Calorimetric Studies on the Interaction of Horse Ferricytochrome *c* and Yeast Cytochrome *c* Peroxidase†

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ABSTRACT: The binding of horse ferricytochrome *c* to yeast cytochrome *c* peroxidase at pH 6.0 in 8.7 mM phosphate buffer (0.0100 M ionic strength) is characterized by a small, unfavorable enthalpy change (+1.91 ± 0.16 kcal mol^{-1}) and a large, positive entropy change (+37 ± 1 eu). The free energy of binding depends strongly upon ionic strength, increasing from −9.01 to −4.51 kcal mol^{-1} between 0.0100 and 0.200 M ionic strength. The increase in free energy is due solely to the change in entropy over this ionic strength range, with the entropy change decreasing from 37 ± 1 to 22 ± 3 eu between 0.0100 and 0.200 M ionic strength. The observed enthalpy change remains constant over the same ionic strength range. At 0.0100 M ionic strength, complex formation is accompanied by the release of 0.54 ± 0.11 proton, causing a variation in the observed enthalpy of reaction depending upon the buffer. After accounting for proton binding to the buffer, the corrected values for the enthalpy and entropy of binding are +2.84 ± 0.26 kcal mol^{-1} and +21 ± 3 eu, respectively. At 0.05 M ionic strength, the observed change in heat capacity, ΔC_p, for the reaction between horse ferricytochrome *c* and cytochrome *c* peroxidase is essentially zero, 1.6 ± 9.6 cal mol^{-1} K^{-1}. The corrected ΔC_p for binding is −28 ± 10 cal mol^{-1} K^{-1} after accounting for proton binding to the buffer. No evidence for formation of a 2:1 horse ferricytochrome *c*/cytochrome *c* peroxidase complex was obtained in this study.

The cytochrome *c*/cytochrome *c* peroxidase system has been extensively investigated as a model for electron transfer between heme proteins. Both cytochrome *c* and cytochrome *c* peroxidase (CcP)[1] are single-subunit, single-heme proteins whose crystallographic structures are known (Finzel et al., 1984; Bushnell et al., 1990; Louie & Brayer, 1990). The electron transfer process involves complex formation between the two proteins, followed by intracomplex electron transfer. Crystallographic structures of complexes between yeast CcP and both horse and yeast cytochrome *c* have been determined by Pelletier and Kraut (1992). This gives unprecedented structural information about a putative electron transfer complex between physiologically-related proteins, and the cytochrome *c*/CcP system has attracted considerable attention in the area of biological electron transfer.

In spite of the seemingly simple properties of cytochrome *c* and CcP, the study of this system has turned out to be complex. Two major unresolved questions concerning electron transfer in the C^{3+}/CcP system still exist. The first concerns the very unusual kinetic properties and the wide differences in apparent electron transfer rates obtained using different experimental techniques (Kang et al., 1977; Hazzard et al., 1988; Summers & Erman, 1988; Geren et al., 1991; Erman et al., 1991; Hahm et al., 1992, 1993; Nuevo et al., 1993). The second concerns the nature of complex formation between the two proteins and whether CcP can bind two molecules of cytochrome *c* simultaneously.

Beetlestone (1960) provided the initial evidence that complex formation between cytochrome *c* and CcP occurred by demonstrating that ferricytochrome *c* was a competitive inhibitor of ferrocytochrome *c* in the CcP-catalyzed reaction. Mochan and Nicholls used sedimentation velocity and gel-filtration chromatography to detect complex formation between cytochrome *c* and CcP (Mochan & Nicholls, 1971; Nicholls, 1974). Over the years, a variety of techniques have been used to investigate the interaction of cytochrome *c* and CcP, including sedimentation velocity (Mochan & Nicholls, 1971), sedimentation equilibrium (Dowe et al., 1984), gel-filtration (Mochan & Nicholls, 1971; Kang et al., 1977; Kornblatt & English, 1986), perturbation of the electronic absorption spectrum (Erman & Vitello, 1980), perturbation of the NMR chemical shift positions of the cytochrome *c* heme methyl resonances (Gupta & Yonetani, 1973; Satterlee et al., 1987a,b; Yi et al., 1993a,b), fluorescence quenching (Leonard & Yonetani, 1974; Kornblatt & English, 1986; Vitello & Erman, 1987; Corin et al., 1991; McLendon et al., 1993), and pH-stat measurements (Mauk et al., 1994).

Electrostatic interactions play a significant role in the binding of CcP and cytochrome *c*. The binding affinity increases substantially with decreasing ionic strength (Vitello & Erman, 1987), consistent with the association of oppositely charged proteins. The isoelectric points of CcP and cytochrome *c* are 5.25 (Yonetani, 1967) and 10.0 (Marini et al., 1980), respectively. Titrations at low ionic strength (~0.01 M) using spectroscopic techniques such as fluorescence quenching (Vitello & Erman, 1987; McLendon et al., 1993), perturbation of absorbance (Erman & Vitello, 1980), and NMR chemical shifts (Gupta & Yonetani, 1973; Satterlee et al., 1987a,b; Yi et al., 1993b) have demonstrated 1:1 complex formation. Binding studies between horse cytochrome *c* and CcP have been carried out between 0.0035

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[1] Abbreviations: CcP, cytochrome *c* peroxidase; C^{3+}, ferricytochrome *c*; MES, 2-(*N*-morpholino)ethanesulfonic acid; *I*, ionic strength in molarity; eu, entropy unit, equal to cal mol^{-1} K^{-1}.

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and 0.20 M ionic strength at pH 6, with reported K_D values varying from less than 2 nM to 0.45 mM.

The first indication that a 2:1 cytochrome c/CcP complex may be involved in the mechanism of CcP catalysis came from the work of Margoliash and co-workers (Kang et al., 1977). Kang et al. observed a biphasic cytochrome c concentration dependence of the steady-state velocity and subsequently used small-zone gel-filtration experiments to show that more than one cytochrome c could bind to CcP. A second study involving gel-filtration and fluorescence quenching to investigate the interaction of both cytochrome c and porphyrin−cytochrome c with various CcP derivatives also reported binding of multiple cytochrome c molecules by CcP (Kornblatt & English, 1986). However, the existence of 2:1 cytochrome c/CcP complexes was not universally accepted for two reasons. It appeared that gel-filtration chromatography overestimated the amount of cytochrome c bound to CcP and gave K_D values which were significantly smaller than values obtained by any of the spectroscopic techniques. In addition, it was noted that porphyrin−cytochrome c had a strong tendency to bind to surfaces, suggesting significant nonspecific binding.

In general, the issue of 2:1 binding has been neglected in the interpretation of most experiments involving CcP and cytochrome c. Recent studies by Hoffman and co-workers have revived the question of the participation of 2:1 complexes in the mechanism of CcP catalysis and electron transfer between cytochrome c and CcP (Stempf & Hoffman, 1993; Zhou & Hoffman, 1993, 1994). These investigators use either CcP or cytochrome c in which the heme iron has been replaced by zinc (and in some cases by magnesium). The zinc−CcP is photoexcited by a short flash of light, and the kinetics of the slowly decaying zinc porphyrin triplet state are monitored. The decay rate increases in the presence of ferricytochrome c, but not in the case of ferrocytochrome c, indicating a quenching pathway which involves electron transfer from the photoexcited triplet state to bound ferricytochrome c. Hoffman and co-workers propose a two-binding-site model to explain the quenching of zinc−CcP by ferricytochrome c. At pH 7.0, in a 2.5 mM potassium phosphate buffer (ionic strength ~0.005 M), they obtain values of 0.1 μM and 130 μM for K_{D1} and K_{D2}, respectively. Their mechanism further requires that quenching by ferricytochrome c bound at the low-affinity site of zinc−CcP be 400 times more efficient than by ferricytochrome c bound at the high-affinity binding site. The K_{D1} values obtained in these studies are consistent with values obtained in the spectroscopic studies, and the K_{D2} values are plausible.

Mauk et al. (1994) have investigated proton uptake and release linked to CcP/yeast iso-1 ferricytochrome c binding. This equilibrium study required a two-binding-site model to account for the proton stoichiometry between pH 5.5 and 7.75, at 0.05 M ionic strength. Values of K_{D1} vary with pH, with values ranging between 0.7 to 5 μM and K_{D2} values varying between 0.360 mM to greater than 1 mM over this pH range. Above 0.10 M ionic strength, the data could be analyzed in terms of only 1:1 complex formation, indicating that at the higher ionic strength binding at the secondary site was too weak to be detected. Below 0.05 M ionic strength and at high pH (7.75), the two-binding-site model was inadequate in fitting the data.

We have recently completed a detailed investigation of the ionic strength dependence of the CcP-catalyzed oxidation of yeast iso-1 ferrocytochrome c by hydrogen peroxide, which required a two-binding-site mechanism in order to explain the kinetic data (Matthis et al., submitted for publication). On the other hand, the kinetics of the CcP-catalyzed oxidation of horse ferrocytochrome c are incompatible with a two-binding-site mechanism (Kim et al., 1990). In order to reconcile the discrepancies in the literature concerning both the affinity and stoichiometry of CcP for cytochrome c from different species, we have initiated a thermodynamic investigation of the interaction of cytochrome c and CcP. In this report, we present calorimetric data for the interaction between horse ferricytochrome c and yeast CcP at pH 6.0 as a function of concentration, temperature, and buffer composition.

MATERIALS AND METHODS

CcP was isolated as previously described (Vitello et al., 1990). PZ values for the multiple preparations used in this study ranged between 1.2 and 1.3. Absorbance ratios at 408/380 nm and 620/647 nm were near 1.5 and 0.76, respectively, indicating that the CcP preparations were five-coordinate, high-spin Fe(III) forms. CcP concentrations were determined using an absorptivity of 98 mM^{-1} cm^{-1} at 408 nm (Vitello et al., 1990). Horse ferricytochrome c (C^{3+}) was obtained from Sigma Chemical Co. (type VI) and used without further purification. The concentration in solution was determined at 410 nm using an absorptivity of 106.1 mM^{-1} cm^{-1} (Margoliash & Frohwirt, 1959). Three different buffer systems were used at pH 6.0 (phosphate, cacodylate, and MES). The buffer ions were kept below 10 mM to minimize interactions with the proteins, and the ionic strength was adjusted by addition of KNO_3. Protein solutions were prepared by adding the protein to the desired buffer, adjusting the pH as necessary with dilute HNO_3 or KOH, and then dialyzing against a minimum of three changes of buffer at a buffer/sample ratio of >100. Small pH adjustments were sometimes required, especially at low ionic strength, to bring the solutions to a common pH for the calorimetry experiments. Reagent grade chemicals and deionized laboratory-distilled water were used for the preparation of all solutions.

Isothermal titration calorimetry experiments were carried out using a MicroCal ITC titration calorimeter (MicroCal, Inc., Northampton, MA) using procedures previously described (Wiseman et al., 1989). The reaction cell volume of the instrument is 1.34 mL, and the titrant was added in 5, 10, or 25 μL increments. The reference cell contained 0.1% aqueous sodium azide. The injection syringe was rotated at 400 rpm for the duration of each experiment, and the time between injections was 480 s. The temperature of the cooling-plates surrounding the titration and reference cells was maintained at least 5 °C below the temperature of the titration and reference cells (Wiseman et al., 1989). Cytochrome c was treated as the ligand and normally placed in the syringe with CcP in the cell. Reverse titrations were conducted with CcP in the syringe and C^{3+} in the cell, in which case C^{3+} was still considered to be the ligand for data analysis. Following the procedure recommended by MicroCal, each titration was integrated manually using the data before and after each peak to establish a base line. The heat change that accompanied the addition of buffer to solutions of either CcP or C^{3+} in the cell was negligible. However, corrections were necessary to account for the heat change

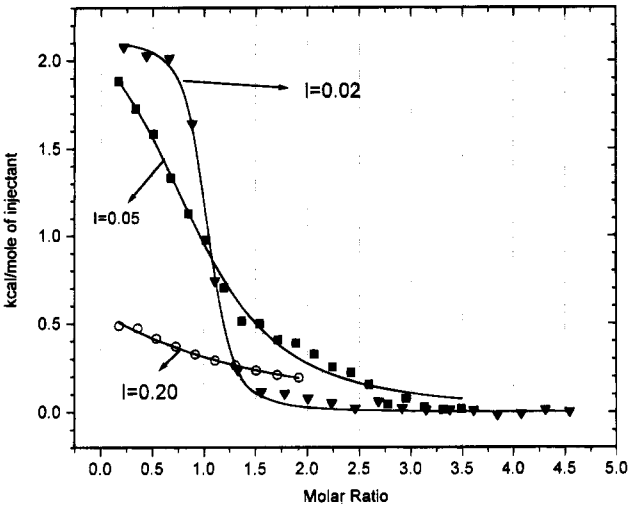

FIGURE 1: Integrated data (corrected for the heat of dilution) for the binding of horse C^{3+} to CcP at pH 6.0, 26 °C, in 0.0100 M phosphate buffer with the ionic strength adjusted with KNO_3. Filled triangles, 0.0200 M *I*, 39.6 μM CcP in cell, 2.32 mM C^{3+} in syringe, $C = 59$. Filled squares, 0.050 M *I*, 43.7 μM CcP in cell, 1.97 mM C^{3+} in syringe, $C = 5$. Open circles, 0.200 M *I*, 561 μM CcP in cell, 5.26 mM C^{3+} in syringe, $C = 1$. The solid lines represent the best-fit analysis for a 1:1 binding site model.

that resulted when CcP or C^{3+} was placed in the syringe and diluted into the cell containing just buffer.

Data analysis to determine binding parameters was conducted using a nonlinear iterative procedure utilizing the Marquardt algorithm (Bevington, 1969). Both one-binding-site and two-binding-site models were tested.

RESULTS

The shape of a calorimetric titration curve is sensitive to both the enthalpy and free energy changes that accompany ligand binding to macromolecules (Kresheck & Hargraves, 1972). Experimental conditions can be adjusted to optimize the determination of either quantity (Wiseman et al., 1989). This is normally accomplished by varying the initial macromolecule concentration in the cell with respect to the ligand concentration in the syringe. Accurate thermodynamic data can be determined experimentally when the product of the total macromolecule concentration in the cell and the equilibrium association constant ranges from about 1 to 1000. This quantity is defined as *C* by Wiseman et al. (1989).

Ionic Strength. The dependence of the thermodynamic parameters on ionic strength for the binding of C^{3+} to CcP was investigated. Representative results at low, medium, and high ionic strength are given in Figure 1. The points are experimental, and the calculated curves represent the best-fit of the data for 1:1 binding. A summary of the results obtained from titrations ranging from an ionic strength of 0.0100 to 1.00 M is given in Table 1. As expected from previous studies (Erman & Vitello, 1980; Vitello & Erman, 1987), the association becomes weaker with increasing ionic strength, reflecting the electrostatic contributions to the binding process. The enthalpy change for the binding is essentially independent of ionic strength over the range investigated, indicating that the ionic strength dependence of the binding affinity is determined by entropic changes.

Temperature. The results obtained for the determination of the enthalpy change and binding constant for the 1:1 interaction of CcP with C^{3+} at pH 6.0 in 0.0100 M phosphate buffer, ionic strength adjusted to 0.050 M with KNO_3, at various temperatures are given in Table 2. The values listed were derived from single titrations at each temperature. Estimated errors for each data set are included. The entropy change for the reaction was calculated from the equation $\Delta G = \Delta H - T\Delta S$, and these values are also included. The striking behavior of these data is their lack of temperature dependence between 12 and 32 °C. The value obtained for the heat capacity change from the enthalpy data was 1.6 ± 9.6 cal mol^{-1} K^{-1} assuming that ΔC_p was not temperature-dependent between 12 and 32 °C.

Table 1: Effect of Ionic Strength on the Equilibrium Association Constant and Thermodynamic Parameters from the Titration of Cytochrome *c* Peroxidase with Horse Cytochrome *c* at 26 ± 1 °C at pH 6.0[a]

ionic strength[b] (M)	$K_A \times 10^{-4}$ (M^{-1})	ΔH (cal mol^{-1})	ΔS (eu)
0.0100 (3)	390 ± 110	1910 ± 160	37 ± 1
0.0200 (1)	150 ± 50	2050 ± 50	36 ± 1
0.0300 (1)	78 ± 20	2230 ± 60	34 ± 1
0.050 (8)	12 ± 5	2320 ± 230	31 ± 1
0.100 (2)	0.9 ± 0.1	2320 ± 260	26 ± 1
0.200 (3)	0.2 ± 0.1	1960 ± 700	22 ± 3
1.00 (1)	ND[c]	ND[c]	ND[c]

[a] Buffers contained 8.7−10.0 mM potassium phosphate with added KNO_3 to adjust the ionic strength. [b] The number of titrations at each ionic strength is given in parentheses. [c] No detectable interaction.

Table 2: Thermodynamic Data for the Temperature Dependence of the Association of Horse Cytochrome *c* with Cytochrome *c* Peroxidase in pH 6.0 Phosphate/KNO_3 Buffer, 0.050 M Ionic Strength

temp (°C)	$K_A \times 10^{-4}$ (M^{-1})	ΔH (cal mol^{-1})	ΔS (eu)
12.1	10.1 ± 1.2	2360 ± 50	31 ± 3
14.5	5.6 ± 0.7	2350 ± 80	30 ± 4
16.8	10.9 ± 1.4	2100 ± 60	30 ± 4
20.1	5.7 ± 0.8	2560 ± 70	30 ± 4
23.2	7.3 ± 0.7	2490 ± 60	30 ± 3
26.6	6.9 ± 2.0	2480 ± 50	30 ± 9
27.4	11.0 ± 2.0	2570 ± 110	32 ± 6
30.1	11.8 ± 1.6	2080 ± 60	30 ± 4
32.2	3.8 ± 0.4	2370 ± 80	29 ± 3

Buffers. Recent studies (Mauk et al., 1994) have shown that proton uptake and release (depending upon the experimental pH) accompany complex formation between CcP and C^{3+}. Therefore, the observed enthalpy of interaction may be expected to depend upon the enthalpy of ionization (proton uptake upon complex formation) or protonation (proton release upon complex formation) of the buffer. Three buffer systems with different enthalpies of ionization were used to examine this effect. The enthalpies of ionization of the buffers were as follows: phosphate, 1.2 kcal mol^{-1}; cacodylate, −0.56 kcal mol^{-1}; and MES, 4.0 kcal mol^{-1} (Jelesarov & Bosshard, 1994; Good et al., 1966). Representative data for individual titrations with each buffer system at pH 6.0 and an ionic strength of 0.0100 M at 26 ± 1 °C are given in Figure 2, and a summary of all the results obtained is given in Table 3. Average values for each buffer system are recorded in parentheses. The observed enthalpy of mixing, ΔH^{obs}, may be corrected for the enthalpy of protonation of the buffer, ΔH^{prot}, in order to obtain the enthalpy of binding, ΔH^{bind}, according to the equation:

$$\Delta H^{obs} = \Delta H^{bind} + n\Delta H^{prot} \quad (1)$$

where *n* represents the number of protons released by the

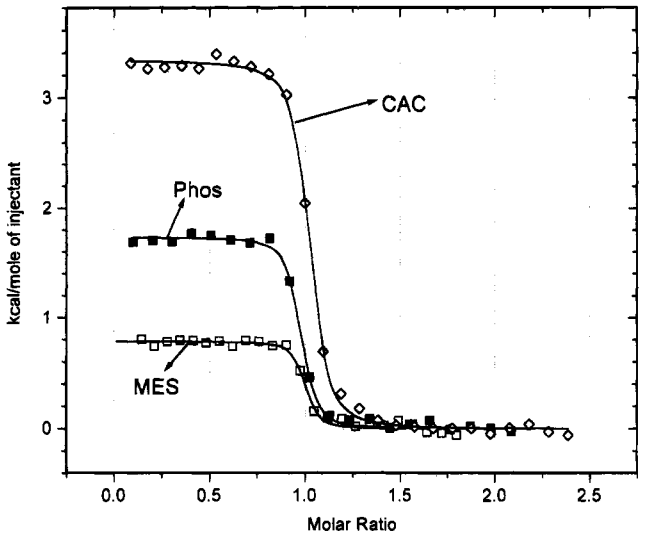

FIGURE 2: Integrated data (corrected for the heat of dilution) for the binding of horse C^{3+} to CcP at 0.0100 M ionic strength, 26 °C, in different buffers. Open squares, MES, 226 μM CcP in the cell, 1.97 mM C^{3+} in the syringe, $C = 340$. Filled squares, phosphate, 49.8 μM CcP in the cell, 1.34 mM C^{3+} in the syringe, $C = 224$. Open diamonds, cacodylate, 194 μM CcP in the cell, 2.80 mM C^{3+} in the syringe, $C = 378$. The solid lines represent the best-fit analysis for a 1:1 binding site model.

Table 3: Thermodynamic Data Obtained from the Titration of Cytochrome *c* Peroxidase with Horse Cytochrome *c* at pH 6.0 in 0.0100 M Ionic Strength Phosphate, Cacodylate, and MES Buffers at 26 ± 1 °C

buffer	$[C^{3+}]^a$ (mM)	$[CcP]^a$ (μM)	$K_A \times 10^{-6}$ (M^{-1})	ΔH (cal mol^{-1})
MES	1.90	226	1.5 ± 0.7	880 ± 20
	1.90	226	1.5 ± 1.2	700 ± 30
				(790 ± 120)[b]
cacodylate	4.35	172	3.4 ± 1.1	3010 ± 50
	4.35	172	1.5 ± 0.5	2710 ± 50
	2.80	194	2.2 ± 0.5	3420 ± 60
	2.80	194	1.7 ± 0.8	3440 ± 120
	0.559	47.9	8.6 ± 1.5	3370 ± 30
	0.280	23.9	9.9 ± 1.2	3530 ± 30
	0.140	12.0	4.8 ± 2.8	3730 ± 190
				(3320 ± 340)[b]
phosphate	1.34	49.8	4.5 ± 2.5	1790 ± 60
	1.34	49.8	4.5 ± 1.7	1840 ± 30
	0.67	24.9	2.6 ± 1.1	2090 ± 80
				(1910 ± 160)[b]

[a] Initial cytochrome *c* concentration in the syringe and initial CcP concentration in the cell. [b] Average value for each buffer.

protein and taken up by the buffer as a result of complex formation. At pH 6.0, 0.0100 M ionic strength, the value of n is 0.54 ± 0.11, and ΔH^{bind} equals 2840 ± 260 cal mol^{-1}.

Similarly, the observed value of ΔC_p, ΔC_p^{obs}, may also be corrected for the effect of protonation of the buffer, ΔC_p^{prot}, according to eq 2. ΔC_p^{bind} is the contribution to the

$$\Delta C_p^{obs} = \Delta C_p^{bind} + n\Delta C_p^{prot} \quad (2)$$

change in heat capacity due to the interaction of CcP with C^{3+}. For phosphate buffer between 15 and 35 °C, the value of ΔC_p^{prot} extrapolated to zero ionic strength is 54 ± 1 cal mol^{-1} K^{-1} (Bates & Acree, 1943). Using this value and the determined value of n, ΔC_p^{bind} is found to be −28 ± 10 cal mol^{-1} K^{-1}.

Table 4: Enthalpy Values Obtained from Reverse Titrations of Cytochrome *c* (in the Cell) and Cytochrome *c* Peroxidase (in the Syringe) at 26 ± 1 °C, pH 6.0 Phosphate Buffer, 0.0100 M Ionic Strength

$[C^{3+}]$ (mM)	injections[a] (μL)	[CcP] (mM)	ΔH (cal mol^{-1})
5.26	25 (5)	0.561	1960 ± 50
2.01	10 (25)	0.197	2100 ± 50
1.80	10 (25)	0.197	2010 ± 100
1.01	10 (25)	0.197	2370 ± 140
0.899	10 (10)	0.483	1930 ± 100
0.503	10 (25)	0.197	2130 ± 90
0.449	10 (10)	0.483	2330 ± 80
0.252	10 (25)	0.197	2150 ± 70
			av 2100 ± 160

[a] The number of injections for each titration is given in parentheses.

Reverse Titrations. Determination of the stoichiometry of the reaction between CcP and horse C^{3+} is one of the goals of our current studies. Previous investigations indicate that CcP has a high-affinity binding site for C^{3+}, giving a 1:1 C^{3+}/CcP complex. There is conflicting evidence on the existence of a low-affinity binding site which would give rise to a 2:1 C^{3+}/CcP complex. In order to achieve the high concentrations of C^{3+} needed to saturate a potential low-affinity site, reverse titrations were carried out in which high concentrations of C^{3+} were placed in the cell and titrated with CcP in the syringe. For these reverse titrations, the initial enthalpy change will reflect the sum of the enthalpy change for binding at the high-affinity site and the enthalpy for any binding at a secondary site. The initial enthalpy changes for the reverse titrations can be compared to the initial changes in the normal titrations which reflect the enthalpy change at the high-affinity binding site.

The results of nine reverse titrations of C^{3+} with CcP in pH 6.0 phosphate buffer, 0.0100 M ionic strength, are given in Table 4. The enthalpy values represent the intercept of a least-squares fit of the titration data corrected for the dilution of CcP into buffer. The standard deviation of the data for each individual titration and also the average value of ΔH for the nine titrations are included in Table 4. The enthalpies do not exhibit a systematic dependence on cytochrome *c* concentration, suggesting that a secondary site is not being titrated. The average value of ΔH for the nine reverse titrations is 2100 ± 160 cal mol^{-1}, which may be compared to the value of 1910 ± 160 cal mol^{-1} reported in Table 1 for normal titrations using C^{3+} in the syringe. The difference in these enthalpy changes is 190 ± 230 cal mol^{-1}, meaning, that if binding at a secondary site occurs, the enthalpy of interaction at the secondary site is essentially zero.

DISCUSSION

Formation of the 1:1 Horse C^{3+}/CcP Complex. The data shown in Figures 1 and 2 demonstrate that the enthalpy change upon binding of horse ferricytochrome *c* to yeast CcP is consistent with 1:1 stoichiometry under the conditions of the calorimetric experiments. The equilibrium association constants derived from these calorimetric studies are consistent with previous investigations using spectroscopic techniques to monitor complex formation (Figure 3). The calorimetric studies have the advantage of determining the enthalpy of the reaction and, in combination with the equilibrium constant, the entropy of the reaction.

The enthalpy of association between these two proteins is dependent upon the buffer components (Figure 2), indicating

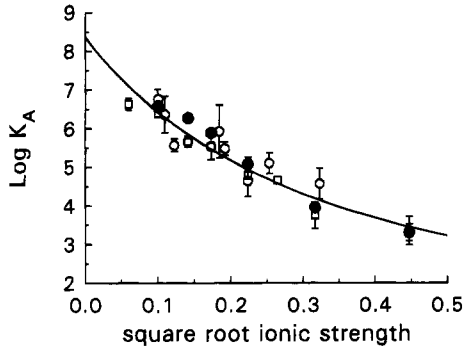

FIGURE 3: log K_A, the equilibrium association constant, plotted as a function of the square root of ionic strength. Filled circles, values determined by calorimetry, 26 ± 1 °C, Table 1. Open circles, values determined by perturbation of the optical absorption spectrum upon complex formation at 25 ± 1 °C; data from Erman and Vitello, (1980). Open squares, values determined from fluorescence quenching experiments using modified CcP in which the heme was replaced by protoporphyrin IX at 25 ± 1 °C; data from Vitello and Erman (1987). All values were obtained at pH 6.0 ± 0.1. The calorimetric data were obtained in 8.7−10 mM potassium phosphate buffers using KNO_3 to adjust the ionic strength while the spectroscopically determined values were determined in 10 mM cacodylate buffers with added KNO_3. The solid line is calculated using eqs 8 and 9 of the text. The extrapolated values of log K_A at zero and infinite ionic strength are 8.40 ± 0.17 and −0.3 ± 0.5, respectively.

that protons are released into solution upon binding of ferricytochrome *c* to the high-affinity binding site on CcP as previously observed for the interaction between yeast iso-1 ferricytochrome *c* and CcP (Mauk et al., 1994). Deconvolution of the observed enthalpy change into an enthalpy of binding and a contribution due to the protonation of the buffer, eq 1, gives a corrected ΔH^{bind} of 2840 ± 260 cal mol^{-1}. The buffer-dependent enthalpy change indicates that 0.54 ± 0.11 proton is released during complex formation between horse C^{3+} and CcP at pH 6.0. The binding is endothermic, indicating an unfavorable enthalpic contribution to the free energy of association at the high-affinity site. The binding is dominated by a favorable entropy of binding. At the lowest ionic strength investigated in this study, 0.0100 M, the observed entropy change is +37 ± 1 eu. Correcting for the entropy of protonation of the buffer (Bates & Acree, 1943) gives an entropy of binding of +21 ± 3 eu.

Many electron transfer reactions occur via electrostatically-stabilized protein−protein complexes. The few thermodynamic studies on the association of electron transfer proteins suggest that these systems have similar thermodynamic parameters (Table 5). The first five entries are for complex formation between oppositely-charged proteins, and the equilibrium association constant decreases considerably with increasing ionic strength. Proton release appears to be a common feature of complex formation. The enthalpies of binding, after correction for enthalpies of ionization of the buffer components, appear to be small and usually unfavorable (Table 5). In all cases, complex formation is characterized by large positive observed entropy changes ranging from +26 to +44 eu. The last entry in Table 5 is interesting in that both methanol dehydrogenase (MDH) and cytochrome c_{551i} have a net negative charge at pH 7.5. Strong binding is observed at high ionic strength where dipole−dipole forces between the two proteins dominate the interaction (Harris et al., 1994). The coupling of proton uptake or release to complex formation has not been studied in this system, and only the observed enthalpy change is available. However, the observed enthalpy change is small and unfavorable, +2.4 kcal/mol, and the entropy change is large and positive, +24 eu, just as for those complexes formed between proteins of opposite charge.

The small, unfavorable enthalpy change and large, positive entropy of binding upon complex formation are consistent with the idea of release of water of hydration from the binding interface. If significant apolar surface area is involved at the interaction interface, the association is characterized by large negative changes in the heat capacity of the system. Observed heat capacity changes for three of the protein systems in Table 5 have been determined. Both the ferredoxin/ferredoxin:NADP reductase and the methanol dehydrogenase/cytochrome c_{551i} have large negative values for ΔC_p. However, ΔC_p is essentially zero for the horse C^{3+}/CcP system. The observed heat capacity change for the CcP/C^{3+} system is due, in part, to the release of protons upon complex formation and binding of the released protons by the buffer. Corrections for proton release and buffer effects give a value for ΔC_p^{bind} of −28 ± 10 cal mol^{-1} K^{-1}, still significantly smaller than the values for the ferredoxin/ferredoxin:NADP reductase and the methanol dehydrogenase/cytochrome c_{551i} systems.

Heat Capacity Change for Formation of the 1:1 Horse C^{3+}/CcP Complex. Perhaps the most striking finding of this investigation is not that the free energy change for 1:1 complex formation is entropy-controlled, but that ΔS is not accompanied by the expected heat capacity change. Four different methods can be used to predict thermodynamic parameters for interacting protein systems.

Based on model compound behavior for typical hydrophobic processes (Sturtevant, 1977), ΔC_p is expected to be about 4 times greater than the unitary entropy change, ΔS_u, and of opposite sign. The unitary entropy change is about

Table 5: Thermodynamic Parameters for Protein−Protein Complexes Involved in Electron Transfer

proteins	pH	buffer	*I* (M)	temp (°C)	log K_A (M^{-1})	ΔH^{obs} (kcal/mol)	ΔH^{bind} (kcal/mol)	ΔS^{obs} (eu)	n^a	$\Delta C_p^{obs\ b}$ [cal/(mol·K)]
CcP/C^{3+} [c]	6.0	KPhos/KNO_3	0.050	26	5.08	+2.3	+2.8	+31	0.54	+1.6
CcP/yC^{3+} [d]	6.0	KNO_3	0.100	25	5.39	+0.2	+0.2	+26	0.46	
b_5/C^{3+} [e]	7.0	KPhos	0.001	25	6.60	+0.2	+0.6	+33	0.3	
Fd/C_3 [f]	7.7	Tris/HCl	0.010	10	6.11	+3.4	+4.5	+44	0.1	
Fd/FNR [g]	7.5	Tris/HCl	0.050	27	6.81	+11.0	−0.3	+30	1.0	−163
MDH/C_{551i} [h]	7.5	Tris/HCl/NaCl	0.280	20	3.43	+2.4		+24		−110

[a] Number of protons released upon complex formation. [b] Observed values of ΔC_p not corrected for proton release and buffer effects. [c] This work. [d] CcP and yeast iso-1 cytochrome *c* (Mauk et al., 1994; pH-stat measurements). [e] Tryptic fragment of bovine cytochrome b_5 and horse C^{3+} (Mauk et al., 1982, 1991; spectroscopic and pH-stat measurements). [f] Ferredoxin I and cytochrome c_3 from *Desulfovibrio desulfuricans* (Guerlesquin et al., 1987; calorimetric studies). [g] Ferredoxin I and ferredoxin:$NADP^+$ reductase from spinach (Jelesarov & Bosshard, 1994; calorimetric studies). [h] Methanol dehydrogenase and cytochrome c_{551i} from *Paracoccus denitrificans* (Harris & Davidson, 1993; kinetically-determined K_A).

8 eu larger than ΔS^{bind} (the observed entropy change corrected for buffer protonation). The unitary entropy change for the binding of horse cytochrome *c* to CcP at 0.050 M ionic strength is 23 eu. If the CcP/C^{3+} reaction conformed to the hydrophobic model, we would expect ΔC_p to be about -92 cal mol^{-1} K^{-1}. The discrepancy between the value of -92 cal mol^{-1} K^{-1} expected for hydrophobic interactions and the experimental value of -28 cal mol^{-1} K^{-1} for ΔC_p^{bind} can be attributed to polar interactions in the binding reaction.

Murphy and Freire (1992) have used changes in the values of the apolar and polar surface areas to estimate ΔC_p and ΔH for protein reactions. The relationships derived from model compounds are

$$\Delta C_p = 0.45\Delta_{apolar} - 0.26\Delta_{polar} \quad (3)$$

and

$$\Delta H = 35\Delta_{polar} - \Delta T\delta C_p \quad (4)$$

where Δ_{apolar} and Δ_{polar} represent the change in area for the apolar and polar surface, respectively. ΔT is equal to 100 minus the temperature of interest in degrees centigrade.

The X-ray structure of the CcP/C^{3+} complex (Pelletier & Kraut, 1992) can be used to estimate the change in surface area of CcP and C^{3+} due to complex formation (Lee & Richards, 1971; Lee, 1991). The results of these calculations (K. P. Murphy, private communication) indicate that 593 Å^2 of apolar surface and 470 Å^2 of polar surface are buried. Using these values and eqs 3 and 4, ΔC_p and ΔH are estimated to be -145 cal mol^{-1} K^{-1} and -5600 cal mol^{-1}, respectively.

A third approach is to estimate ΔC_p from just the change in nonpolar surface area (Spolar et al., 1992):

$$\Delta C_p = 0.25\Delta_{apolar} \quad (5)$$

This method gives a calculated value of -148 cal mol^{-1} K^{-1} for ΔC_p.

Finally, it is possible to estimate the ΔH of association from the number of water molecules excluded as a result of complex formation (Weber, 1993):

$$\Delta H = (\Delta H_{ww} + \Delta H_{pp} - 2\Delta H_{pw})n \quad (6)$$

The subscripts ww, pp, and pw represent interactions at the solvent−solvent, protein−protein, and protein−solvent interfaces, respectively, and n corresponds to the number of water molecules excluded from the interface (two hydrogen bonds per molecule). Values given by Weber for ΔH_{ww}, ΔH_{pp}, and ΔH_{pw} are -7000, -760, and -3840 cal mol^{-1}, respectively. The number of displaced water molecules was obtained by dividing the total accessible area buried upon complex formation by the surface area of one water molecule (10 Å^2; Weber, 1993). The resulting value of ΔH is -8900 cal mol^{-1} using an average value for the buried surface area, 1107 Å^2 (1150 Å^2, Jelesarov & Bosshard, 1994; or 1063 Å^2, K. P. Murphy, private communication).

The estimates of each of the four methods are in good agreement with each other, giving ΔC_p values between -92 and -148 cal mol^{-1} K^{-1} and ΔH values between -5600 and -8900 cal mol^{-1}. However, the predicted values do not agree with the experimental values (corrected for proton release) of -28 ± 10 cal mol^{-1} K^{-1} and $+2840 \pm 260$ cal mol^{-1} for ΔC_p^{bind} and ΔH^{bind}, respectively. We conclude that the model studies do not account for all of the interactions (long and short range) that are responsible for the tight binding between CcP and C^{3+}. These interactions are both hydrophobic and electrostatic in nature, and are accompanied by the release of solvent molecules that gives rise to a positive ΔS. However, the expected negative ΔC_p due to hydrophobic bond formation (Sturtevant, 1977) could be balanced by a significant positive contribution to ΔC_p by dehydration of the protein interface and release of electrostricted water (Ha et al., 1989). If this is true, it suggests that additional model compound data may be needed to describe ion pair formation (and rupture) involving protein side chains.

Ionic Strength Dependence of K_A for the 1:1 Horse C^{3+}/CcP Complex. The value of K_A decreases substantially with increasing ionic strength as expected for the interaction between oppositely-charged proteins (Figure 3). The ionic strength dependence of K_A can be associated with changes in the activity of the reacting species with increasing ionic strength (eq 7) (Record et al., 1978). In eq 7, K_A^0 is the

$$K_A^0 = K_A\frac{\gamma_{pc}}{\gamma_p\gamma_c} \quad (7)$$

equilibrium association constant at zero ionic strength, K_A is the observed equilibrium association constant at any ionic strength, and γ_i is the activity coefficient of species i, with subscripts p, c, and pc representing CcP, C^{3+}, and the complex, respectively. Taking the logarithm of both sides of the equation and rearranging, we obtain eq 8:

$$\log K_A = \log K_A^0 + \log \gamma_p + \log \gamma_c - \log \gamma_{pc} \quad (8)$$

The activity coefficient of a spherical protein in terms of its net charge and dipole moment has been given by Koppenol (1980) (eq 9). The activity coefficient depends

$$\ln \gamma_i = -\frac{Z_i^2e^2}{2DkT}\frac{\kappa}{1+\kappa a_i} - \frac{\kappa^2}{2DkT}\left[\frac{3\mu_i^2}{4a_i(1+\kappa a_i+\kappa^2a_i^2/3+\kappa^2b_i^2/6a_i)}\right] \quad (9)$$

upon three molecular parameters: Z_i, the net charge on species i; μ_i, the dipole moment of species i; and b_i, the radius of species i; a_i is the distance of closest approach of a buffer ion to species i and is taken as $b_i + 1.5$ Å. The parameter κ depends upon the ionic strength and has units of reciprocal length. Equation 10 gives the value for κ in aqueous solution at 298 K in units of Å^{-1}:

$$\kappa = \sqrt{\frac{8\pi N_A e^2 I}{1000DkT}} = 0.329\sqrt{I} \quad (10)$$

We have estimated the radius of CcP and cytochrome *c* to be 21.5 and 15.5 Å, respectively, from the partial specific volumes and diffusion coefficients (Margoliash & Schejter, 1966; Ellfolk, 1967; Dowe & Erman, 1985). The net charges on CcP and horse ferricytochrome *c* at pH 6.0 were estimated as -4.0 and $+6.2$, respectively, from pH titration curves (Conroy & Erman, 1978; Marini et al., 1980). The dipole moment of CcP has been calculated by Northrup et al. (1986)

and varies between 416 and 587 D depending upon the model used. We have used the magnitude of the dipole moment calculated from the all-atom partial charge model of Northup et al. (1986) with the heme propionates charged. This gives a value of 520 D as the magnitude of the CcP dipole. Koppenol and Margoliash (1982) have calculated the dipole moment for horse ferricytochrome *c* to be 325 D.

In the nonlinear least-squares regression of the data to eq 8, two adjustable parameters are used: log K_A^0 and the dipole moment of the complex, μ_{pc}. Using the parameters described in the preceding paragraph, the best-fit values are 8.40 ± 0.17 and 430 ± 310 D for log K_A^0 and μ_{pc}, respectively. These are the parameters used to calculate the theoretical line shown in Figure 3.

The value of log K_A^0 and the fit of the data over the ionic strength range 0.0100−0.200 M are relatively insensitive to the choice of dipole moments for CcP and cytochrome *c*. Keeping the magnitude of μ_p at 520 D and varying μ_c between 200 and 400 D caused less than a 7% change in the sum of squared residuals (the difference between calculated and observed values of log K_A), and the value of log K_A^0 varied between 8.32 and 8.41. Similarly, keeping μ_c at 325 D and varying μ_p between 400 and 600 D had little effect on the fit (<2%) or log K_A^0 (8.37−8.43). However, if both μ_p and μ_c were at their lower limits, 400 and 200 D, respectively, the fit was substantially poorer with an increase of about 34% in the residual sum of squares, and log K_A^0 decreased to 8.14.

The insensitivity of log K_A^0 and the fit over the ionic strength range 0.0100−0.200 M to the dipole moments of the species is to be expected since the first term in eq 9 (the monopole term) dominates at low ionic strength, with the second (dipole) term becoming important at higher ionic strength (>0.1 M; van Leeuwen, 1983). We have only one data point above 0.1 M ionic strength, and no interaction could be detected at 1.00 M ionic strength (Table 1).

The free energy of interaction between CcP and C^{3+} can be divided into electrostatic and nonelectrostatic contributions (eq 11). Assuming that $\Delta G°_{es}$ is dependent upon and that

$$\Delta G° = \Delta G°_{es} + \Delta G°_{nes} \qquad (11)$$

$\Delta G°_{nes}$ is independent of ionic strength allows us to estimate both contributions by extrapolation of log K_A to zero and infinite ionic strength. At infinite ionic strength, $\Delta G°_{es}$ will be equal to zero, and only the nonelectrostatic contributions will stabilize the complex. At zero ionic strength, $\Delta G°_{es}$ will have its maximum value. The extrapolated values for log K_A are 8.40 ± 0.17 and −0.3 ± 0.5 at zero and infinite ionic strength, respectively. $\Delta G°_{nes}$ equals +0.4 ± 0.7 kcal mol^{-1} while the maximum value of $\Delta G°_{es}$ at zero ionic strength is −11.9 ± 0.8 kcal mol^{-1}. Although the extrapolations of log K_A may be questioned, these calculations indicate that electrostatic interactions dominate the binding of CcP and horse C^{3+}.

Evidence for a 2:1 Horse C^{3+}/CcP Complex. Evidence for a low-affinity binding site on CcP for both horse and yeast iso-1 cytochrome *c* has been reported (Stempf & Hoffman, 1993; Zhou & Hoffman, 1993, 1994; Mauk et al., 1994). The strongest evidence for a secondary site comes from the interaction of yeast iso-1 cytochrome *c* with CcP. In addition, the kinetics of CcP-catalyzed oxidation of yeast iso-1 ferrocytochrome *c* by hydrogen peroxide are best described by a two-binding-site model (Matthis et al., submitted for publication). However, the kinetics of the CcP-catalyzed oxidation of horse ferrocytochrome *c* by hydrogen peroxide are incompatible with the two-binding-site model used for the yeast iso-1 cytochrome *c* (Kim et al., 1990).

We have been particularly interested in determining whether or not there is any evidence for a secondary binding site on CcP for horse cytochrome *c* in the calorimetric titrations. Both one-binding-site and two-binding-site models were used in fitting the titration data. In all cases, a one-binding-site model was sufficient to fit the data within experimental error. No significant improvement in fit occurred when a two-binding-site model was used. All the results presented in Tables 1−4 are those obtained from fitting the titration data to a one-site model.

Stemp and Hoffman (1993) suggest that CcP has a similar affinity for cytochrome *c*'s from both fungal and mammalian sources at the secondary binding site. We have utilized the data of Stempf and Hoffman, as well as those of Zhou and Hoffman (1993, 1994) (horse C^{3+}) and Mauk et al. (1994) (yeast iso-1 C^{3+}), to estimate that the equilibrium association constant for the 2:1 C^{3+}/CcP complex should be about 5 × 10^3 M^{-1} at pH 6.0, 0.0100 M ionic strength. The reverse titrations utilized C^{3+} concentrations ranging from 0.252 to 5.26 mM (Table 4). If horse C^{3+} binds to the secondary site on CcP with an association constant of 5 × 10^3 M^{-1}, then between 56% and 96% of the secondary site should be occupied in the initial phase of the reverse titrations. Since the reverse titrations show no systematic dependence upon C^{3+} concentration and give essentially the same enthalpy of interaction as the normal titration in which C^{3+} binds predominantly at the high-affinity site, we conclude either that the enthalpy of interaction is zero (+190 ± 230 cal mol^{-1}) or that the equilibrium association constant for binding of horse C^{3+} to the secondary site is substantially smaller than 200 M^{-1} in phosphate buffer, pH 6.0, 0.0100 M ionic strength.

We can put additional constraints upon formation of a 2:1 horse C^{3+}/CcP complex. Mauk et al. (1994) have shown that when yeast iso-1 C^{3+} binds to CcP at the high-affinity binding site, 0.65 proton is released and greater than 0.2 proton is bound when yeast iso-1 C^{3+} binds at the low-affinity binding site (pH 5.9, 0.050 M ionic strength). The value of 0.65 proton released for binding of yeast C^{3+} at the high-affinity binding site is similar to the value we obtain from the calorimetric data for binding of horse C^{3+} at the high-affinity binding site, a release of 0.54 ± 0.11 proton at pH 6.0, 0.0100 M ionic strength. If binding of horse C^{3+} to the low-affinity binding site involves proton uptake as Mauk et al. (1994) have demonstrated for yeast iso-1 C^{3+}, then there should have been an indication of secondary binding in the experiments shown in Figure 2, in which different buffers (with different enthalpies of ionization) were used. If the observed enthalpy of interaction at the secondary site is zero (+190 ± 230 cal mol^{-1}) in phosphate buffer, the observed enthalpy change would have to be significantly different in either cacodylate or MES buffer if proton uptake is associated with binding at the secondary site. Again assuming that the equilibrium association constant for the secondary site is about 5 × 10^3 M^{-1}, about 53% and 63% of the secondary sites should have been filled during the titrations in MES and cacodylate buffers, respectively (Figure 2). If >0.2 proton is taken up during binding at the

secondary site, there should be a difference of >900 cal mol^{-1} in the observed enthalpy change for binding to the secondary site in cacodylate and MES buffer. No such difference is seen.

Our data provide no evidence for 2:1 complex formation between horse C^{3+} and CcP. Although we cannot disprove the existence of a 2:1 complex, we can place limits on the thermodynamic quantities associated with binding of horse C^{3+} to a secondary site on CcP. If horse C^{3+} binds to a secondary site on CcP with an affinity similar to that of yeast iso-1 C^{3+} as suggested by Stempf and Hoffman (1993), then both ΔH_{bind} and proton uptake have to be essentially zero at pH 6.0, 0.0100 M ionic strength. The alternative is that the association equilibrium constant is substantially smaller than 200 M^{-1}, more than 25 times weaker than current estimates (Stempf & Hoffman, 1993; Zhou & Hoffman, 1993, 1994).

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