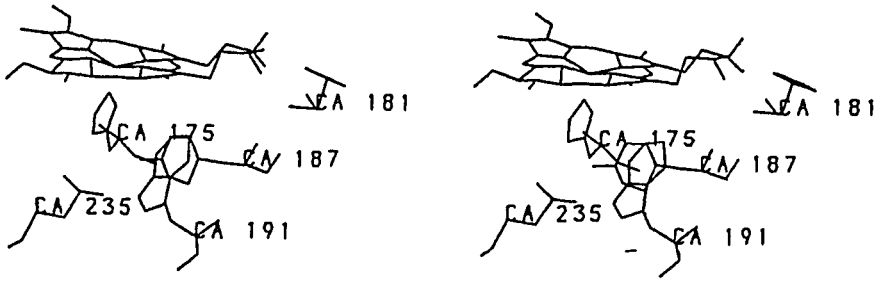
Utilizing the ability to express cytochrome c peroxidase in E. coli as a wild-type protein, it has become possible to induce site-specific mutations in the enzyme and isolate sufficient quantities of protein to perform biochemical and biophysical studies (Fishel et al., 1987; Mauro et al., 1988; Miller et al., 1988, Erman et al., 1989). (Due to the presence of a methionine and an isoleucine residue at the n-terminus of CcP, resulting from the expression of the cloned yeast protein in E. coli, the cloned species will be designated as MI-CcP whereas the enzyme which is isolated from baker's yeast will be designated as CcP, consistent with the notation employed in previous chapters). In this chapter we compare the results pertaining to the one-electron reduction of Compound I from the native enzyme isolated from yeast, the native enzyme cloned into E. coli, and site-specific mutants of this latter species by ferrous cytochrome c and free flavin reductants.

One of the site-specific mutants of CcP is a species in which histidine-181, which is exposed on the enzyme surface, has been converted to a glycine, denoted as MI,H181G-CcP. The location of His-181 relative to the peroxidase heme is shown in Figure V-1a. In the hypothetical model of the cytochrome c:CcP complex proposed by Poulos and co-workers (Poulos and Kraut, 1980; Poulos

Figure V-1. Stereo views of His-181 and Trp-191 in Cytochrome c Peroxidase.

Side (a) and front (b) views, relative to the site of cytochrome c binding in the hypothetical complex, showing the orientation of His-181 and Trp-191 relative to the heme of CcP. Also shown are the distal His-175, Asp-235, and Tyr-187.

a)



b)

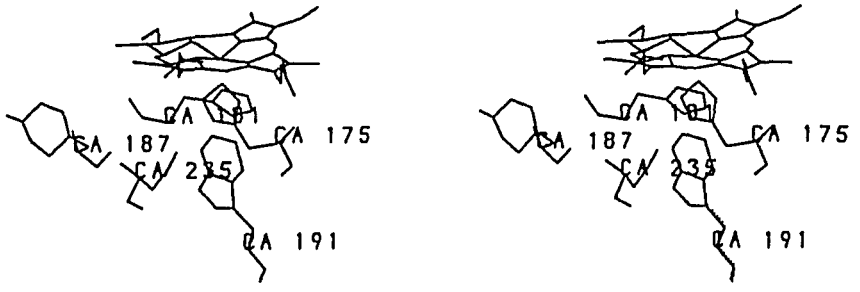


Figure V-1. Stereo views of His-181 and Trp-191 in Cytochrome c Peroxidase.

and Finzel, 1984) this histidine residue was predicted to serve an obligatory role in the electron transfer pathway from the cytochrome c heme to that of CcP. While chemical modification studies (Bosshard et al., 1984) have suggested a role for His-181 in the steady-state oxidation of ferrous cytochrome c by CcP Compound I, these studies did not unambiguously prove that modification of this residue had been achieved.

The other mutant species of CcP in which tryptophan 191 has been converted to phenylalanine, MI,W191F-CcP, was investigated even though this residue has not typically been considered important to the electron transfer pathway. Tryptophan 191 lies deeply buried within the protein matrix orthogonal to the CcP heme and is both co-planar and within van der Waals contact with the proximal histidine 175 which is an axial ligand of the heme Fe and within hydrogen bonding distance with Asp-235 (Poulos and Kraut, 1980) as shown in Figure V-1b. Based on comparisons of high resolution x-ray crystallographically determined structures of the ferric and Compound I species of CcP, Edwards et al. (1987) noted that when CcP is oxidized to Compound I, small changes in electron density were observed at Trp-191 and in other nearby residues which are part of an outer surface β-sheet structure which is in the general vicinity of the proposed cytochrome c interaction domain. Thus, it was

suggested that these structural changes might arise from a coupling of the redox related changes in the enzyme structure with the π-π interactions between the indole and imidazole rings of Trp-191 and His-175. Furthermore, it has also been suggested that Trp-191 might participate in a highly extended π-orbital system which enables cytochrome c peroxidase to efficiently stabilize the oxidized amino acid(s) in Compound I (Poulos and Finzel, 1984; Finzel et al., 1984; Edwards et al., 1987; Chance et al., 1986). Therefore, even though this residue has not been previously regarded as being important in the electron transfer pathway between cytochrome c and CcP, a role in the redox properties of the enzyme was suspected.

In the chapter which follows, it is demonstrated that when His-181 is converted to a glycine a moderate decrease (47 %) of the electron transfer rate constant is observed. However, the rate constant is sufficiently large at high ionic strengths (1850 s^{-1}) to suggest that the presence of a histidine at this location on the surface of CcP is, in fact, not mandatory for the reduction of Compound I by ferrous cytochrome c. On the other hand, no intracomplex electron transfer could be observed between ferrous cytochrome c and the Compound I species of the MI-CcP W191F mutant on time scales $\leq$ 10 seconds.

Results and Discussion

Reduction of Horse Cyt c(III) in 1:1 Complexes with Ferric MI-CcP, and MI,W191F-CcP.

The steric accessibility of cytochrome c(III) complexed to either baker's yeast CcP or MI-CcP at low ionic strength can be probed using the kinetics of cytochrome c reduction by electrostatically neutral lumiflavin semiquinone. Figure V-2a shows plots of k_{obs} vs. cytochrome c(III) concentration for LFH· reduction of horse cyt c(III) complexed with the ferric forms of native CcP, MI-CcP or MI,W191F-CcP at μ = 8 mM. Rate constants determined from these plots are given in Table V-1. Also given in Table V-1 are the rate constants for reduction of free cytochrome c and cytochrome c complexed to baker's yeast CcP(III). For complexes involving MI-CcP and the W191F mutant, decreases of 60% and 70%, respectively, in the cytochrome c reduction rate constant relative to the free protein were observed, while there was a 74% decrease for horse cytochrome c(III) complexed with native CcP. As the percent decrease in the rate constant is determined mainly by the relative steric accessibility of the cytochrome c heme (Meyer et al., 1983; Tollin et al., 1987), we conclude that there is only a slight difference between the relative cytochrome c heme accessibilities in complexes with the three different CcP species. Thus, the Trp to Phe mutation apparently has

Figure V-2. Plots of kobs vs. concentration for the reduction of horse cytochrome c in 1:1 complexes with MI- and MI,W191F-CcP.

a) LFH· reduction of cytochrome c(III) in complexes with ferric species of MI-CcP (O) and MI,W191-CcP (●). Reduction kinetics were measured at 575 nm.

b) LFH· reduction of cytochrome c(III) in complexes with the Compound I species of MI-CcP (O) and MI,W191F-CcP (●)

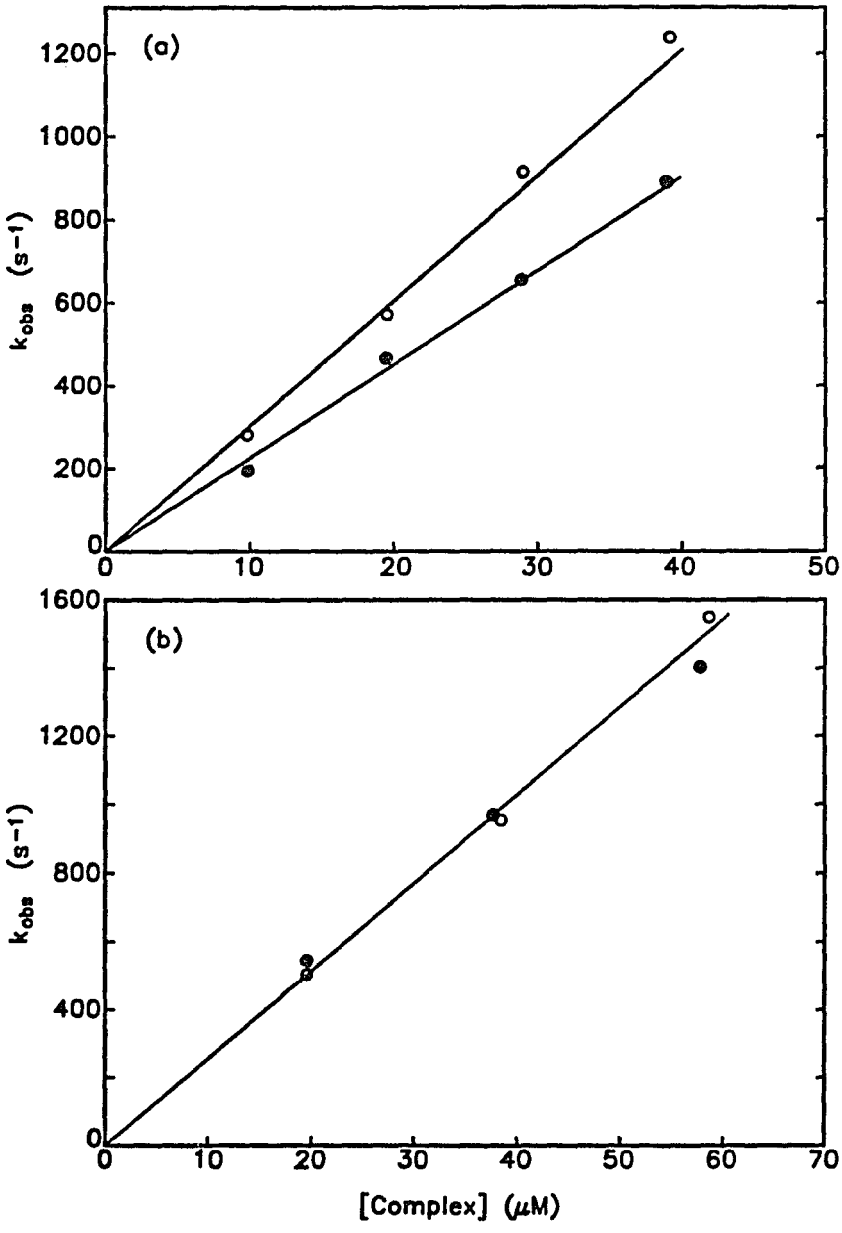


Figure V-2. Plots of kobs vs. concentration for the reduction of horse cytochrome c in 1:1 complexes with MI- and MI,W191F-CcP.

Table V-1

Second-order Rate Constants for Reduction of Horse Cytochrome c(III) in 1:1 Complexes with Native, MI- and MI,W191F CcP(III).

CcP Species and Oxidation State		$k_{II.2}$ x 10^{-7}[a] (M^{-1} s^{-1})
Native	(III)	1.9[b]
	(IV,R+.)	3.0
MI	(III)	3.0
	(IV,R+.)	2.6
MI,W191F	(III)	2.2
	(IV,R+.)	2.6
(Free cyt c(III))		7.4[b]

[a] Data obtained from plots of k_{obs} vs. concentration shown in Figure V-2.
[b] Data from Chapter II.

little or no effect on the degree of exposure of the cytochrome c heme group within the complex with CcP. When the two cloned enzymes were subjected to steady-state analysis by Mauro et al. (1988) values for the cytochrome c Km value for MI and MI,W191F (10.2 and 12.8 μM, respectively) were also found to be quite similar. Taken together, the two sets of data indicate that the association of and complexes resulting from the binding of cytochrome c with baker's yeast, MI-, and MI,W191F-CcP are quite similar.

As has been shown in Chapter IV, when native CcP is converted to the oxy-ferryl species, there is an increase in the steric accessibility of cytochrome c which is electrostatically complexed to the peroxidase at low ionic strength, relative to that found in the complex with ferric CcP. Such results have been found for horse, yeast iso-1 and iso-2 cytochrome c(III). In the case of the yeast cytochromes a 7-fold increase was observed, while for the horse cytochrome only a 1.5-fold increase was found. Figure V-2b shows plots of k_{obs} vs. complex concentration for the reduction of horse cytochrome c(III) by LfH$^{\cdot}$ in the complexes with Compound I of MI-CcP and MI,W191F-CcP. Table V-1 lists the rate constants obtained from these plots as well as the value obtained for native CcP(IV,R+.). For the two CcP species expressed in E. coli, there was no

significant change in the rate constant for reduction of cytochrome c upon conversion of the peroxidase to Compound I. As stated above, a 1.5-fold increase was observed in the complex with native CcP. Thus, again it appears that the accessibility of the ferric heme of horse cytochrome c is similar in complexes with the Compound I species of native, MI-, and MI,W191F-CcP, consistent with the idea that the interaction domains of these three peroxidase species with horse cytochrome c are similar, but not necessarily identical.

Reduction of Baker's Yeast CcP, MI-CcP, MI,W191-CcP, and MI,H181G-CcP Compound I by Ferrous Cytochrome c

Preliminary steady-state assays of MI-CcP and MI,W191-CcP by Dr. Matt Mauro indicated that whereas the MI-CcP behaved in a manner very similar to baker's yeast CcP, the W191F mutant had a catalytic activity which was diminished by 3000 fold at pH 7 (Mauro et al., 1988). In order to test for direct one-electron transfer from ferrous cytochrome c to the Compound I species of the cloned enzymes we performed laser flash photolysis experiments as described in previous chapters. In order to illustrate the dramatic effects observed for the W191F mutant, Figure V-3 shows kinetic decay curves obtained during these studies.

Figure V-3a shows the transient decay curve obtained following photochemical generation of LFH$^{\cdot}$ at μ = 8 mM. A

Figure V-3. Laser flash transient decay curves for reactions involving cytochrome c(III), MI-CcP(IV,$R^{+}\cdot$) and MI,W191F-CcP(IV,$R^{+}\cdot$) monitored at 550 nm.

a) Transient decay curve obtained upon laser excitation of a solution containing 50 μM lumiflavin and 0.5 mM EDTA in 4 mM phosphate buffer at pH 7.

b) Decay curve for the solution of (a) to which had been added 20 μM cytochrome c(III).

c) Decay curve for the solution of (b) to which had been added 20 μM MI-CcP(IV,$R^{+}\cdot$).

d) Decay curve for the solution of (b) to which had been added 20 μM MI,W191F-CcP(IV,$R^{+}\cdot$).

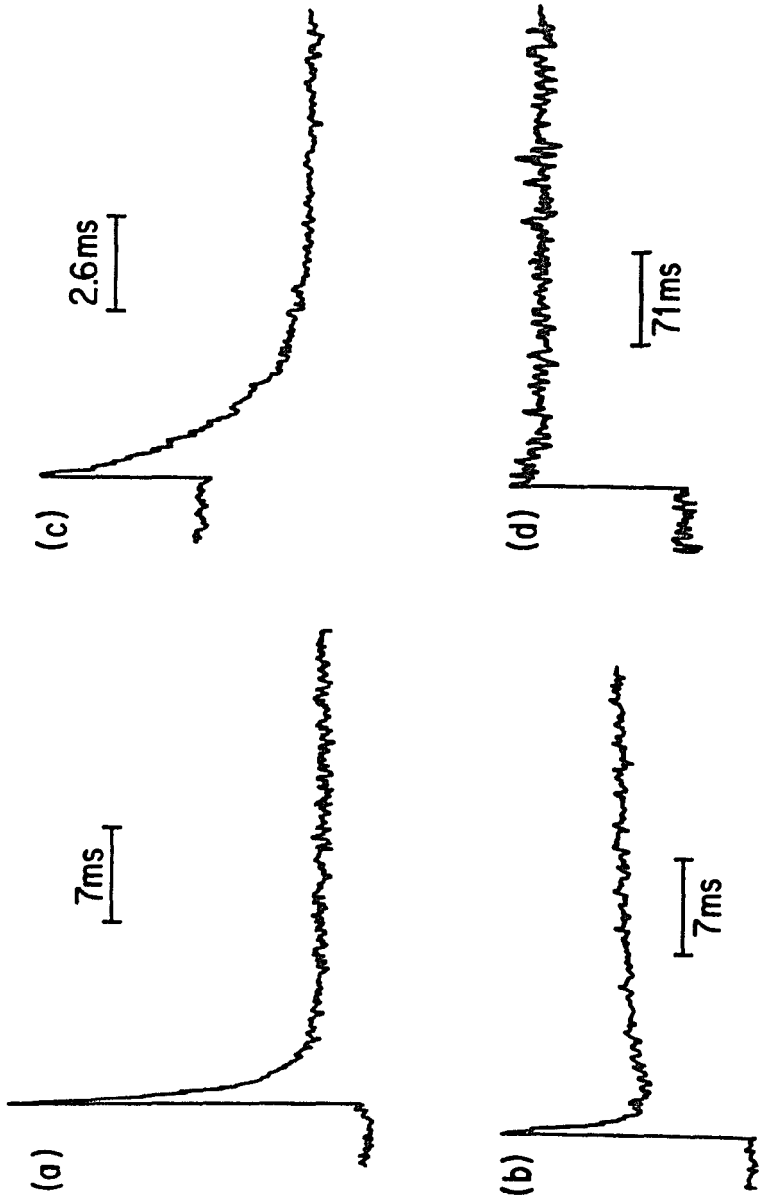


Figure V-3. Laser flash transient decay curves for reactions involving cytochrome c(III), MI-CcP(IV,$R^{+}\cdot$) and MI,W191F-CcP(IV,R^{+}.) monitored at 550 nm.

rapid rise in A_{550}, consistent with LFH· production, is followed by a non-exponential decay to the pre-flash baseline due to flavin disproportionation. As described in Chapter II, only slight changes in this decay curve are observed when baker's yeast CcP(IV,R^{+}·) is added. Figure V-3b shows the decay curve obtained after cytochrome c(III) addition; the rise in A_{550} corresponding to the production of cytochrome c(II). Upon addition of MI-CcP(IV,R^{+}.) to the solution of Figure V-3b, there is a marked decay of the signal below the preflash baseline as shown in Figure V-3c, in a manner similar to that observed with baker's yeast CcP. When MI,W191F-CcP(IV,R+·) was added to the solution containing cytochrome c, there was no change in the decay curve relative to that of cytochrome c reduction alone as shown in Figure V-3d. Thus, the decay curve obtained with the W191F mutant indicates that even on time scales ~30 times greater than that required for MI-CcP, there apparently is no electron transfer from ferrous cytochrome c to MI,W191F-CcP(IV,R^{+}.). Redox titrations performed by Dr. T. E. Meyer at the University of Arizona on the ferric species of the W191F mutant gave a mid-point potential of -202 mV, whereas values of -194 mV and -190 mV have been determined for the MI- and native CcP species (Mauro et al., 1988; Conroy et al., 1976). Thus, the inability of the W191F mutation to accept electrons from ferrous

cytochrome c most likely is not related to a major change in the redox properties of the heme Fe per se. This interpretation must be tempered with the recognition that these redox potential determinations were not performed on the Compound I species. In the absence of any data to the contrary, however, we assume that there will be comparable differences in redox potentials in the Compound I species, and given this assumption, the widely disparate kinetic differences can not be accounted for by the relatively small differences in $E_{m,7}$ (Meyer et al., 1983).

Reduction of MI-CcP by ferrous cytochrome c was performed at ionic strengths of 8 and 30 mM. Rate constants obtained from the data, when modelled according to equations II.3 and II.4 for the μ = 8 mM data and equations III.1-III.3 for the μ = 30 mM data, are given in Table V-2. At μ = 8 mM the values for the limiting first-order rate constant ($k_{II.4}$) are quite similar, consistent with the argument that the nature of the two complexes formed are most likely similar, but not necessarily the same. At μ = 30 mM, there is a marked difference in that the rate-limiting first-order rate constant, $k_{III.3}$, ca. one-half of that for baker's yeast CcP. The association constant, $K_{III.2}$, for the two species is, however, quite similar. The data indicate, therefore, that unlike the complexes produced at the lower ionic strength, in the

Table V-2

Rate Constants for Reduction of Native[a] and MI-CcP(IV,R+.)[b] by Horse Cytochrome c(II) at μ = 8 and 30 mM.

	μ (mM)	$k_{III.2}$ x 10-7 (M^{-1} s^{-1})	$K_{III.2}$ x 10-5 (M-1)	$k_{II.4}$ or $k_{III.3}$ (s^{-1})
Native	8	---	---	750
	30	1.31	1.35	3220
MI	8	---	---	850
	30	1.0	1.0	1350

[a] Values for native CcP are from Chapter II and III.
[b] Values are from the plots of k_{obs} vs. concentration shown in Figure V-3. Values for data obtained at μ = 30 mM were calculated from computer modelling according to the mechanism given in equations III.1-III.3.

transient complexes formed upon collision of cytochrome c with CcP at μ = 30 mM the orientation and/or prosthetic group distances for the two CcP species may be significantly different.

Comparison of the Direct Reduction of Native and H181G Compound I by Reduced Free Flavins.

In order to kinetically characterize the MI,H181G-CcP mutant we performed studies designed to elucidate whether removal of the bulky histidine 181, which lies below the two heme propionate carboxyl groups of CcP (Poulos et al., 1980), modifies the accessibility of small reductants to that enzymes prosthetic group. In Chapter II it was shown that in the absence of ferric cytochrome c native yeast Compound I is not reduced by neutral flavin semiquinones at pH 7, specifically lumiflavin or 5-deazariboflavin, despite a very high driving force of ~1 V for the reaction. However, it was also suggested that native Compound I does react with the fully reduced lumiflavin produced by disproportion of the semiquinone species ($k \approx 10^9$ M^{-1} s^{-1}; Vaish and Tollin, 1971). It was suggested that the rate constant for direct reduction of native CcP(IV,$R^{+\cdot}$) by flavin semiquinone must therefore be $< 5 \times 10^6$ M^{-1} s^{-1}. Based on the studies of Meyer et al. (1983) the rate constant for the fully reduced species would be smaller than this value. However, reduction of Compound I from

Figure V-4. Plots of kobs vs Compound I concentration for reduction of baker's yeast CcP, MI-CcP, and MI,H181G-CcP by LFH_2 and ferrous cytochrome c.

a) Plots for the reduction of Compound I of baker's yeast (◆) and MI,H181G-CcP (O) by LFH_2 at pH 6. Experiments were performed in phosphate buffer (μ = 8 mM) containing 0.5 mM EDTA and 70 μM lumiflavin.

b) Plots for the reduction of Compound I species of baker's yeast (◆), MI-(O), and MI,H181G-CcP (O) by ferrous cytochrome c. For all reactions in (b), the concentration of ferric cytochrome c was held constant at 30 μM in 100 mM phosphate buffer (μ = 114 mM) containing 0.5 mM EDTA and 90 μM 5-deazariboflavin. The solid lines represent best fits from the nonlinear regression analysis based on the mechanism described in the text.

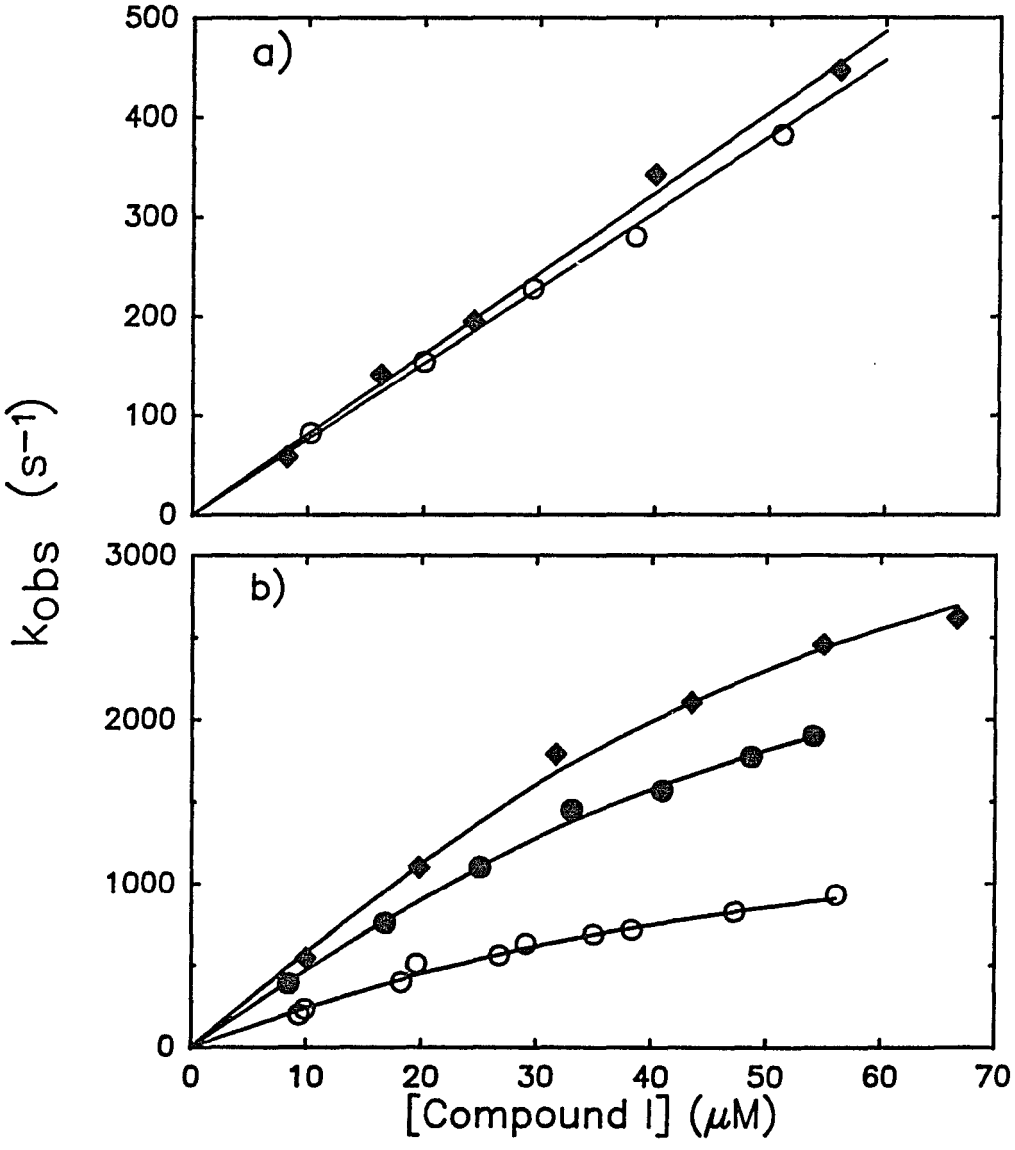


Figure V-4. Plots of kobs vs Compound I concentration for reduction of baker's yeast CcP, MI-CcP, and MI,H181G-CcP by LFH_2 and ferrous cytochrome c.

Table V-3

Second-order Rate Constants for the Reduction of Native and MI,H181G-CcP Compound I by Fully Reduced Free Flavins.

Protein	$k \times 10^{-6}$ M^{-1} s^{-1}
Native	8.1 (pH 6)
MI,H181G	7.6 (pH 6)

Data obtained from the plots of kobs vs. [Compound I] shown in Fig. V-4. Experimental conditions: 5.4 mM phosphate buffer (μ = 8 mM), pH 6, containing 0.5 mM EDTA and 70 μM lumiflavin.

baker's yeast and MI,H181G-CcP by fully reduced lumiflavin (LFH_2) can be readily observed at pH 6.0. Figure V-4a shows plots of k_{obs} vs [Compound I] for these experiments. The second-order rate constants determined from these plots are given in Table V-3. The rate constants are the same within the error of the experiment (± 10%), suggesting that the accessibility of the CcP heme to small exogenous reductants is not significantly affected by the conversion of His-181 to a glycine. When similar experiments were performed with 5-DRFH·, no measurable reduction of either Compound I species by this flavin was observed. This is not surprising since oxidation of 5-$DRFH_2$ is quite slow relative to that of other flavins (Edmondson et al., 1972). Under the conditions of these experiments, we cannot distinguish between a one or two-electron reduction of Compound I. The spectral changes coincident with the reduction of Compound I are consistent with reduction of the Fe(IV) heme; however, we have no way to determine whether or not simultaneous reduction of the amino acid radical, R.+, occurs).

Reduction of Native, MI-, and MI,H181G-Ccp Compound I by Reduced Cytochrome c at pH 6.0.

In order to determine the effect of the His-181 to Gly mutation on kinetics of electron transfer between ferrous cytochrome c and Compound I, flash photolysis experiments

were performed in the presence of ferric cytochrome c and 5-DRF at pH 6 and μ = 114 mM. The results are presented in Figure V-4b. As described in previous chapters for reactions between various mitochondrial cytochromes and native CcP k_{obs} shows a saturation-type dependence on Compound I concentration, with k_{obs} reaching a limiting value at high Compound I concentrations. Kinetic parameters derived from these plots are given in Table V-4.

It is acknowledged that there could be a possible contribution of competitive binding of ferric cytochrome c with Compound I to the overall mechanism:

$$\text{cyt c(III)} + \text{CcP(IV,R}\cdot^{+}\text{)} \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} \text{cyt c(III)-CcP(IV,R}\cdot^{+}\text{)}$$

The equilibrium constant for this reaction is given by $K_i = k_i/k_{-i}$. Summers and Erman (1988) have found that $K_i = 1 \times 10^6$ M^{-1} at μ = 10 mM, pH 7.5; essentially the same value was determined for the association constant for ferrous cytochrome c. Assuming that the inhibition and association constants also have similar values at μ = 114 mM ($\sim 3 \times 10^4$ M^{-1}), the percentage of free compound I which is present prior to the laser flash is calculated to be 61, 71, and 76% of the total compound I concentration at 10, 30, and 50 μM, respectively. Since the predicted effect of competitive binding of ferric cytochrome c is therefore

Table V-4

Kinetic Parameters for the Reduction of Native, MI and H181G CcP Compound I by Ferrous Horse Cytochrome c

CcP Species	$k_{III.2} \times 10^{-7}$ (M^{-1} s^{-1})	$k_{III.3}$ (s^{-1})	$K_{III.2} \times 10^{-4}$ (M^{-1})
Native	9.6	4500	3.4
MI	8.0	3450	3.3
MI,H181G	6.7	1850	2.3

Data obtained from nonlinear least-squares fits to the plots shown in Figure V-4 according to the mechanism given in equations III.1-III.3
Experimental Conditions: 100 mM phosphate buffer (μ = 114 mM), pH 6, containing 0.5 mM EDTA and 90 μM 5-deazariboflavin. Cytochrome c concentration was held constant at 30 μM.

small, it has not been included in the computer modeling.

The effect of the H181G mutation on the intracomplex electron transfer rate is relatively small, as the first-order electron transfer rate constant, $k_{III.3}$, for Compound I of the mutant protein was 41% of that of baker's yeast CcP and 53% that of the parental MI-CcP. The similarity observed in the apparent association rate constant, $k_{III.2}$, and the overall association equilibrium constant, $K_{III.2}$, for the bimolecular reaction between the three Compound I species and ferrous cytochrome c suggest that there is only a small effect of the H181G mutation on the initial formation of the electron transfer complex. It should also be noted that the values for the association constant given in Table V-3 are in good agreement with reported values for the binding of ferric cytochrome c to native ferric CcP at pH 6.0 and μ = 100 mM (Erman and Vitello, 1980).

Relevance of His-181 and Trp-191 to the Hypothetical Model for the Cytochrome c : CcP Complex.

In the case of the H181G mutation, the rate of electron transfer from cytochrome c(II) to Compound I is diminished in the mutant protein, when compared to both the native and MI species. However, the magnitude of the decrease in the rate constant is not as large as would be expected if His-181 had an obligatory role in the electron transfer pathway as suggested by Poulos and co-workers. Furthermore, the

decrease in rate constant for H181G is insignificant when compared to the virtual non-reducibility of the Phe-191 mutant, where no electron transfer could be observed on time scales ≤ 1 sec. The decrease in the rate constant in the Gly-181 mutant may reflect a change in the binding geometry of the two proteins in the transiently formed complex. Thus, His-181 may play a role of stabilizing the two proteins in the transiently formed complex which permits a more favorable electron transfer pathway. Consistent with this argument is the fact that the catalytic activity of MI,H181G-CcP is one-half that of the MI-CcP (Miller et al., 1988). It is important to note that we cannot exclude the possibility that removal of the imidazole side chain at residue 181 results in an electron transfer by an alternate and kinetically equivalent route, although there is no evidence to support this notion. It is also interesting to note that X-ray diffraction and spectral properties of the peroxidase are relatively unaffected by the His-181 to Gly mutation, despite the large difference in sizes of the two residues (Miller et al., 1988).

Accounting for the almost complete lack of reactivity of the W191F mutant is challenging. Based on visible, EPR, and vibrational spectroscopic measurements (Mauro et al., 1988; Smulevich et al., 1988) as well as X-ray

crystallographic data (Edwards et al., 1988) it has been suggested that Trp-191 plays an important role in H_2O_2-induced changes in the protein structure. It has been argued by Mauro et al. (1988) that by the combination of hydrogen bonding to Asp-235 and His-175, Trp-191 helps to induce a "strain" on the heme Fe in the oxy-ferryl species. Based on X-ray data this "strain" is manifested as a 0.2 Å movement toward the proximal His-175 ligand (Edwards et al., 1987). This proximal-ligand-dependent tension which may be necessary to allow rapid iron relaxation into the resting ferric position may not be present in the phenylalanine mutant due to loss of the hydrogen bonding capabilities of a tryptophan at this position. Thus, small adjustments in the CcP structure induced upon binding cytochrome c may be important for transiently enhancing the "strain" placed on the heme Fe in the Trp-191 protein, which could potentially lead to a lowered transition-state energy for the reduction process. Lacking these structural interactions, the Phe-191 could not utilize the same mechanism.

A second role for Trp-191 might lie in its ability to act as an electron conduit into the oxy-ferryl heme. Although this residue is not located on the surface of the protein, it is co-planar with Tyr-187, a residue which forms one of the sides of the peroxide access channel. Thus, Trp-191

may act as an important link between a surface residue and the deeply buried heme. Recent work of Mayo et al. (1986) and Kuki and Wolynes (1987) on rutheniated myoglobin has suggested that an indole ring placed between the ruthenium and the heme Fe can play a role as an electron transfer enhancer. Unfortunately, the Poulos-Kraut model cannot account for such a role for Trp-191 since this residue lies significantly below the binding domain between the two proteins. Therefore, further elucidation of the role played by Trp-191 will have to await further modification of aromatic residues which are within close proximity to Trp-191 and are located on the surface of CcP.

CHAPTER VI

CONCLUDING REMARKS

When these studies were undertaken, there was a great deal of reliance in the literature on the hypothetical model for the cytochrome c:CcP complex in order to either predict the results from or understand the data collected from various biophysical and biochemical experiments. As our studies progressed, however, it became obvious that the Poulos-Kraut model was not completely consistent with the kinetic data and that the model was, at minimum, an over-simplification of the structures that the complexes could have in solution. The data presented in the previous chapters clearly show that the ability of proteins to transfer electrons efficiently is not necessarily dependent on their propensity to form tightly bound electrostatically-stabilized complexes which supposedly maximize specific charged pair interactions. Quite to the contrary, in the case of the cytochrome c:CcP complex, the transiently formed intermediate which occurs at higher ionic strength leads to greater rates of electron transfer. These results are logical since the proteins must function within the mitochondrial milieu where an ionic strength of ~145 mM is found. Thus, the reliance of model builders on the interactions of specific amino acids, whether they are evolutionarily conserved or not, in stabilizing a transient

complex should be suspect at the outset, in the absence of experimental data to support such a proposal.

The findings that His-181 of CcP did not play an obligatory role in electron transfer from ferrous cytochrome c to CcP, whereas the highly buried Trp-191 was essential for electron transfer, were perhaps the most startling findings of these investigations. In both cases, the results were either not predicted by or in complete disagreement with the Poulos-Kraut model. The inability of the Trp-191 mutant to accept electrons from cytochrome c has spawned interest in identifying which residues located on the surface of CcP near Trp-191 may be the actual site of electron entry into the protein.

A fundamental question which arises from our investigations concerns the utility of computer-generated hypothetical complexes in general. It is a natural, and perhaps logical, tendency to generate such models in order to better understand the structure function relationships of proteins, to understand known biochemical or biophysical properties of the proteins in question, or to predict the outcome of proposed investigations. One must be ever mindful, however, of the fact that these are models, after all, and that they may not represent the situation found in nature. When need be, the models will have to be modified to account for experimental findings.

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