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FORMATION OF ELECTROSTATICALLY-STABILIZED COMPLEX AT LOW IONIC STRENGTH INHIBITS INTERPROTEIN ELECTRON TRANSFER BETWEEN YEAST CYTOCHROME c AND CYTOCHROME c PEROXIDASE

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Electron transfer from yeast ferrous cytochrome c to H_2O_2-oxidized yeast cytochrome c peroxidase has been studied using flash photoreduction methods. At low ionic strength (μ < 10 mM), where a strong complex is formed between cytochrome c and peroxidase, electron transfer occurs rather slowly ($k \sim 200 s^{-1}$) However, at high ionic strength where the electrostatic complex is largely dissociated, the observed first-order rate constant for peroxidase reduction increases significantly reaching a concentration independent limit of $k \sim 1500\ s^{-1}$. Thus, at least in some cases, formation of an electrostatically-stabilized complex can actually impede electron transfer between proteins.

Rapid progress is being made in understanding long distance electron transfer between proteins, from theory (1,2), model studies (3-10) and studies of reaction between specific protein pairs (11-22). An important adjunct of protein-protein studies has been the characterization of binding between specific physiological partners, like cytochrome c (cyt c) and cytochrome c peroxidase (CcP). Thermodynamic binding data (23,24), studies involving chemical modification or covalent cross-linking of the proteins (25-30), site-directed mutagenesis of specific surface residues (31,32), and spectral (24,33-35) perturbations resulting from complexation are providing an increasingly detailed understanding of the factors which control recognition between redox protein partners, as well as the ability to refine models proposed by computer graphic techniques (36,37). Such hypothetical models provide an important guide to experimental design. The features of such a model for the cyt c:CcP couple include stabilization by specific ionic interactions (36,37). Thus, Erman showed that the binding

Abbreviations: cyt c(II), cyt c(III): ferrous and ferric forms of cytochrome c, respectively; CcP(III) and CcP(IV,R$^{\bullet+}$): ferric and the peroxide-oxidized species of cytochrome c peroxidase (i.e. compound I);cyt b_5: trypsin solubilized cytochrome b_5; EDTA: ethylaminediaminetetraacetic acid.

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constant between cyt c(III) and CcP(III) is quite high at low ionic strength ($K = 6 \times 10^6$ M^{-1} at μ = 10 mM) but decreases significantly at high ionic strength ($K = 2 \times 10^3$ M^{-1} at μ = 200 mM) (24).

Recently we have used flash photolysis techniques to probe protein electron transfer (11-20). The basis of these experiments is the rapid (<1 μs) in situ formation of a strong reductant by the laser flash (flavin semiquinone at concentrations < 0.1 μM) (20), followed by second-order electron transfer to the redox protein and, where applicable, intramolecular or intracomplex first-order processes. One result of such studies was that in the absence of cyt c, direct reduction of CcP(IV,$R^{\bullet+}$) by flavin semiquinones occurs only slowly, whereas in the presence of cyt c(III) a rapid first-order electron transfer within the cyt c:CcP(IV,$R^{\bullet+}$) complex (18), preformed at low (μ < 10 mM) ionic strength could be directly observed. The observed first-order rate constant was found to vary with the cyt c species (i.e. tuna, horse, yeast iso-2). In the present report, we present data which suggests that the electrostatically stabilized complex formed at low ionic strength is not the most "optimal" for rapid electron transfer.

Materials and Methods

CcP was isolated from baker's yeast (Red Star) as previously described (18). Cyt c was purified from yeast according to (38). The ferryl form of CcP was prepared by titration with H_2O_2 immediately prior to the kinetic experiments. For low ionic strength studies (μ = 8 mM) a 3 mM phosphate buffer containing 0.5 mM EDTA and 100 μM lumiflavin at pH 7 was used. The concentration of cyt c was in slight excess of that of CcP. For the high ionic strength experiments (μ = 260 mM), a 0.1 M phosphate buffer at pH 7 containing 0.025 M EDTA and 100 μM 5-deazariboflavin was used. In this case, CcP concentration was varied and total [cyt c] was held constant at 30 μM. 5-deazariboflavin was used to ensure that cyt c reduction was not rate-limiting at the concentration used in these experiments. Under these conditions, the concentration of cyt c(II) produced per flash was < 0.1 μM (19,20), and thus pseudo-first-order conditions were maintained. The kinetics of CcP(IV,$R^{\bullet+}$) reduction were followed by monitoring the absorbance decrease at 550 nm (cf. ref. 18 for details). At both ionic strengths, the observed rate constants for cyt c(III) reduction were much larger than those for CcP(IV,$R^{\bullet+}$) reduction within the complex.

Results and Discussion

Figure 1 shows plots of kobs vs. concentration for the reduction of CcP(IV,$R^{\bullet+}$) by yeast iso-1 and iso-2 cyts c(II) at low and high ionic strengths. Concentration independent values of 150 and 250 s^{-1} for iso-2 and iso-1 cyts c, respectively, were obtained at low ionic strength (μ = 8 mM) (Table 1). These first-order rate constants at low μ were independent of the chemical nature of the flavin semiquinone reductant, i.e. lumiflavin or 5-deazariboflavin.

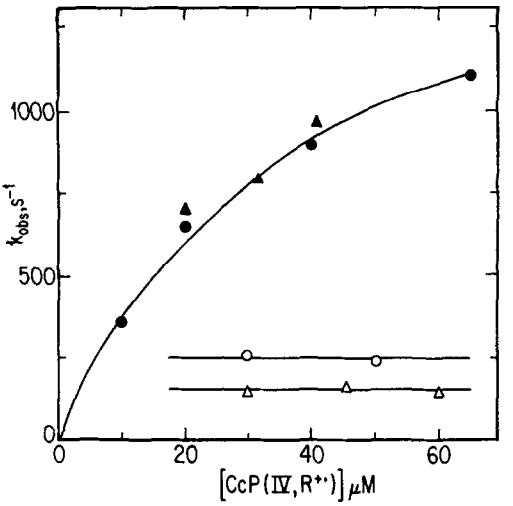

Figure 1. Kinetics of reduction of CcP(IV,R•+) by yeast iso-1 and iso-2 cyts c(II). Data obtained at low ionic strength (3 mM phosphate, 0.5 mM EDTA at pH 7; μ = 8 mM) upon reduction of cyt c by lumiflavin semiquinone: (○)iso-1 and (△)iso-2 (data from ref. 6f). Data obtained at high ionic strength (0.1 M phosphate, 0.025 M EDTA at pH 7; μ = 260 mM) upon reduction of cyt c by 5-deazariboflavin semiquinone: (●)iso-1 and (▲)iso-2. The solid line for the high ionic strength data is the theoretical curve obtained from non-linear least squares analysis of the data based on the mechanism given in the text.

As a part of more detailed studies of the physiologically relevant yeast cyt c reactions with CcP, we have studied the effect of ionic strength on the electron transfer rate constants. At high ionic strength (μ = 260 mM) the preformed cyt c:CcP complex is dissociated at the experimental concentrations used (24) and essentially all cyt c is free in solution. Rapid photoreduction of a small amount (<0.1 μM) of free cyt c by laser-produced 5-deazariboflavin semiquinone ($k = 2 \times 10^9$ $M^{-1}s^{-1}$) (14), is followed by a pseudo-first-order reoxidation of the cyt c(II) by CcP(IV,R•+). In the present experiments, we have directly monitored this second step, at several independent wavelengths. The concentration dependecies of the observed first-order rate constants for reduction of the peroxidase by the two cyt c species are plotted in Fig. 1. The non-linear

Table 1: Intramolecular rate constants and binding equilibrium constants for electron transfer from reduced cytochromes c to CcP(IV,R•+)

cytochrome c	μ = 8 mM	μ = 260 mM	
	k (s^{-1})	k_3[a] (s^{-1})	$K^b \times 10^{-3}$ (M^{-1})
iso-1	250	1460	5.1
iso-2	150[c]	1480	6.0

[a] k_3 and K were determined from non-linear least squares fits to the data of Figure 1 based on the mechanism given by the equation in the text.
[b] $K = k_1/k_2$.
[c] value from ref. 18.

concentration dependence is consistent with the formation of a transient complex at high CcP(IV,R$^{\bullet+}$) concentrations, with the intracomplex electron transfer step (k_3) becoming rate-limiting (19,32):

$$\text{cyt } \underline{c}\text{(II)} + \text{CcP(IV,R}^{\bullet+}\text{)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{complex} \xrightarrow{k_3} \text{cyt } \underline{c}\text{(III)} + \text{CcP(III)}$$

Kinetic parameters determined from non-linear least squares analysis of the data based on the above mechanism are given in Table 1. The simple preequilibrium kinetic models often used to interpret protein electron transfer (1) predict that the rate of electron transfer from cyt c to CcP within an intermediate complex should decrease at high u if the electrostatically-stabilized complex is optimized for electron transfer. However, as the plots of Fig. 1 and the values listed in Table 1 show, in the case of cyt c and CcP such a decrease is not observed. Instead, the limiting rate constant for electron transfer at μ = 260 mM greatly exceeds that at μ = 8 mM, reaching an extrapolated value of 1460 s^{-1} for the intracomplex electron transfer when [CcP] > 30 μM. Clearly, formation of electrostatically-stabilized yeast iso-1 or iso-2 cyt c:CcP complexes inhibits electron transfer. The value of K obtained from this theoretical fit (5 x 10^4 M^{-1}) is appreciably larger than the value of 2 x 10^3 M^{-1} obtained by Erman and Vitello (24). However, the present kinetic results refer to the interaction between cyt c(II) and CcP(IV,R$^{\bullet+}$), whereas the value determined by Erman and Vitello was obtained using cyt c(III) and CcP(III) under different experimental conditions. There is no a priori reason to expect that these two association constants should be the same.

The present results have precedent in the well-studied steady state kinetics (39,40) of the cyt c:CcP system and offer insight into such kinetics. It has been shown in steady state measurements with yeast iso-1 cyt c that maximal turnover rates are obtained not at low ionic strength, but at intermediate ionic strength (40) (μ ~ 150 mM). The decrease in observed turnover rate at low μ was attributed to a rate-limiting product dissociation step (40). The present single turnover studies generally confirm these results but suggest an alternate interpretation: the low rate at low μ is not due to product dissociation (which cannot affect the present results) but rather to a direct influence of ionic strength on the processes which control the electron transfer step.

There are at least two possible explanations for such inhibition. The well-characterized complex formed at low ionic strength could represent a species which is not structurally optimized for electron transfer, analogous to a "dead end" complex, and which must dissociate or rearrange to form the "active" complex found at high ionic strength. Alternatively,

if the product complex is destabilized more by higher u than is the reactant complex, then the reaction will be driven toward completion (i.e. ΔG will increase), causing an associated increase in k_3 (1,2,41). In any event, the present results unambiguously demonstrate that the complexes between electron transfer proteins formed at low ionic strength may not necessarily be the optimal structures for electron transfer reactions. In this vein, we note that qualitatively similar results are found for the the horse cyt c:CcP (19) and cyt c:cyt b_5 complexes (41). Greater insight into the nature of the productive complex and the transition state may be available by detailed studies of site-specific mutant proteins. Such studies will be reported in due course.

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