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PROBING THE ROLE OF SURFACE RESIDUES IN THE COMPLEX FORMED BETWEEN CYTOCHROME C PEROXIDASE AND CYTOCHROME C

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Abstract The "lock and key" ionic-interaction model[1] for the protein electron transfer complex between cytochrome c (cytc) and cytochrome c peroxidase (ccp) has been tested. Complex formation between these two proteins was probed by constructing site-directed mutations on the surface of ccp and preparing a fluorescent derivative of ccp, magnesium-porphyrin ccp (Mgccp). Data from fluorescence titrations, steady-state kinetics, chromatography and competition binding experiments suggest that this model only partially accounts for the electrostatic elements of binding. Aspartic acid 37 on the ccp surface, as predicted, plays a significant role in complexation and activity; when changed to lysine a significant decrease in the activity and binding to cytc is observed. Similar surface mutations at positions 79 and 217 show less dramatic effects.

Keywords: Cytochrome c and cytochrome c peroxidase, mutagenesis, complex formation, electron transfer, fluorescence

INTRODUCTION

One model of the binding between cytc and ccp, which are physiological redox partners, has been proposed by Poulos and Kraut.[1] Using computer-graphics modeling studies based upon electrostatic and hydrogen bonding interactions and the crystal structures of the individual proteins, they suggest that a region of positively charged lysines on cytc and a region of negatively charged residues on ccp are involved in the binding surface of the complex. The proposed interactions of residues believed to be important to the complex, as predicted by the modeling studies, include lysine residues 13, 27, and 72 of tuna cytc with aspartic acid residues 37, 79, and 217 of ccp, respectively. The proposed involvement of these residues is consistent with previous studies involving chemical modification in which these residues were found to be protected from modification when the proteins were complexed. In order to better understand the process of biological electron transfer, with specific emphasis on protein recognition and interaction, this model has been tested thermodynamically and kinetically[2-5] over the last few years.

SITE-DIRECTED MUTAGENESIS

Site directed mutagenesis can be used as an effective method of probing the binding interface of the cytc:ccp complex. By making specific replacements at positions purported to constitute key recognition sites, the nature of the binding interactions and the validity of the Poulos and Kraut model can be assessed. Ccp mutants were expressed in and isolated from E. coli and involve substitution of positively charged lysine residues for the negatively charged aspartic acid residues 37, 79, and 217. It is expected that this change in charge (+2) will perturb the binding of ccp to cytc without significantly disturbing the structure of the protein. The corresponding cytc mutants, provided by Fred Sherman's lab and involving the following replacements of lysyl residues, Gln32, Ile18, and Arg77, were studied previously.[3]

BINDING OF THE COMPLEX

Binding studies using fluorescence titrations were performed on the native and mutant proteins in both cytochrome c and ccp. The binding of the complex was monitored by fluorescence quenching. By substituting Mg porphyrin for the Fe porphyrin in native ccp, a fluorescent probe (Mgccp) is made. The fluorescence is quenched upon addition of Fecytc, thereby allowing the binding constant and stoichiometry of the complex to be measured.

The binding of Mgccp with cytochrome c showed a very clear 1:1 stoichiometry in the complex (Figure 1). The binding constant of the cytc:Mgccp complex was measured and found to be $2.9 \pm 0.1 \times 10^7\ M^{-1}$ in 10mM phosphate pH 7 buffer and $2.0 \pm 0.1 \times 10^7\ M^{-1}$ in 50mM pH 6 buffer. The binding of native and mutant ccp to cytc was also studied using (H_2) free base porphyrin cytochrome c as the fluorescent probe. The relative binding strength of the native and mutant ccp proteins follow the order ccp 217 > wild type > ccp79 > ccp37 (Figure 2).

Competition binding studies permit a direct means of visualizing the relative binding affinities of native and mutant ccps. If the sites mutated play a significant role in complex formation, it seems reasonable to expect that native and the various peroxidase mutants could exhibit different binding constants to iso-1-cytc. If two types of ccp (Mgccp and a mutant ccp) are present in solution with cytc an equilibrium of both types of complexes (Mgccp:cytc and mutant ccp:cytc) will be established reflecting the relative binding constants of the competing species.

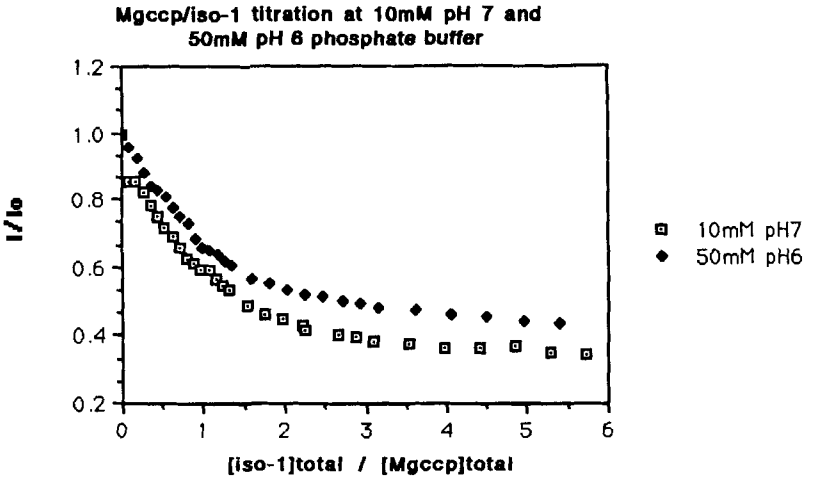

Figure 1. Fluorescence titration binding curves of Mgccp with cytc.

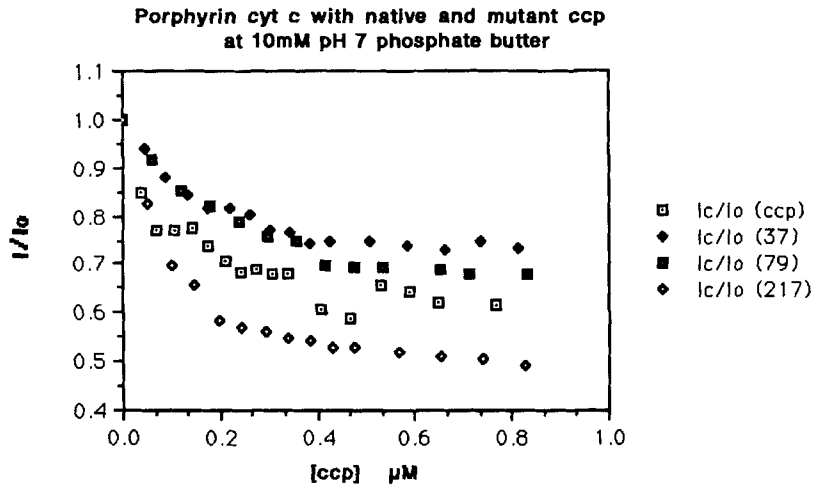

Figure 2. Fluorescence titration binding curves of H_2cytc with native and mutant ccp.

Experimentally, it is observed that when Mgccp is bound to Fe(III)iso-1-cytc in a 1:1 ratio, the addition of excess ccp of a different, nonfluorescent variety causes competitive binding between Mgccp and the second ccp. When the mutant (nonfluorescent) ccp binds competitively to the cytc, a recovery of fluorescence intensity is observed due to the increase of free Mgccp in solution. Since K_1, the binding constant of Mgccp with iso-1-cytc, is known through fluorescence titration binding experiments,

K_2, the binding constant of the second ccp to iso-1-cytc can be calculated from this competition titration experiment. As shown in Figure 3, ccp37 did not compete effectively compared to the other ccp mutants. This result suggests that, as predicted in the model, ccp37 plays a significant role in complexation. In contrast, the ccp79 and ccp217 mutants were more effective in the competition experiment, suggesting that these residues are less important for recognizing the cytc binding interface.

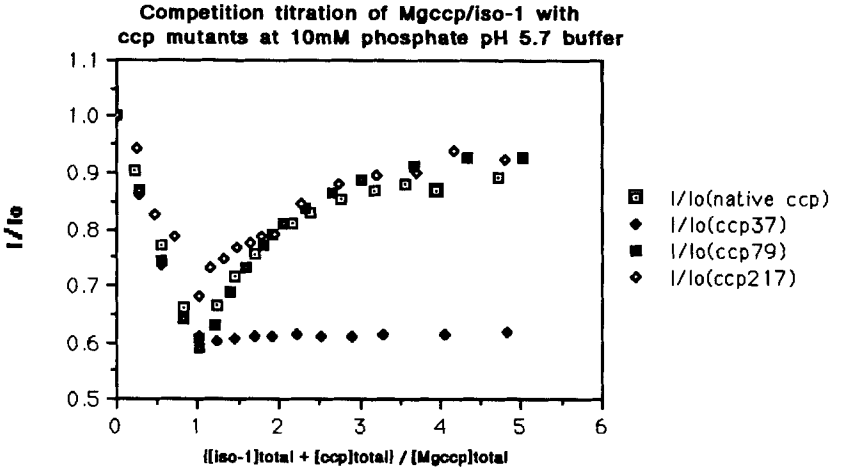

Figure 3. Competition titration curves of Mgccp/iso-1 with ccp mutants.

STEADY-STATE KINETICS

Steady-state kinetic measurements were conducted using horse heart cytc. Initial rates were measured for peroxidase catalyzed turnover of reduced cytc in the presence of excess (180μM) H_2O_2. The conversion of reduced cytc (FeII) was monitored spectrally at 550 nm and was corrected for direct oxidation of cytc. Corrected rates were then plotted as a function of [Fe(II)cytc] and fitted to the Michaelis-Menten equation. All of the peroxidases were fitted using similar K_ms but different ccp normalized maximum velocities, or turnover numbers, as shown in Table 1. Erman[6] has shown that the simple Michaelis-Menten equation is inadequate to fully describe the steady-state kinetics. However, the resulting turnover numbers extracted from such a simple treatment provides a measure of the relative catalytic efficiencies of the different peroxidases. Mutations of Asp → Lys at positions 79 and 217 showed catalytic activity similar to that of native ccp, but the same mutation at position 37 exhibited an order of magnitude less activity.

As stated above, this mutant also showed decreased binding affinity for cytc. Is the decreased activity a result of decreased binding or a decrease in electron transfer ?

Table 1. Steady State Turnover Numbers of Cytc by Ccp (Compound I)

Peroxidase	[cytc:ccp/sec]
WT* (Yeast)	321
cloned WT (*E. coli*)	389
D37K	26
D79K	249
D217K	312

Table 2. Limiting Electron Transfer Rates at PH 6 (μ = 114 mM)

Peroxidase	$K_{et}(s^{-1})$
WT (*E. coli*)	1940
D37K	140
D79K	230
D217K	2150

*WT stands for wild type. The cloned version behaves the same but has two amino acid differences from the protein isolated from yeast.

INTRACOMPLEX ELECTRON TRANSFER RATES

Measurements were conducted to obtain the single turnover (electron transfer) rates of cytc oxidation with flavin reduced cytc within a complex. Flash photolysis experiments were performed as described previously[7]. The radical semiquinone of 5-deazariboflavin generated in the experiment selectively reduces Fe(III) cytc in solution at pH 6 with equimolar concentrations of peroxide and ccp. Equimolar equivalents of peroxide produce a species of ccp which is oxidized two equivalents above the Fe(III) resting state, compound I and designated ccp[Fe(IV), R·+]. In this state the enzyme is poised to oxidize the Fe(II) cytc substrate. In a typical experiment (Figure 4) a rapid increase in the absorbance at 550 nm is observed, designating the semiquinone reduction of Fe(III) cytc, followed by a slower decay of the absorbance. The slower decay corresponds to the reoxidation of cytc by the peroxidase and the reduction of compound I back to the resting state. The kinetics are pseudo first order in ccp, as very low concentrations of reduced cytc are generated. The pseudo first order decay rates as a function of [ccp] constituted a hyperbolic response function, as might be expected from a two state reaction mechanism. Fits of this data to a hyperbola yielded the plateau or limiting electron transfer rates, which presumably correspond to the electron transfer rates in the complex (Table 2). From these limiting rates it is clear that Asp → Lys mutations at positions 37 and 79 substantially

decrease the observed rates. Wild type ccp and D217K gave similar rates. It should be noted that compound I of D79K was found to be significantly less stable than the other peroxidases and might explain the slower electron transfer rate observed for that enzyme. This is not the case for D37K. However, preliminary measurements at a much lower ionic strength (ca. μ = 8 mM), where formation of an electrostatic complex is probably forced, show that the electron transfer rates for D37K are increased to ca. 800 s^{-1}, a respectable rate. Binding data are needed to support this hypothesis.

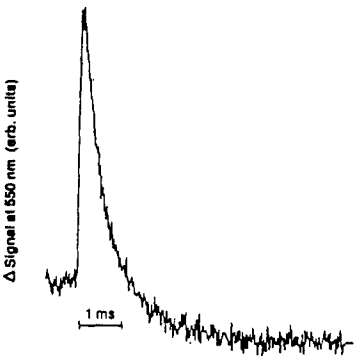

Figure 4. Transient trace from flash photolysis of 5-deazariboflavin, compound I and cytc. [Compound I] = [cytc] = 10 μM in pH 6 phosphate buffer, μ = 114 mM.

CONCLUSION

Complex formation between cytc and ccp is a critical prerequisite for electron transfer between these two molecules. Identical amino acids placed at different positions on the surface of peroxidase may play very different roles in protein-protein recognition, an essential component of biological specificity. This role may be distinct from the role that the same amino acid plays in electron conduction during the electron transfer step. When Asp 37 is changed to Lys a marked decrease in binding and electron transfer is observed for this protein to cytc at μ = 114 mM. However, if an electrostatic complex is presumably forced at lower ionic strength the electron transfer rate, although less than that observed for wild type, is much faster. Asp 37 appears to be important for protein-protein recognition but may not be critical to electron conduction between the two metal centers of the ccp/cytc complex.

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