Interactions between Yeast Iso-1-cytochrome *c* and Its Peroxidase[†]

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ABSTRACT: Isothermal titration calorimetry was used to study the formation of 19 complexes involving yeast iso-1-ferricytochrome *c* (Cc) and ferricytochrome *c* peroxidase (CcP). The complexes comprised combinations of the wild-type proteins, six CcP variants, and three Cc variants. Sixteen protein combinations were designed to probe the crystallographically defined interface between Cc and CcP. The data show that the high-affinity sites on Cc and CcP coincide with the crystallographically defined sites. Changing charged residues to alanine increases the enthalpy of complex formation by a constant amount, but the decrease in stability depends on the location of the amino acid substitution. Deleting methyl groups has a small effect on the binding enthalpy and a larger deleterious effect on the binding free energy, consistent with model studies of the hydrophobic effect, and showing that nonpolar interactions also stabilize the complex. Double-mutant cycles were used to determine the coupling energies for nine Cc−CcP residue pairs. Comparing these energies to the crystal structure of the complex leads to the conclusion that many of the substitutions induce a rearrangement of the complex.

Iso-1-cytochrome *c* (Cc)[1] from the yeast *Saccharomyces cerevisiae* and Cc peroxidase (CcP) are physiological redox partners. Cc, a 12.5 kDa single-domain protein with a covalently bound heme *c*, is the penultimate electron-transfer protein of the eukaryotic respiratory chain whose main function is to accept electrons from Cc reductase and donate them to Cc oxidase (*1*). CcP is a 35 kDa monomer with a noncovalently bound heme, which undergoes a two-electron oxidation by peroxide (*2*). The oxidized CcP is re-reduced to its resting state by two ferroCc molecules in consecutive one-electron steps.

The Cc−CcP complex is one of the most well studied protein complexes. An important role for electrostatic interactions in the CcP reaction was recognized 60 years ago from studies of ionic strength dependence (*3*). A high-affinity Cc−CcP complex was postulated in 1964 (*4*) and observed in 1971 (*5*). Chemical modification studies in the 1970s identified the positively charged residues on Cc that are important for forming the high-affinity complex (*6*), while chemical cross-linking and protection studies helped to identify the important negatively charged residues on CcP (*7*, *8*). These results have been confirmed by more recent site-directed mutagenesis studies (*9*−*17*).

The pioneering molecular modeling study of Poulos and Kraut (*18*) suggested a structure for the complex. A refined version of this model (*2*), as well as the results from Brownian dynamics simulations (*19*) and analyses of electron-transfer pathways (*20*), is generally consistent with the results from experiment (*6*−*17*). However, steady-state kinetic experiments (*21*), electron-transfer studies (*20*, *22*), potentiometric titration experiments (*23*), and a recent isothermal titration calorimetry (ITC) study (*10*) have identified a second, weak Cc binding site on CcP that was predicted by simulation data (*19*, *20*).

A great deal is now known about the structure of the Cc−CcP complex. In 1987, Poulos et al. reported the production of crystals with a 1:1 molar Cc:CcP ratio, but defined electron density for the Cc could not be located (*24*). In 1992, Pelletier and Kraut reported high-resolution crystal structures for the horse Cc−CcP complex and the yeast iso-1-Cc−CcP complex (*25*). Both complexes possess one well-defined binding site on each protein, and the residues shown to be important for promoting high-affinity binding are found at the crystallographically defined binding sites.

The goal of the present studies is to quantify the thermodynamics of several interprotein interactions between yeast iso-1-ferriCc and ferriCcP. The interactions involve D34, V197, and E290 on CcP and K87, A81, and K72 on Cc. These Cc residues are part of the crystallographically defined CcP binding site in both the yeast (Figure 1) and horse Cc−CcP complexes, but the Cc binding sites on CcP are slightly different (*25*). We used ITC (*26*) to measure the thermodynamic parameters, protein variants to assess the contribution of individual residues, and double-mutant cycles (*27*) to examine specific side chain−side chain interaction energies (*28*−*30*).

To ensure that our analyses of the high-affinity site on CcP are not complicated by binding to other sites (*10*, *20*−*23*), we also studied CcP variants with amino acid changes at D217, L182, and D148. Modeling (*24*), dynamics simulations (*19*), and electron-transfer pathway analyses (*20*) suggest that D217 and L182 define part of the Cc binding site. The simulations also suggest that D148 is part of a second Cc binding site. As shown in Figure 1, these side chains are adjacent to the crystallographically defined site,

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[1] Abbreviations: Cc, cytochrome *c*; CcP, cytochrome *c* peroxidase; DMG, dimethylglutaric acid; ITC, isothermal titration calorimetry; WT, wild-type.

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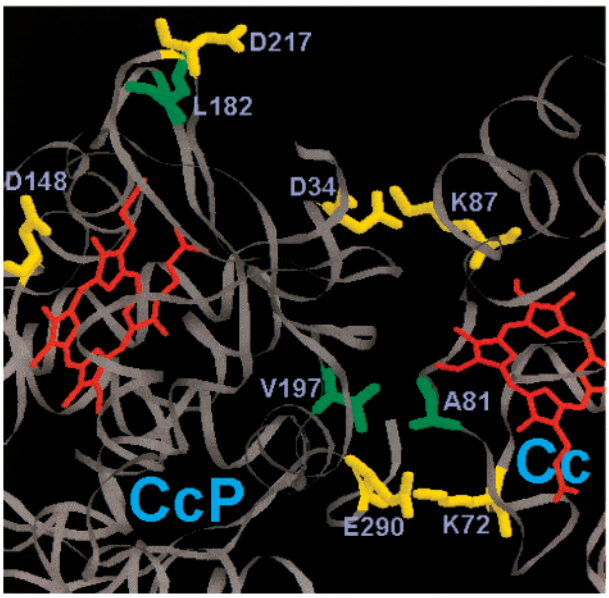

FIGURE 1: Portion of the binding interface taken from the crystal structure of the yeast iso-1-Cc−CcP complex (*25*). The residues studied here are numbered, and their side chains are colored yellow (charged side chains) or green (nonpolar side chains). The porphyrin rings are colored red.

with the closest residue (L182) more than 13 Å from the defined site.

MATERIALS AND METHODS

Protein Variants. Cc and CcP refer to the ferri forms of these recombinant proteins expressed in *Escherichia coli*. The C102T variant of recombinant yeast iso-1-Cc is termed the wild-type (WT) protein; all other Cc variants also carry this mutation. The Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to construct the mutants (extension temperature of 70 °C, 16 cycles per reaction). Cc mutants were constructed on the pBTR2(C102T) plasmid, which carries the gene for the C102T mutant (*31*). The original plasmid (*32*) was a gift from G. Mauk. CcP mutants were constructed on a plasmid containing the WT CcP gene, which was a gift from T. Poulos. The DNA sequence of the entire coding region of each mutant was confirmed by automated DNA sequencing. No additional mutations were found.

Sample Preparation. Recombinant Cc and its variants were expressed and purified from freshly transformed BL21 DE3 *E. coli* cells as described by Morar et al. (*31*). Recombinant CcP and its variants were expressed and purified from *E. coli* by using a modified version (*33*) of a published protocol (*34*). The ratio of the absorbance at 408 nm to that at 280 nm ranged between 1.20 and 1.30, showing that the CcP has a five-coordinate high-spin heme (*35*). Aliquots of CcP crystals were stored in deionized, distilled water at −80 °C and dissolved in buffer before use.

ITC. Experiments were performed with a Microcal MCS calorimeter (Northampton, MA). Solutions were buffered with 50 mM dimethylglutaric acid (DMG) (Sigma, St. Louis, MO), adjusted with NaOH to pH 6.0. Cc was treated as the ligand and injected with a 250 μL syringe, which was rotated at 300 or 400 rpm for the duration of each experiment. The injection volume was 5−15 μL depending on the protein concentrations, but was constant for each experiment. The time between injections was 360 s. Experiments were performed at 25 °C, with an external water bath temperature of 20 °C. The calorimeter was calibrated by titrating 2′-CMP into ribonuclease A as recommended by Microcal. Other details are given by Wang and Pielak (*33*).

Table 1: Thermodynamic Parameters for Protein Pairs That Test the Crystallographically Defined Binding Site[a]

		WT CcP	D34A CcP	V197A CcP	E290A CcP
WT Cc	no.	5	3	3	3
	ΔH	−2.6 ± 0.1	−0.9 ± 0.1	−2.3 ± 0.2	−5.8 ± 0.3
	ΔG	−8.1 ± 0.1	−9.0 ± 0.7	−6.0 ± 0.2	−1.9 ± 0.2
	$\Delta\Delta H$	0 ± 0.1	1.7 ± 0.1	0.3 ± 0.2	−3.2 ± 0.3
	$\Delta\Delta G$	0 ± 0.1	−0.9 ± 0.7	2.1 ± 0.2	6.2 ± 0.2
K87A Cc	no.	2	3	3	3
	ΔH	−0.8	−0.7 ± 0.1	−0.9 ± 0.2	0 to −0.4[b]
	ΔG	−7.2	−7.9 ± 0.3	−6.6 ± 0.5	nd
	$\Delta\Delta H$	1.8 ± 0.4	1.9 ± 0.1	1.7 ± 0.2	2.2 ± 0.3
	$\Delta\Delta G$	0.9 ± 0.5	0.2 ± 0.3	1.5 ± 0.5	nd
K72A Cc	no.	3	2	3	3
	ΔH	−1.1 ± 0.1	∼0.3 [b]	−0.5 ± 0.1	−1.2 ± 0.1
	ΔG	−7.8 ± 0.2	nd	−5.3 ± 0.7	−7.0 ± 0.2
	$\Delta\Delta H$	1.5 ± 0.1	2.9 ± 0.5	2.1 ± 0.4	1.4 ± 0.1
	$\Delta\Delta G$	0.3 ± 0.2	nd	2.8 ± 0.7	1.1 ± 0.2
A81G Cc	no.	3	1	2	2
	ΔH	−2.4 ± 0.3	−0.8	−2.2	∼−0.3[b]
	ΔG	−6.2 ± 0.4	−8.2	−6.0	nd
	$\Delta\Delta H$	0.2 ± 0.3	1.8 ± 0.3	0.4 ± 0.3	2.3 ± 0.3
	$\Delta\Delta G$	1.9 ± 0.4	−0.1 ± 0.5	2.1 ± 0.5	nd

[a] Experiments were performed at 25 °C in 50 mM DMG buffer (pH 6.0). The units for ΔH, ΔG, $\Delta\Delta H$, and $\Delta\Delta G$ are kilocalories per mole. no., number of ITC experiments. nd, not determined. [b] For these protein pairs, the heats were too small to give a reliable fit.

Error Analysis. Uncertainties in ΔH and ΔG are reported as the standard deviation of the mean from repetition of the experiment. In all instances, the sample standard deviations obtained from the quality of the fits were smaller than the sample standard deviations obtained from repeating the experiment. This observation indicates that the data are well fit by the model, and suggests that the uncertainties arise from random errors. For experiments that were performed fewer than three times, the uncertainties in ΔH and ΔG were assumed to be ±0.3 and ±0.7 kcal mol^{-1}, respectively, the largest measured uncertainties for experiments performed three or more times (Table 1). The uncertainties in $\Delta\Delta G$ and $\Delta\Delta H$ were estimated by using standard propagation procedures (*36*). The uncertainty in $\Delta\Delta S$ was estimated by using standard procedures to propagate the uncertainties in $\Delta\Delta H$ and $\Delta\Delta G$ through a modified form of the Gibbs equation [$\Delta\Delta S = (\Delta\Delta H - \Delta\Delta G)/T$]. Uncertainties for comparing the distribution of $\Delta\Delta G$ values and $\Delta\Delta H$ values were computed as the sample standard deviation.

Coupling Energies. $\Delta\Delta H_c$ and $\Delta\Delta G_c$ were calculated by using eq 1 (*29*).

$$\Delta\Delta J_c = \Delta J_{X,Y} - \Delta J_{X\rightarrow A/G,Y} - \Delta J_{X,Y\rightarrow A/G} + \Delta J_{X\rightarrow A/G,Y\rightarrow A/G} \quad (1)$$

where J represents H or G, X and Y represent the WT residues in Cc and CcP, respectively, and A/G represents a change to alanine or glycine. The contribution of the coupling entropy to $\Delta\Delta G_c$ ($-T\Delta\Delta S_c$) was calculated by using the

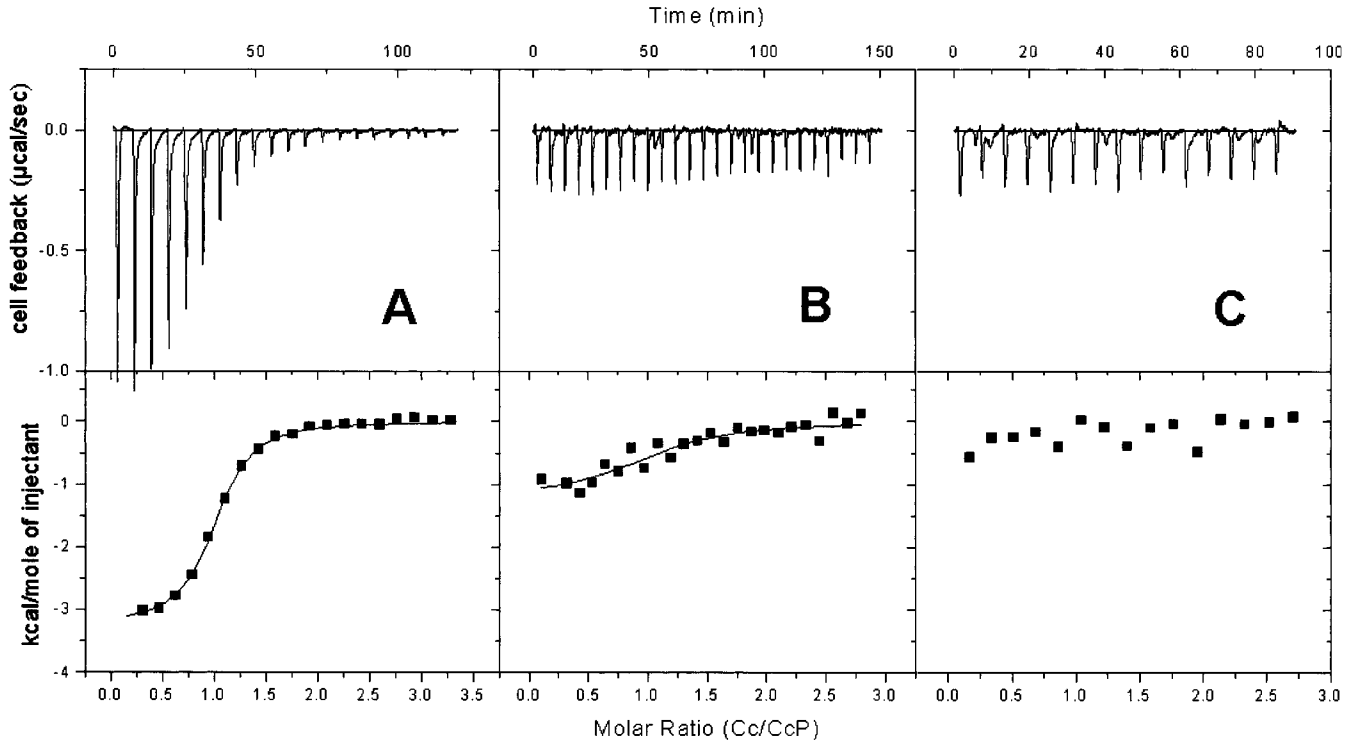

FIGURE 2: ITC data for iso-1-Cc−CcP binding (25 °C, 50 mM DMG, pH 6.0). The upper panels show data after baseline correction. The lower panels show integrated data corrected for heat of dilution (■) and fits to a 1:1 binding model (—). Panel A shows the titration of 1.85 mM WT Cc (8.0 μL injections) into 70 μM CcP. Panel B shows the titration of 0.95 mM K72A Cc (6.0 μL injections) into 40 μM E290A CcP. Panel C shows the titration of 0.95 mM K72A Cc (12.0 μL injections) into 42 μM D34A CcP.

Table 2: Thermodynamic Parameters for Protein Pairs Designed To Test Other Cc Binding Sites on CcP[a]

		D217A CcP	L182A CcP	D148A CcP
WT CcP	no.[b]	3	3	3
	ΔH	-3.0 ± 0.2	-4.0 ± 0.3	-2.7 ± 0.2
	ΔG	-7.7 ± 0.1	-7.4 ± 0.2	-7.8 ± 0.1
	$\Delta\Delta H$	-0.3 ± 0.2	-1.4 ± 0.4	-0.1 ± 0.2
	$\Delta\Delta G$	0.4 ± 0.2	0.7 ± 0.2	0.3 ± 0.2

[a] Conditions are the same as those described in Table 1. [b] Number of ITC experiments.

equation $\Delta\Delta G_c = \Delta\Delta H_c - T\Delta\Delta S_c$. Uncertainties were estimated by using standard procedures (*36*).

RESULTS AND DISCUSSION

Three ITC data sets are shown in Figure 2. Panel A shows high-quality data representative of most of the experiments. Panel B shows the lowest-quality data that could be reliably fit to the binding model. Panel C shows a data set that could not be fit. The parameters derived from all the titrations are summarized in Tables 1 and 2. ΔG, ΔH, and ΔS represent standard state values, where the standard state refers to 1 M reactants and products at pH 6.0, and are presented in terms of complex formation. $\Delta\Delta G$, $\Delta\Delta H$, and $\Delta\Delta S$ are presented as the values for a complex minus the values for the WT Cc−WT CcP complex. The results are reported as averages when multiple experiments were performed. See Materials and Methods for a complete description of error analysis.

Authentic versus Recombinant Yeast Iso-1-Cc. Authentic yeast iso-1-Cc, which contains a trimethylated lysine at position 72 (*26*), was used in the crystallographic study (*25*). This protein also contains a free cysteine at position 102, which causes covalent homodimer formation in solution (*37*). In the studies reported here, we used a recombinant version of the C102T variant expressed in *E. coli* (*32*). The C102T variant from yeast is structurally identical to the authentic WT protein (*38*−*40*), but is not prone to dimerization (*41*). The *E. coli*-expressed C102T variant is also monomeric and structurally identical to the C102T variant from yeast (*42*), but K72 is unmodified (*32*). This lack of modification does not affect in vivo stability or function (*43*, *44*), but it does decrease the p*K* of the alkaline transition from ~8.6 to ~8.0 (*32*). This change in p*K* does not affect our results because the pH used in the present studies, 6.0, is much lower than the p*K*.

We previously studied the interaction between authentic C102T iso-1-Cc and CcP (*33*). Under the same conditions used here, the stoichiometry, enthalpy, and free energy of complex formation are 0.97 ± 0.02, -6.2 ± 0.3 kcal mol^{-1}, and -8.1 ± 0.1 kcal mol^{-1}, respectively. Comparing these values to those for the *E. coli*-expressed protein (Table 1) shows that deleting the K72 methyl groups increases ΔH by 3.6 ± 0.1 kcal mol^{-1}, but has no effect on ΔG.

Removing the K72 methyl groups makes the binding surface of recombinant iso-1-Cc more like that of horse Cc, but the thermodynamics of complex formation remains distinct. Specifically, binding is exothermic for the reactions of WT CcP with authentic C102T iso-1-Cc (*33*), recombinant C102T iso-1-Cc (Table 1), and variants of recombinant C102T iso-1-Cc (Tables 1 and 2), but binding is endothermic for the reactions of CcP and its variants with horse Cc (*11*, *45*). Therefore, removing the K72 methyl groups does not convert the iso-1-Cc−CcP complex into the horse Cc−CcP complex.

Stoichiometries. Although 2:1 Cc−CcP binding has been detected by other techniques (*20*−*23*), and in ITC studies

of the horse Cc−CcP complex (*10*), we find no evidence of 2:1 binding for the yeast Cc−CcP complex under the conditions used here. We fit our ITC data to models with both 1:1 and 2:1 stoichiometries. In all instances where sufficient heat was evolved to allow analysis, the data were well fit by a 1:1 model, and could not be fit by a 2:1 model. The average stoichiometry for five trials involving the WT proteins is 1.06 ± 0.04, and the average for all 53 titrations is 1.02 ± 0.01, with a range of 0.88−1.16. Furthermore, if a low-affinity site existed, disrupting an interaction at the high-affinity site might be expected to increase the contributions from lower-affinity sites (*10*), but such contributions are absent. Given the protein concentrations used here, we could not detect a second binding site if $|\Delta H| \leq$ approximately 0.5 kcal mol^{-1} or if $\Delta G \geq$ approximately −1.5 kcal mol^{-1}. However, our data provide no information about possible kinetic roles for weak sites.

Other Binding Sites. Our goal is to analyze the crystallographically defined yeast Cc binding site on CcP. To ensure that the high-affinity site on CcP does not include residues from the other proposed sites discussed in the introductory section, we studied the reactions between Cc and the D217A, L182A, and D148A variants of CcP. As shown in Table 2, the D217A and D148A variants do not affect binding. We conclude that D217 and D148 are not part of the high-affinity binding site. The L182A variant has a small effect on ΔG and a larger effect on ΔH, but L182 is the closest of these three residues to the crystallographically defined site. Taken together, these observations suggest that our analysis will not be affected by binding at these other sites. We now turn to analysis of the crystallographically defined binding site (Table 1).

Analysis of ΔG, ΔH, *and* ΔS. Complex formation is exothermic in all instances where binding could be evaluated, but $|\Delta H|$ is greater than $|\Delta G|$, with one exception (the WT Cc−E290A CcP complex). Therefore, complex formation is mostly driven by a favorable entropy change. Given the entropic penalty incurred by bringing two molecules together, this favorable entropic contribution suggests an important role for solvent reorganization.

We could not determine ΔG values for three of the 16 protein combinations shown in Table 1 (K87A Cc−E290A CcP, K72A Cc−D34A CcP, and A81G Cc−E290A CcP) because ΔH approaches zero (Figure 2C). When ΔH is near zero, weak binding cannot be differentiated from strong entropy-driven binding. We assume that binding is weak for these three complexes because other protein combinations harboring at least one of these variants yield interpretable ITC data. The three instances all involve the change of at least one charged residue, consistent with the idea that electrostatic interactions are important for stabilizing the complex.

Analysis of $\Delta\Delta G$, $\Delta\Delta H$, *and* $\Delta\Delta S$. As shown by the data in Table 1, nearly all the targeted residues stabilize the complex. From this observation, we conclude that the high-affinity site includes the crystallographically defined site, a conclusion consistent with all studies of complex formation between Cc and CcP (*6*−*17*, *46*−*48*).

The data in Table 1 also provide more specific information about the contribution of charged and nonpolar residues located at the interface. Changing a charged residue to alanine causes a constant and destabilizing change in ΔH, but the change in ΔG depends on the location of the residue. In seven of the eight examples in which one charged residue is changed to alanine, ΔH increases by an average of 1.8 kcal mol^{-1} with a standard deviation of only ±0.3 kcal mol^{-1}. This sign of $\Delta\Delta H$ suggests that complementary electrostatic interactions impart enthalpic stabilization. The constant value of $\Delta\Delta H$ is more difficult to explain, but this finding suggests that individual charge−charge interactions contribute equally to ΔH. The situation is completely different for $\Delta\Delta G$, which ranges from 2.1 to −0.9 kcal mol^{-1}. The large range shows that the entropic contribution depends on the location of the substituted residue.

The data for formation of the WT Cc−E290A CcP complex are the exception to the above observation about ΔH. For this pair, ΔH *decreases* by 3.2 ± 0.7 kcal mol^{-1} compared to that of the WT pair. The residue following E290 in CcP is D291. We speculate that changing E290 to alanine allows D291 to form a new, enthalpically stabilizing interaction with a positively charged residue on Cc. This new interaction would be expected to disrupt the alignment or the solvation of the two proteins. Such a disruption would be expected to decrease the stability of the complex, as is observed. As discussed in the section about coupling energies, mutation-induced rearrangements appear to be present in several of the complexes, especially those involving the E290A variant.

Burying nonpolar residues at the interface also stabilizes the complex, but for these residues, there is a uniform contribution to ΔS. Table 1 shows data for three complexes where one, two, or three methyl groups have been removed but the charge on the complex is held constant (WT Cc−V197A CcP, A81G Cc−WT CcP, and A81G Cc−V197A CcP). For these complexes, both $\Delta\Delta H$ and $\Delta\Delta G$ are constant, 0.3 ± 0.1 and 2.0 ± 0.1 kcal mol^{-1}, respectively, but $\Delta\Delta H$ is nearly zero. These results are consistent with what is known about the hydrophobic effect (*49*); ΔH is nearly zero for transferring a nonpolar group from water to a nonpolar liquid at room temperature, while the free energy of transfer is unfavorable. Like the model studies, burial of nonpolar groups stabilizes the complex by decreasing the entropy of complex formation.

In summary, both electrostatic and nonpolar residues are important for stabilizing the complex. However, it is not possible to state which type of interaction is more important because the effect of changing charged residues depends on the location of the charge.

Coupling Energies. Nine interprotein interactions (Figure 1) were quantified by using double-mutant cycles (*27*−*30*). In this approach, the side chains of both residues are truncated and the thermodynamics of the interactions between all four protein pairs is determined. Equation 1 gives the coupling free energy, $\Delta\Delta G_c$, and the coupling enthalpy, $\Delta\Delta H_c$. The entropic component, $-T\Delta\Delta S_c$, is evaluated from $\Delta\Delta H_c$ and $\Delta\Delta G_c$ by using the Gibbs equation. The results are shown in Table 3.

Provided that the assumptions inherent in this analysis are valid, a negative coupling energy suggests that the interaction stabilizes the complex, while a positive value suggests destabilization. Comparing coupling energies to the crystal structure of the complex then reveals relationships between energetics and structure. Such analyses have provided

Table 3: Coupling Energies[a]

residue pair (Cc−CcP)	$\Delta\Delta H_c$	$-T\Delta\Delta S_c$	$\Delta\Delta G_c$
K87−D34	-1.6 ± 0.3	1.8 ± 1.0	0.2 ± 0.9
K87−V197	-0.4 ± 0.4	-1.1 ± 0.9	-1.5 ± 0.7
K87−E290	3.6 ± 0.5	nd	nd
K72−D34	-1.0 ± 0.3	nd	nd
K72−V197	0.3 ± 0.3	0.1 ± 0.8	0.4 ± 0.8
K72−E290	3.1 ± 0.4	-8.5 ± 0.5	-5.4 ± 0.4
A81−D34	-0.1 ± 0.4	-1.0 ± 1.0	-1.1 ± 1.0
A81−V197	-0.1 ± 0.5	-1.8 ± 0.8	-1.9 ± 0.7
A81−E290	5.3 ± 0.5	nd	nd

[a] The units for coupling energies are kilocalories per mole. nd, not determined because one of the parameters is missing (see Table 1).

valuable information about protein complexes (*28*−*30*), but it is important consider the assumptions, which are discussed below.

(1) The Structure of the Individual Proteins Does Not Change upon Complex Formation. If a structural change occurs, then the coupling energies contain contributions from both the structural change and binding. This complication is probably not important for the Cc−CcP complex because, except for a few surface side chains, the structures of Cc and CcP in the complex are the same as the structures of the individual proteins (*25*).

(2) The Amino Acid Substitutions Do Not Alter the Structure of the Individual Proteins. This assumption should be valid because the substitutions are on the protein surface. That is, the substituted side chains interact more with the solvent than with the protein interior.

(3) The Model Used To Fit the ITC Data Reflects the Equilibrium Mechanism of Complex Formation. This assumption is important for interpreting $\Delta\Delta G_c$ and $-T\Delta\Delta S_c$ (interpreting $\Delta\Delta H_c$ is model-independent because ΔH is the difference between the horizontal baselines in Figure 2B). The fact that the data can only be fit by a 1:1 model (Figure 2) suggests that our simple binding model is appropriate. It must be borne in mind, however, that the calorimetric enthalpy for the WT pair does not equal the van't Hoff enthalpy (*33*), an observation also made for other reactions (*50*). These differences suggest the model used to determine the van't Hoff enthalpy is inappropriate, but the source of the difference is unknown (*51*). For the purposes of this paper, we assume that the simple model is correct.

(4) The Crystal Structure of the Complex Is the Same as Its Solution Structure. Although this assumption can only be completely tested by determining both structures, an indication of its validity comes from comparing $\Delta\Delta G_c$ to the distance between the two residues. In several studies, residues more than 8 Å apart do not exhibit significant $\Delta\Delta G_c$ values (*28*−*30*). A second indication comes from comparing the structure to the results of solution-based cross-linking studies. The observation that interprotein interactions present in the crystal are also present in cross-linked areas supports the assumption of structural identity. The results from cross-linking the horse Cc−CcP complex are consistent with its crystal structure (*7*, *8*).

(5) The Amino Acid Changes Do Not Induce a Rearrangement of the Structure or Solvation of the Complex. There is no easy way to define structural changes short of structural analysis, but comparing $\Delta\Delta G_c$ to the distance between the residues provides confidence. We have attempted to avoid structural changes by substituting large residues with small residues so as not to increase steric bulk. Changes in hydrophobic solvation are also difficult to prove, but because they are often accompanied by enthalpy−entropy compensation (*52*), the observation that $|\Delta\Delta H_c|$ is much greater than $|\Delta\Delta G_c|$ provides an indication (*29*). Changes in the solvation of charged groups can also complicate interpretation if the substitutions affect complex-induced changes in pK (*53*, *54*). Such effects cannot be ruled out because we studied complex formation at a single pH.

Additional Caveats. Interpretation can be difficult even when the above assumptions seem valid. For instance, Pons et al. (*30*) showed that interpreting $\Delta\Delta G_c$ for the interaction between oppositely charged residues separated by less than 8 Å in terms of electrostatics is confounded by the fact that nonpolar interactions are also disrupted by changing a large charged side chain to a small uncharged side chain. Furthermore, Lockless and Ranganathan have observed evolutionarily conserved coupling between residues separated by more than 8 Å (*55*). Finally, Mark and van Gunsteren have warned against over interpreting $\Delta\Delta G_c$ and $\Delta\Delta S_c$ because decomposition of $\Delta\Delta G_c$ in terms of specific interactions is not possible (*56*), but this idea is controversial (*29*, *57*, *58*). With these issues in mind, we turn to the analysis of the coupling energies (Table 3).

K87−D34. $\Delta\Delta G_c$ for this interaction is indistinguishable from zero, despite a significant and stabilizing $\Delta\Delta H_c$. In studies of the barnase−barstar complex, the observation of negative $\Delta\Delta H_c$ values for residues more than 8 Å apart despite the fact that $\Delta\Delta G_c$ is near zero was taken as evidence for local solvation changes accompanied by enthalpy−entropy compensation (*29*). Taken at face value, these observations suggest that the side chains must be separated by more than 8 Å. However, inspection of the structures shows that they are separated by 3.8 Å in the yeast Cc complex (Figure 1) and by 5.3 Å in the horse Cc complex. These observations suggest either that the crystal structure and the solution structure of the complex are different or that a rearrangement has occurred in one or more of the variant-containing complexes. Since an interaction between K87 and D34 is suggested in many other studies (*2*, *6*−*8*, *19*, *20*, *25*), a rearrangement seems more likely because nearby residues (K86 on Cc and D33 and E35 on CcP) could replace the K87−D34 interaction.

K87−V197. $\Delta\Delta G_c$ for the K87−V197 pair is -1.5 ± 0.7 kcal mol^{-1}, suggesting that this interaction stabilizes the complex. However, inspection of the structures shows that the side chain atoms on these residues are separated by more than 16.7 Å. These results suggest either that the solution and crystals structures differ, that the residues are involved in a long-range interaction, or that at least one of the amino acid substitutions causes a rearrangement. Unfortunately, there is no way to decide between these alternatives.

K87−E290. $\Delta\Delta G_c$ could not be calculated because ΔG for the K87A Cc−E290A CcP variant cannot be determined (Table 1). If we assume that ΔG is greater than or equal to the estimated detection limit (-1.5 kcal mol^{-1}), $\Delta\Delta G_c$ would be at least -0.5 kcal mol^{-1}. In the crystal structures, the side chains are separated by more than 16.7 Å. These observations suggest that the interaction is unimportant because the residues are far apart, but analysis of $\Delta\Delta H_c$ provides additional insight. The positive value of $\Delta\Delta H_c$ (3.6

$\pm$ 0.5 kcal mol^{-1}) suggests that the interaction is enthalpically *destabilizing*. One would expect that interactions between oppositely charged residues would be enthalpically stabilizing even at distances of more than 8 Å (*29*). We conclude that at least one of the substitutions causes a rearrangement. A rearrangement is consistent with the presence of other nearby charged residues (K86 on Cc and E291 on CcP).

K72−D34. $\Delta\Delta H_c$ is negative, but $\Delta\Delta G_c$ could not be calculated because ΔG for one complex could not be determined (Table 1). If it is assumed that ΔG is greater than or equal to the detection limit, $\Delta\Delta G_c$ would be $\geq$7.2 kcal mol^{-1}, an extremely large and positive value. In both crystal structures, the atoms on these residues are separated by more than 16.9 Å. These observations suggest either that the crystal and solution structures differ and the K72−D34 interaction *destabilizes* the complex or that at least one of the amino acid substitutions causes a rearrangement. A rearrangement seems more likely because, even if the structures differ, a lysine−aspartate interaction would be expected to stabilize the complex. A third explanation, that salt bridges impart specificity, not stability, is also reasonable, but a rearrangement seems more likely because of the local availability of other charged residues (K73 on Cc and D33 and E35 on CcP).

K72−V197. For this pair, all the coupling energies are indistinguishable from zero. The distance between the nearest γ-carbon on V197 and the side chain nitrogen atom of L72 is 5.2 Å in the yeast Cc complex. The simplest explanation is that the K72−V197 interaction is not important for complex formation.

K72−E290. This residue pair gives the most negative measured $\Delta\Delta G_c$ (-5.4 ± 0.4 kcal mol^{-1}). The shortest distance between the side chain nitrogen atom of K72 and the nearest side chain oxygen atom of E290 is 10.3 Å in the yeast Cc complex and 3.2 Å in the horse complex. The simplest explanation is that K72 and E290 form a stabilizing interaction because the orientation of the K72 side chain in the yeast Cc complex in solution is slightly different from its orientation in the crystal. As for the K87−E290 interaction, $\Delta\Delta H_c$ is large and positive, which is unexpected for an interaction between complementary charged residues. We conclude that a rearrangement has occurred because of the other charged residues in the vicinity of both K72 and E290.

A81−D34. $\Delta\Delta G_c$ is slightly stabilizing, and atoms on these residues are separated by more than 16.7 Å in both structures. We conclude that the interaction between A81 and D34 is not important.

A81−V197. This interaction involves two uncharged residues. $\Delta\Delta G_c$ is -1.9 ± 0.7 kcal mol^{-1}, and all the stabilization arises from $\Delta\Delta S_c$. In the yeast Cc complex, the β-carbon of A81 is 4.4 Å from the nearest γ-carbon of V197 (the distance between the closest γ-carbon of I81 of horse Cc and the nearest γ-carbon of V197 is 7.4 Å). A large entropic contribution and a small enthalpic contribution are exactly what is expected from the hydrophobic effect (*49*). We conclude that the interaction stabilizes the complex by the mutual and specific burial of nonpolar surface.

A81−E290. $\Delta\Delta G_c$ for this pair could not be calculated because ΔG for the A81G Cc−E290A CcP complex could not be determined (Table 1). If it is assumed that ΔG is greater than or equal to the detection limit, $\Delta\Delta G_c$ would be at least -1.5 kcal mol^{-1}. The closest approach between the oxygen atoms on E290 and the β-carbons of A81 is more than 9.4 Å. Given this distance, the large positive $\Delta\Delta H_c$, and our conclusions about other E290 interactions, it seems likely that one of the amino acid substitutions causes a rearrangement.

CONCLUSIONS

The high-affinity binding sites on Cc and CcP correspond to the crystallographically defined interface between the two proteins, and two alternative Cc binding sites on CcP do not interfere with our study of the high-affinity site. Comparison of the data for the variants that probe the high-affinity site to the data for the recombinant WT complex reveals two patterns. First, the enthalpic effect of changing a charged residues is constant and destabilizing, but the entropic effect depends on the location of the substitution. The one exception suggests that some of the substitutions change the structure or the solvation of the complex. Second, changing nonpolar residues imparts an inconsequential enthalpy change and a larger destabilizing entropy change, as is expected from model studies of the hydrophobic effect. Coupling energies were determined for nine residue pairs. For seven pairs, the data are most consistent with mutation-induced rearrangements of the complex caused by the availability of other charged residues close to the sites of the amino acid substitutions.

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REFERENCES

1. Pettigrew, G. W., and Moore, G. R. (1987) *Cytochromes c: Biological Aspects*, Springer-Verlag, London.
2. Poulos, T. L., and Finzel, B. (1984) *Pept. Protein Rev. 4*, 115−171.
3. Altschul, A. M., Abrams, R., and Hogness, T. R. (1940) *J. Biol. Chem. 136*, 777−794.
4. Nicholls, P. (1964) *Arch. Biochem. Biophys. 106*, 25−48.
5. Mochan, E., and Nicholls, P. (1971) *Biochem. J. 121*, 69−82.
6. Millett, F., and Durham, B. (1996) in *Cytochrome c: A Multidisciplinary Approach* (Scott, R. A., and Mauk, A. G., Eds.) pp 573−592, University Science Books, Sausalito, CA.
7. Waldmeyer, B., and Bosshard, H. R. (1985) *J. Biol. Chem. 260*, 5184−5190.
8. Bechtold, R., and Bosshard, H. R. (1985) *J. Biol. Chem. 260*, 5191−5200.
9. Hazzard, J. T., McDonough, C. A., and Tollin, G. (1994) *Biochemistry 33*, 13445−13454.
10. Leesch, V. W., Bujons, J., Mauk, A. G., and Hoffman, B. M. (2000) *Biochemistry 39*, 10132−10139.
11. Erman, J. E., Kresheck, G. C., Vitello, L. B., and Miller, M. A. (1997) *Biochemistry 36*, 4054−4060.
12. Hake, R., McLendon, G., Corin, A., and Holzschu, D. (1992) *J. Am. Chem. Soc. 114*, 5442−5443.
13. Corin, A. F., Hake, R. A., McLendon, G., Hazzard, J. T., and Tollin, G. (1993) *Biochemistry 32*, 2756−2762.
14. Corin, A. F., McLendon, G., Zhang, Q., Hake, R. A., Falvo, J., Lu, K. S., Ciccarelli, R. B., and Holzschu, D. (1991) *Biochemistry 30*, 11585−11595.
15. Hake, R., Corin, A., and McLendon, G. (1997) *J. Am. Chem. Soc. 119*, 10557−10558.

16. Mei, H., Wang, K., McKee, S., Wang, X., Waldner, J. L., Pielak, G. J., Durham, B., and Millett, F. (1996) *Biochemistry 35*, 15800−15806.
17. Miller, M. A., Lin, R.-Q., Hahm, S., Geren, L., Hibdon, S., Kraut, J., Durham, B., and Millett, F. (1994) *Biochemistry 33*, 8686−8693.
18. Poulos, T. L., and Kraut, J. (1980) *J. Biol. Chem. 255*, 10322−10330.
19. Northrup, S. H., Boles, J. O., and Reynolds, J. C. L. (1988) *Science 241*, 67−70.
20. Nocek, J. M., Zhou, J. S., DeForest, S., Priyadarshy, S., Beratan, D. N., Onuchic, J. N., and Hoffman, B. M. (1996) *Chem. Rev. 96*, 2459−2489.
21. Kang, C. H., and Margoliash, E. (1977) *J. Biol. Chem. 252*, 919−926.
22. Mei, H., Wang, K., Peffer, N., Weatherly, G. T., Cohen, D. S., Miller, M., Pielak, G. J., Durham, B., and Millett, F. (1999) *Biochemistry 38*, 6846−6854.
23. Mauk, M. R., Ferrer, J. C., and Mauk, A. G. (1994) *Biochemistry 33*, 12609−12614.
24. Poulos, T. L., Sheriff, S., and Howard, A. J. (1987) *J. Biol. Chem. 262*, 13881−13884.
25. Pelletier, H., and Kraut, J. (1992) *Science 258*, 1748−1755.
26. Pierce, M. M., Raman, C. S., and Nall, B. T. (1999) *Methods 19*, 213−221.
27. Fersht, A. (1999) *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, pp 129−131, Freeman, New York.
28. Schreiber, G., and Fersht, A. R. (1995) *J. Mol. Biol. 248*, 478−486.
29. Frisch, C., Schreiber, G., Johnson, C. M., and Fersht, A. R. (1997) *J. Mol. Biol. 267*, 696−706.
30. Pons, J., Rajpal, A., and Kirsch, J. F. (1999) *Protein Sci. 8*, 958−968.
31. Morar, A. S., Kakouras, D., Young, G. B., Boyd, J., and Pielak, G. J. (1999) *J. Biol. Inorg. Chem. 4*, 220−222.
32. Pollock, W. B., Rosell, F. I., Twitchett, M. B., Dumont, M. E., and Mauk, A. G. (1998) *Biochemistry 37*, 6124−6131.
33. Wang, X., and Pielak, G. J. (1999) *Biochemistry 38*, 16876−16881.
34. Fishel, L. A., Villafranca, J. E., Mauro, M., and Kraut, J. (1987) *Biochemistry 26*, 351−360.
35. Dasgupta, S., Rousseau, D. L., Anni, H., and Yonetani, T. (1989) *J. Biol. Chem. 264*, 654−662.
36. Taylor, J. R. (1982) *An Introduction to Error Analysis*, University Science Books, Mill Valley, CA.
37. Betz, S. F., and Pielak, G. J. (1992) *Biochemistry 31*, 12337−12344.
38. Gao, Y., Boyd, J., Pielak, G. J., and Williams, R. J. P. (1991) *Biochemistry 30*, 1928−1934.
39. Gao, Y., Boyd, J., Williams, R. J., and Pielak, G. J. (1990) *Biochemistry 29*, 6994−7003.
40. Brayer, G. D., and Murphy, M. E. P. (1996) in *Cytochrome c: A Multidisciplinary Approach* (Scott, R. A., and Mauk, A. G., Eds.) pp 103−166, University Science Books, Sausalito, CA.
41. Cutler, R. L., Pielak, G. J., Mauk, A. G., and Smith, M. (1987) *Protein Eng. 1*, 95−99.
42. Boyd, J., Dobson, C. M., Morar, A. S., Williams, R. J. P., and Pielak, G. J. (1999) *J. Am. Chem. Soc. 121*, 9247−9248.
43. Holzschu, D., Prinipio, L., Conklin, K. T., Hickey, D. R., Short, J., Rao, R., McLendon, G., and Sherman, F. (1987) *J. Biol. Chem. 262*, 7125−7131.
44. Ceesay, K. J., Rider, L. R., Bergman, L. W., and Tuck, M. T. (1994) *Int. J. Biochem. 26*, 721−734.
45. Kresheck, G. C., Vitello, L. B., and Erman, J. E. (1995) *Biochemistry 34*, 8398−8405.
46. Miller, M. A., Geren, L., Han, G. W., Saunders, A., Beasley, J., Pielak, G. J., Durham, B., Millett, F., and Kraut, J. (1996) *Biochemistry 35*, 668−673.
47. Pappa, H. S., Tajbaksh, S., Saunders, A. J., Pielak, G. J., and Poulos, T. L. (1996) *Biochemistry 35*, 4837−4845.
48. Pappa, H. S., and Poulos, T. L. (1995) *Biochemistry 34*, 6573−6580.
49. Creighton, T. E. (1993) *Proteins: Structures and Molecular Properties*, pp 157−162, Freeman, NY.
50. Liu, Y., and Sturtevant, J. M. (1997) *Biophys. Chem. 64*, 121.
51. Yokota, A., Takenaka, H., Oh, T., Noda, Y., and Segawa, S.-I. (1998) *Protein Sci. 7*, 1717−1727.
52. Dunitz, J. D. (1994) *Science 264*, 670−670.
53. Baker, B. M., and Murphy, K. P. (1996) *Biophys. J. 71*, 2049−2055.
54. Bradshaw, J. M., and Waksman, G. (1998) *Biochemistry 37*, 15400−15407.
55. Lockless, S. W., and Ranganathan, R. (1999) *Science 286*, 295−299.
56. Mark, A. E., and van Gunsteren, W. F. (1994) *J. Mol. Biol. 240*, 167−176.
57. Brady, G. P., and Sharp, K. A. (1995) *J. Mol. Biol. 254*, 77−85.
58. Boresch, S., and Karplus, M. (1995) *J. Mol. Biol. 254*, 801−807.

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