Extragenic Compensation in Complex Formation: Restoration of Binding of a Charge Reversal Mutant of Cytochrome *c* Peroxidase (D217K) by a Compensatory Charge Reversal in Cytochrome *c* (K77D)

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The complex of cytochrome *c* (Cc) and cytochrome *c* peroxidase (Ccp) has become an instructive paradigm for molecular recognition in electron transfer systems. As such this system is extensively studied.[1−3] The crystal structures of each protein and of a specific Cc:Ccp complex are all known.[4] Detailed thermodynamic and kinetic studies have been performed.[1] The strong ionic strength dependence of binding and reaction suggests that interfacial charges can play important roles in binding.[5] Some controversy remains on the energetic and functional significance of specific structural sites within the complex, including the functional importance of "second site" binding.

In order to clarify the forces involved in site-specific recognition and reactivity, we and others have studied the effects in complex formation of amino acid replacements on the surface of Cc and/or Ccp.[6−8] Earlier studies from this lab focused on a subset of Ccp changes which are suggested to lie in or near two possible binding sites[1,4,7] for cytochrome c.

Since site-specific mutations can have many effects in addition to the intended one (e.g., conformational change) *direct* interpretation of a loss of function by mutation is not always straightforward. However, if function is restored by a specific alteration of the *partner* protein, then the interpretation of such alterations is highly restricted. The present report provides the *first* example of compensatory mutations in the Cc:Ccp paradigm and will provide experimental limits for ongoing theoretical analyses of recognition and binding in this system.

In the context of protein−protein recognition, such "second site" compensation studies are rare. They are of particular interest not only in understanding specific structure−function relationships but also as molecular level examples of "extragenic compensation". One recent elegant example of such extragenic compensation was provided by a study of the barnase−barstar[10] system. Here, a charge reversal in barstar which diminished binding could be compensated by an alternative charge reversal in barnase. For electron transfer complexes, where protein docking is intimately linked to reactivity, no equivalent examples of compensatory mutations exist. Such extragenic mutants allow for distinctions between structure function models which focus on specific stereochemical interactions, including "salt links" vs "patchwise" models in which the ionic strength-dependent recognition involves overall interactions between protein dipoles.

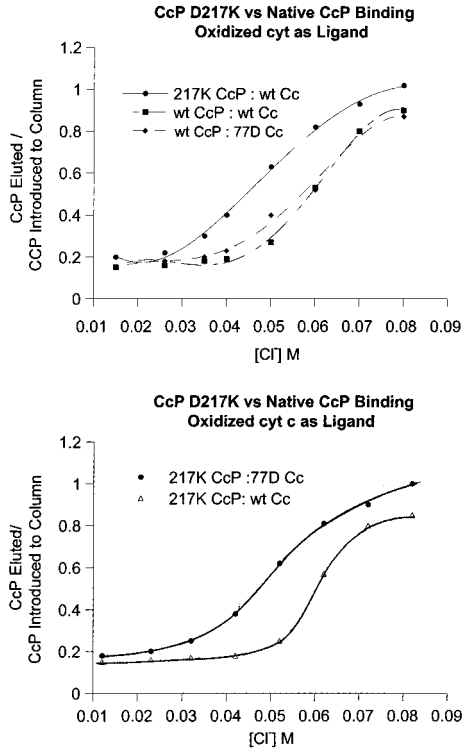

Figure 1. Elution profiles (first derivative) of Ccp from Cc (thiosepharose) affinity columns as a function of (molar) chloride ion concentration.

In the course of mutagenesis studies on the Cc:Ccp system, we discovered a remarkable example of extragenic compensation in Cc:Ccp binding which can directly address structure function issues for this system. Using an affinity chromatography method described in detail previously,[9] one can obtain (relative) binding profiles for the strong 1:1 binding site of Ccp with Cc. In brief, Cc is covalently attached to a thiosepharose column. Ccp binds noncovalently to Cc and is displaced by an ionic strength gradient. The elution profile can be analyzed to provide quantitative binding information.[9] Because under these conditions binding interactions respond cooperatively to ionic strength changes, the elution profiles can be measured with high precision: the elution midpoints are reproducible to better than 5%. Quantitative comparisons in binding are possible over the most sensitive binding region (e.g., 0.04−0.06 M salt). The shift in the binding curves between wild type and mutant Cc corresponds to a greater than a 50-fold change in effective binding at 0.04 M Cl^-. (The data are obviously insensitive to differences above 0.1 M or below 0.02 M.)[12] As described previously (refs 5 and 9), the chromatographic method used here gives binding data which quantitatively agree with those from the equilibrium (fluorescence) binding methods. As shown in Figure 1a, the replacement in Ccp of asparate 217 by lysine (D217K) leads to diminished binding of Ccp to Cc. This, by itself, is only mildly surprising. Residue 217 lies outside the primary interface defined crystallographically. Thus, a simple

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(12) As previously detailed (refs 5 and 9), the chromatographic method used here gives binding data which quantitatively agrees with equilibrium (fluorescence) binding methods.

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"pairwise" model would predict only minimal effects on binding. However, it has been emphasized previously that electrostatic binding can be sensitive to the overall electrostatic field at the interface.[11] Since the molecular replacement D217K alters this field, binding can be correspondingly altered. Single charge effects are well precedented in the Cc:Ccp system.

Important new information is obtained when complex formation of a charge reversal variant of Cc, K77D, is similarly examined. This variant binds to the D217K Ccp variant quite strongly (Figure 1b). Indeed, the binding profile of the D217K Ccp:K77D Cc complex is indistinguishable from the binding profile of the wild type proteins. Thus, the Cc K77D charge replacement *quantitatively* compensates for the loss of binding associated with the Ccp D217K replacement. This provides the first molecular level example of extragenic compensation in an electron transfer protein complex. However, in this case energetic compensation clearly does not imply direct interaction. A direct interaction between Cc residue 77 and Ccp residue 217 is inconsistent with the crystal structure of the complex. As noted above, it is the overall electrostatic fields at each protein surface that govern electrostatic binding.[11] Thus, the perturbation of Ccp by the D217K Ccp replacement appears to be compensated by the change at the Cc surface in the K72D replacement. An important caveat is that the Cc K77D variant might bind better to *any* Ccp of altered change. This is not the case. The Ccp D37K variant binds weakly to wild type Cc. However, the elution profile is unaltered for Ccp D37K on binding to the Cc K77D variant. Thus, the compensation of K77D Cc is specific to Ccp D217K.

In summary, we have observed that a charge reversal mutation in Ccp (D217K) which diminishes binding to Cc can be compensated by a charge reversal in Cc (K77D). Such compensation appears to be specific and quantitatively restores the wild type binding profile measured by affinity chromatography. To our knowledge, the K77D Cc:D217K Ccp pair represents the first example of compensatory mutation in an electron transfer protein complex.

Furthermore, these results demonstrate the subtle energetics of interfacial binding in the Cc:Ccp paradigm for protein-to-protein electron transfer. They provide specific support for models in which electrostatic binding interactions are be modulated by groups which do not interact directly and may lie outside the crystallographically defined interface. Detailed calculations of such interactions remain of interest. The present results place significant limits on any such models.

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