Calorimetric Studies of the Thermal Denaturation of Cytochrome *c* Peroxidase†

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ABSTRACT: Two endotherms are observed by differential scanning calorimetry during the thermal denaturation of cytochrome *c* peroxidase at pH 7.0. The transition midpoint temperatures (t_m) were 43.9 ± 1.4 and 63.3 ± 1.6 °C, independent of concentration. The two endotherms were observed at all pH values between 4 and 8, with the transition temperatures varying with pH. Precipitation was observed between pH 4 and 6, and only qualitative data are presented for this region. The thermal unfolding of cytochrome *c* peroxidase was sensitive to the presence and ligation state of the heme. Only a single endotherm was observed for the unfolding of the apoprotein, and this transition was similar to the high-temperature transition in the holoenzyme. Addition of KCN to the holoenzyme increases the midpoint of the high-temperature transition whereas the low-temperature transition was increased upon addition of KF. Binding of the natural substrate ferricytochrome *c* to the enzyme increases the low-temperature transition by 4.8 ± 1.3 °C but has no effect on the high-temperature transition at pH 7. The presence of cytochrome *c* peroxidase decreases the stability of cytochrome *c*, and both proteins appear to unfold simultaneously. The results are discussed in terms of the two domains evident in the X-ray crystallographic structure of cytochrome *c* peroxidase.

Yeast cytochrome *c* peroxidase (ferrocytochrome *c*:H_2O_2 oxidoreductase, EC 1.11.1.5; CcP)[1] is a moderately sized enzyme (M_r 34 168) consisting of a single polypeptide chain (294 residues) and a single noncovalently bound heme group (Takio et al., 1980; Kaput et al., 1982). The crystallographic structure of the protein has been determined (Poulos et al., 1980) and refined to 0.17 nm (Finzel et al., 1984). The enzyme catalyzes the peroxidatic oxidation of ferrocytochrome *c* (Altschul et al., 1940) and has been receiving increased attention as a well-characterized, structural model for long-range electron transfer between heme proteins (Poulos & Kraut, 1980). Cytochrome *c* and CcP have opposite net charges at neutral pH and associate with high affinity at low ionic strength (Erman & Vitello, 1980; Vitello & Erman, 1987). The enzyme has been extensively studied by spectroscopic and kinetic techniques, generally at room temperature or below. The protein appears to be quite flexible with a number of accessible conformational states (Loo & Erman, 1975; Satterlee & Erman, 1980; Dhaliwal & Erman, 1985). A spectroscopic study of the thermal denaturation of CcP showed complex behavior (Gross & Erman, 1985). To better characterize the thermal stability of the enzyme, a DSC study was undertaken with the effects of pH, ligand binding, and association with cytochrome *c* determined.

Experimental Procedures

Cytochrome *c* peroxidase was isolated from commercial bakers' yeast according to the procedure of Yonetani and co-workers (Yonetani & Ray, 1965; Yonetani et al., 1966). The purity index (ratio of absorbance at 408 to 282 nm) of all preparations used was 1.18 or greater. Concentrations of holo- and apoenzyme were determined spectrophotometrically in the appropriate buffer by using an extinction coefficient of 95 mM^{-1} cm^{-1} at 408 nm (Coulson et al., 1971) and 55 mM^{-1} cm^{-1} at 280 nm (Yonetani, 1967), respectively. Horse heart cytochrome *c* was obtained from Sigma Chemical Co. (type VI) and used without further purification. The concentration of ferricytochrome *c* was determined spectrophotometrically at 410 nm by using an extinction coefficient of 106.1 mM^{-1} cm^{-1} (Margoliash & Frohwirt, 1959). Apocytochrome *c* peroxidase (apoCcP) was prepared from CcP by the acid–butanone method according to Yonetani (1967). Acetate (Ac), phosphate (Phos), and cacodylate (Cac) buffers of the desired molarity were prepared by using KNO_3 to adjust the ionic strength of the final solutions. Reagent-grade chemicals and deionized laboratory-distilled water were used in all cases.

The calorimetric studies were carried out with an MC-1 differential scanning calorimeter (Micro Cal, Northampton, MA) which was interfaced to an IBM 9000 microcomputer system as previously described (Kresheck & Vanderkooi, 1985). The volume of the sample and solvent reference was 0.61 mL, and data were collected between 20 and 90 °C at a heating rate of 1.10 °C/min unless specified otherwise. The transition temperature, t_m, was an average of the temperature of maximum excess specific heat and the temperature corresponding to 50% of the total excess specific heat (normally ±0.1 °C) whenever nonoverlapping transitions occurred. Otherwise, t_m corresponded to the temperature of the maximum heat capacity for the transition. The total transition enthalpy change, Δh_d, in units of calories per gram, was found from the area under the peak corresponding to the excess heat capacity by using an electrical pulse to convert the observed area to units of calories. Numerical integration of peak areas and resolution of overlapping peaks were performed by using a trial and error curve-fitting procedure on the digitized data with the microcomputer using either linear base lines, which connected the points just below and above the observed transition, or sigmoidal base lines (Fukada et al., 1983) when a change in heat capacity of the proteins accompanied the thermal transitions. The latter was observed for the cytochrome *c* but not CcP transitions. It was not possible to distinguish between base-line uncertainty or nonsymmetrical van't Hoff (two-state) peaks due to experimental noise from the presence of additional transitions for some of our data. We

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[1] Abbreviations: DSC, differential scanning calorimetry; CcP, cytochrome *c* peroxidase; C^{3+}, ferricytochrome *c*.

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Table I: Summary of Thermodynamic Data for Cytochrome *c* Peroxidase and Its Derivatives[a]

compound	pH	[CcP] (mg mL^{-1})	t_{m1} (°C)	t_{m2} (°C)	ΔH_1 (kcal mol^{-1}) cal	ΔH_1 (kcal mol^{-1}) van't Hoff	ΔH_2 (kcal mol^{-1}) cal	ΔH_2 (kcal mol^{-1}) van't Hoff
CcP	7.0	1.18–10.3	43.9 ± 1.4 (13)[d]	63.3 ± 1.6 (13)	68 ± 15 (7)	126 ± 15 (7)	96 ± 19 (6)	166 ± 24 (6)
CcP	8.0	8.95–9.78	39.9 ± 1.4 (3)	60.3 ± 0.8 (3)	79 ± 24 (3)	108 ± 12 (3)	105 ± 6 (3)	199 ± 9 (3)
apoCcP	7.0	6.54–13.1		59.7 ± 0.6 (2)			107 ± 29 (2)	195 ± 18 (2)
CcP·F[b]	7.0	4.08–8.16	46.5 ± 1.8 (2)	61.4 ± 2.1 (2)	64 (1)	150 (1)	77 (1)	184 (1)
CcP·CN[c]	8.0	8.95–9.78	38.7 ± 0.7 (2)	65.1 ± 0.4 (2)	91 ± 6 (2)	92 ± 6 (2)	145 ± 14 (2)	197 ± 18 (2)

[a] 10 mM phosphate buffer with added KNO_3 to 0.1 M ionic strength. [b] Fluoride concentration 35–70 mM. [c] Cyanide concentration 1.0 mM. [d] Mean ± standard deviation (number of observations).

chose to favor identifying fewer certain transitions rather than a greater number of less certain transitions for our analysis. The molar calorimetric enthalpy change, ΔH_{cal}, was calculated for cytochrome *c* and cytochrome *c* peroxidase by using molecular weights of 12 360 and 34 168, respectively:

$$\Delta H_{cal} = \Delta h_d M_r \quad (1)$$

The van't Hoff enthalpy change for the thermal transition enthalpy was evaluated from the ratio of T_m (degrees kelvin) to the half-width, $T_{1/2}$:

$$\Delta H_{vH} = 4R(t_m + 273.15)^2/T_{1/2} \quad (2)$$

The best values of $T_{1/2}$, t_m, and Δh_d were obtained by using the same trial and error curve-fitting procedure used to resolve overlapping peaks. The procedure used in curve fitting was as follows. The value of ΔH_{vH} was estimated from the experimental values of T_m and $T_{1/2}$. The value of K_i at a given temperature was determined from eq 3 where $K_m = 1$ when $T_i = T_m$ and

$$K_i/K_m = \exp\left[-\frac{\Delta H_{vH}}{R}\left(\frac{1}{T_i} - \frac{1}{T_m}\right)\right] \quad (3)$$

$K_i = \alpha_i/(1 - \alpha_i)$, where α_i equals the fraction of thermal product, given by

$$\alpha_i = K_i/(1 + K_i) \quad (4)$$

The fractional heat change, q_i, at T_i is obtained from

$$q_i = \alpha_i q_T \quad (5)$$

where q_T is the total observed heat change. Finally, the value for the calculated heat capacity, y_i, which was plotted versus temperature was obtained from the difference

$$y_i = q_i - q_{i-1} \quad (6)$$

The mean standard deviation between 650 observed and calculated points for 2 cytochrome *c* experiments was 3.6% of the maximal value of the excess heat capacity. Similar accuracy was obtained in the analysis of CcP when individual symmetrical peaks were observed. The accuracy is estimated to be about 3-fold less certain when resolution of overlapping peaks was required.

Results

pH and Concentration Dependence. Cytochrome *c* peroxidase is stable at room temperature between pH 4 and about pH 8 (Dowe & Erman, 1985). A previous spectroscopic investigation of the thermal denaturation of the enzyme in this pH region has shown that cytochrome *c* peroxidase precipitates upon heating between pH 4 and 6 while no precipitation was observed upon heating between pH 7 and 8 (Gross & Erman, 1985). These observations were confirmed in this study. The pH dependence of the thermal denaturation was investigated by DSC between pH 4 and 8 in 0.1 M ionic strength buffers where it was observed that the thermal denaturation of CcP is a multistate process with two transitions apparent at all pH values investigated (Figure 1). The midpoint transition temperatures have an average standard deviation of 1.1 °C, and the values for t_{m1} and t_{m2} are plotted as a function of pH in Figure 2.

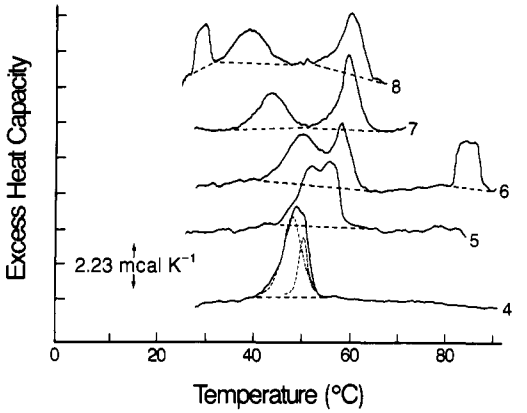

FIGURE 1: DSC curves for the thermal denaturation of CcP at pH values ranging from 4.0 to 8.0 at a scanning rate of 0.55 °C/min. The pH values are given by the numbers to the right of each curve. Buffers were either 10 mM acetate (pH 4 and 5) or 10 mM phosphate (pH 6–8) with added KNO_3 to adjust the ionic strength to 0.1 M. Protein concentration ranged from 9.62 to 9.78 mg mL^{-1}. Electrical calibrations are included in the traces at pH 6 (11.126 mcal) and pH 8 (6.6549 mcal). The dashed lines in the pH 4 curve represent calculated values for the two transitions from the curve-fitting procedure described in the text.

At pH 7 and 8, the two thermal processes are well separated, and the DSC endotherms are nearly symmetrical. Both the calorimetric and van't Hoff enthalpies, along with the transition midpoint temperatures, are collected in Table I for the pH 7 and 8 data. Due to the precipitation of the protein between pH 4 and 6 and the overlapping nature of the transitions, only qualitative enthalpy data have been obtained in this pH region. The total calorimetric enthalpy observed between pH 4 and 6 is 200 kcal/mol with a standard deviation of 41 kcal/mol for seven observations. This is somewhat higher but within experimental error of the total calorimetric enthalpy obtained at pH 7 and 8 (Table I).

The concentration dependence of the thermal denaturation was investigated at pH 7.0. The midpoint transition temperatures are independent of protein concentration between 1.18 and 10.3 mg mL^{-1}, and there is no apparent concentration dependence of the calorimetric or van't Hoff enthalpies.

Reversibility. The thermal denaturation of CcP is irreversible at acidic pH where protein precipitation occurs. At pH 7, denaturation is also irreversible when the sample is heated above the high-temperature transition. However, the process responsible for the low-temperature transition was partially reversible at pH 7. When a sample is heated to 50 °C (above the low-temperature but below the high-temperature transition), cooled to 25 °C, and then rerun, both endotherms are observed but with midpoint transition temper-

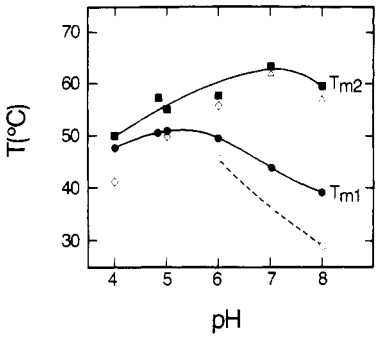

FIGURE 2: pH dependence of the midpoint temperatures for the thermal transitions in CcP. The closed symbols represent the low-temperature transition, t_{m1} (closed circles), and high-temperature transition, t_{m2} (closed squares), observed in the DSC experiments. The open symbols represent transitions observed in a spectrophotometric study of the thermal transitions in CcP (Gross & Erman, 1985). The open circles represent a spectral change in which the Soret maximum changes from 408 nm at low temperature to 414 nm at high temperature. The open triangles represent the transition from the species with a Soret maximum at 414 nm to one with a greatly diminished absorptivity in the Soret region and a Soret maximum of 390 nm. The open diamonds represent the onset of turbidity observed spectrophotometrically.

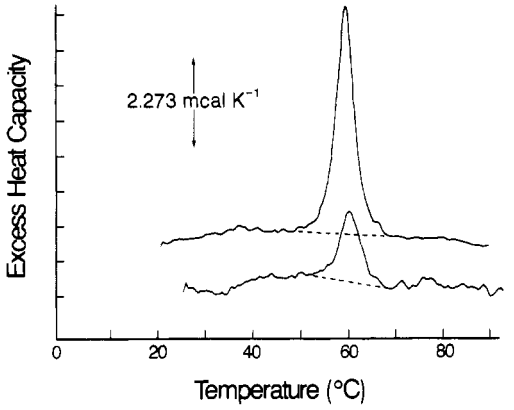

FIGURE 3: DSC curves for the thermal denaturation of apoCcP. Protein concentrations are 6.54 and 13.08 mg mL^{-1} for the lower and upper tracings, respectively. Conditions: pH 7.0, 10 mM phosphate buffer with added KNO_3 to adjust the ionic strength to 1.0 M.

atures 3–5 °C lower and with enthalpy changes about 50% less than those of the native enzyme.

Apocytochrome c Peroxidase. The presence or absence of the heme has a profound influence on the thermal denaturaton of CcP. Single DSC endotherms were observed for the apoenzyme (Figure 3). The maximum excess specific heat exhibited nearly the same concentration dependence as ΔH_{cal}, and yielded values of 0.44 and 0.70 cal K^{-1} g^{-1} for the 6.54 and 13.08 mg mL^{-1} samples, respectively. The average value is 0.57 ± 0.13 cal K^{-1} g^{-1}, corresponding to a 23% error, which may be compared with that of 14% observed by Takahashi and Sturtevant (1981) for the thermal denaturation of subtilisin BPN'. The enthalpy data obtained at pH 7 are collected in Table I. The midpoint transition temperature for the apoprotein is similar to, but slightly lower than, t_{m2} for the holoenzyme. The calorimetric enthalpy for the apoprotein is similar to ΔH_2 of the holoenzyme.

Heme Ligands. The effect of fluoride and cyanide binding to the heme of CcP on the thermal properties of the enzyme was investigated. At pH 7.0, fluoride concentrations from 35 to 70 mM caused a small but significant increase in t_{m1} with no effect on t_{m2}. The change in t_{m1} upon fluoride binding is 2.6 ± 2.3 °C with a 95% confidence level that this is a significant difference based upon the statistical t test (Table I).

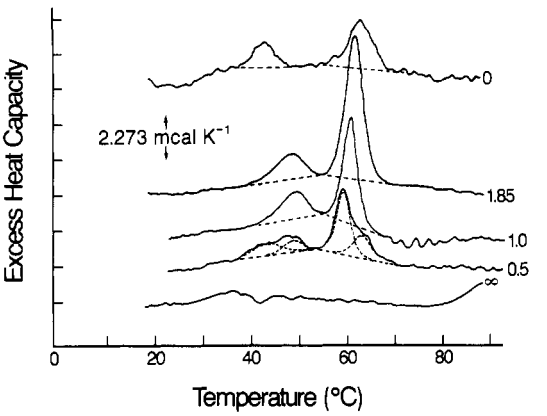

FIGURE 4: DSC curves for the thermal denaturation of mixtures of ferricytochrome c and CcP at pH 7. The buffer was 10 mM cacodylate, 8 mM ionic strength. The mole ratio of cytochrome c to CcP is given at the right of each tracing. The CcP concentration for the four uppre tracings was 8.12 mg mL^{-1}. The cytochrome c concentration for the lower tracing was 5.53 mg mL^{-1}. The dashed lines in the DSC curve at a mole ratio of cytochrome c to CcP of 0.5 represent four transitions calculated through the curve-fitting procedure described in the text.

K_D for the CcP–fluoride complex is 0.3 mM at pH 7 (Erman, 1974a; Lent et al., 1976). The binding affinity for fluoride increases with decreasing pH, and at pH 4.8, K_D has a value of 6 μM. DSC studies of CcP–fluoride mixtures at pH 4.8 showed that above 0.5 mM fluoride, t_{m1} of the native enzyme increased and became coincident with t_{m2}, an increase of about 8 °C. The value of t_{m2} was not significantly altered in the presence of up to 5 mM fluoride at pH 4.8.

The binding affinity of cytochrome c peroxidase for cyanide is greatest at pH values between 7.5 and 8.5 (Erman, 1974b). At pH 8, K_D for the enzyme–cyanide complex is 1 μM (25 °C). Addition of 1 mM cyanide to CcP has no effect on t_{m1} but increases t_{m2} by 4.8 ± 0.9 °C (99% confidence level). The thermodynamic data for denaturation of the CcP–cyanide complex are collected in Table I.

Effect of Cytochrome c Binding. Cytochrome c peroxidase and ferricytochrome c form a 1:1 complex at both pH 6 and pH 7.5 (Erman & Vitello, 1980; Vitello & Erman, 1987). The effect of cytochrome c binding on the thermal denaturation of CcP was investigated at pH 7.0. At pH 7.0, in 10 mM cacodylate buffer (I = 8 mM), ferricytochrome c is significantly more stable to thermal denaturation than CcP. The onset of cytochrome c denaturation is observed at about 80 °C (Figure 4, lower trace). Cytochrome c denaturation is irreversible and accompanied by protein precipitation at this pH.

Additions of ferricytochrome c to CcP at pH 7 alters the denaturation properties of both proteins. When 0.5 molar equiv of cytochrome c is added to CcP, each of the thermal transitions associated with denaturation of the enzyme appears to be split into two components (Figure 4). We attribute this splitting to distinct denaturation of the native enzyme and the cytochrome c–enzyme complex. The binding between cytochrome c and the enzyme is quite strong under these experimental conditions, with the equilibrium dissociation constant estimated to be 0.4 μM at 25 °C (Vitello & Erman, 1987). Addition of stoichiometric or larger amounts of cytochrome c to the enzyme produces DSC scans with two apparent transitions. The low-temperature transition of the cytochrome c–CcP complex is elevated 4.8 ± 1.4 °C (99% confidence level) compared to the native enzyme (Table II). There is no statistically significant change in t_{m2} (−1.4 ± 1.8 °C) although the DSC scan at a cytochrome c:CcP ratio of 0.5 (Figure 4)

Table II: Summary of Thermodynamic Data for the Cytochrome *c*–Cytochrome *c* Peroxidase Complex[a]

compound	I (M)	t_{m1} (°C)	t_{m2} (°C)	ΔH_1 (kcal mol^{-1})		ΔH_2 (kcal mol^{-1})	
				cal	van't Hoff	cal	van't Hoff
CcP	0.008	43.1 ± 0.1 (4)[b]	61.3 ± 1.1 (4)	74 ± 22 (3)	131 ± 28 (3)	113 ± 10 (3)	154 ± 6 (3)
C^{3+}–CcP	0.008	47.9 ± 1.5 (5)	59.9 ± 1.4 (5)	65 ± 8 (4)	135 ± 20 (4)	176 ± 46 (4)	207 ± 22 (4)
CcP	1.008	47.0 (1)	60.4 (1)	64 (1)	118 (1)	73 (1)	196 (1)
C^{3+}–CcP	1.008	46.9 (1)	59.2 (1)	65 (1)	141 (1)	75 (1)	260 (1)

[a] Data for C^{3+}–CcP mixtures with mole ratios of 0.91–9.17; 10 mM cacodylate buffer with and without 1.0 M KNO_3, pH 7.0. [b] Mean ± standard deviation (number of observations).

suggests there is a systematic decrease in t_{m2}. In addition, the second transition is sharper, with the half-width decreasing about 30% in the presence of stoichiometric or larger amounts of cytochrome *c*.

A separate transition for cytochrome *c* denaturation is not observed in the presence of CcP, even in the case where cytochrome *c* is in excess (Figure 4). At the end of these runs, all of the protein is precipitated, suggesting that the thermal stability of cytochrome *c* is reduced in the presence of CcP and that the two proteins unfold simultaneously.

High ionic strength weakens the interaction between cytochrome *c* and the enzyme. At pH 7, 1.0 M ionic strength, no interaction between cytochrome *c* and the enzyme is observed at room temperature (Vitello & Erman, 1987), and cytochrome *c* has no effect upon the thermal denaturation of CcP (Table II). However, t_m for cytochrome *c* is decreased from above 80 to 76 °C in the presence of a stoichiometric amount of CcP even at 1.0 M ionic strength. Comparison of the data for CcP alone indicate that t_{m1} is dependent upon ionic strength, increasing by 3.9 ± 1.1 °C (99% confidence level) upon increasing the ionic strength from 8 mM to 1.0 M. There is no significant effect of ionic strength on t_{m2} (Table II).

Discussion

Nature of the Thermal Transitions. The DSC study of CcP is complicated by protein precipitation observed near the isoelectric point of the enzyme, pI = 5.25 (Yonetani, 1967), and the irreversible nature of the unfolding. The standard deviation in t_m values is 1.1 °C, and the average coefficient of variation in the calorimetric and van't Hoff enthalpies is 18% and 12%, respectively. The standard deviations of the enthalpies determined in this study are higher than normally observed for well-behaved protein systems but similar to the 12–14% standard deviations reported for the core protein of *lac* repressor (Manly et al., 1985) and for yeast hexokinase in the presence of glucose at low ionic strength (Takahashi et al., 1981). Because of these problems, the data should only be considered to give a semiquantitative guide to the thermal unfolding of cytochrome *c* peroxidase.

The DSC experiments clearly show that at least two major thermal transitions are involved in the unfolding of the native structure of CcP (Figure 1). At pH 7 and 8, the two transitions are completely resolved with about 42% of the heat absorbed in the lower temperature transition and 58% of the heat absorbed in the high-temperature transition (Table I). At the first level of interpretation, it is commonly assumed that multiple transitions imply unfolding of unique domains within the protein (Pabo et al., 1979; Ploplis et al., 1981; Privalov, 1982). Taking into account the temperature dependence of the specific enthalpy for protein unfolding (Privalov, 1979), the domains in CcP would be approximately equal in size.

Generally, comparisons of the calorimetric and van't Hoff enthalpies can provide information about the denaturation process. A necessary, but insufficient, condition for a two-state denaturation process is that the calorimetric and van't Hoff enthalpies must be identical. The occurrence of intermediate states, such as the unfolding of independent domains, within a single thermal transition is reflected as an apparent decrease in the van't Hoff enthalpy. Protein–protein interactions and irreversibility sharpen the transition, resulting in a larger apparent van't Hoff enthalpy. For CcP, we generally observe van't Hoff enthalpies which are larger than the calorimetric enthalpies, consistent with the irreversible nature of the denaturation and protein–protein interactions.

There is evidence that the low-temperature transition involves intermediates during the unfolding. This evidence comes from a comparison of the spectroscopic study of the thermal denaturation of CcP (Gross & Erman, 1985) and the DSC studies. Between pH 6 and 8, the enzyme undergoes a spectral transition in which the Soret maximum shifts from 408 to 414 nm, indicating a rearrangement of the protein in the vicinity of the heme. The midpoint temperatures for this spectral transition are plotted in Figure 2. The midpoint of the spectral transition is significantly lower than t_{m1} determined from the DSC curves although there is considerable overlap of the spectral and calorimetric transitions. The van't Hoff enthalpies for the spectral transition vary between 68 (pH 8) and 100 (pH 6) kcal mol^{-1}, suggesting a major unfolding of the protein associated with the spectral changes. It should be noted that this evidence for intermediates states occurring in the low-temperature transition includes pH 7 and 8 where the van't Hoff to calorimetric enthalpy ratio is greater than 1, leading to the conclusion that both intramolecular and intermolecular interactions are occurring in the low-temperature transition.

pH and Ionic Strength Effects. The pH dependence of t_{m1} and t_{m2} provides evidence for the independence of the two major thermal transitions. They each have unique pH dependencies, with t_{m1} having a maximum value near pH 5 while t_{m2} has a maximum at pH 7 (Figure 2). The total calorimetric enthalpy remains essentially constant as a function of pH with a value of 193 ± 37 kcal mol^{-1} between pH 4 and 8. This corresonds to a specific enthalpy for unfolding of 5.6 ± 1.1 cal g^{-1}, which is within the range of values reported for unfolding of globular proteins with transition temperatures between 40 and 60 °C (Privalov, 1979). The variation in ΔH_1 and ΔH_2 does not correlate with the transition temperatures, indicating that this pH variation cannot be assigned to purely heat capacity effects but must be attributed to interaction between domains or to some unknown structural changes of either the folded or the unfolded domain as in the similar case for the thermal unfolding of phosphoglycerate kinase (Hu & Sturtevant, 1987).

The maximum in t_{m1} is near the isoelectric point of the protein, suggesting that this transition is sensitive to electrostatic effects. This suggestion is supported by the ionic strength dependence of t_{m1} at pH 7 (Table II). At low ionic strength (8 mM), t_{m1} has a value of 43.1 °C which increases to 47.0 °C at 1.0 M ionic strength. At pH 7, 25 °C, CcP has a net charge of −9 (Conroy & Erman, 1978) and electrostatic repulsion between various parts of the protein would promote unfolding. Increasing ionic strength decreases the electrostatic

contribution to the unfolding, stabilizing the protein and increasing t_{m1}. There is no significant effect upon the enthalpies for the low-temperature transition at pH 7. Electrostatic effects appear to be much less important for the higher temperature transition in CcP. The pH of maximum stability is about 2 pH units above the isoelectric point, and t_{m2} does not depend upon ionic strength within our experimental error.

Ionic strength effects on thermal denaturation are common. Cytochrome *c* and cytochrome c_1 each unfold in a single, cooperative transition (Privalov & Khechinashivili, 1974; Yu et al., 1983), and increasing ionic strength increases the transition temperature in each of these proteins. KCl has an unusual effect on the transition temperature of ferricytochrome *c* which increases from 82.6 to 86.7 °C between 10 and 75 mM ionic strength but decreases to a value of 84 °C between 75 mM and 0.7 M ionic strength. This probably reflects specific ion binding rather than an ionic strength effect. Yu et al. (1983) also observed that t_m for ferricytochrome c_1 increased from 61 to 69 °C over the ionic strength range 0.01–1.5 M accompanied by an increase in the enthalpy from about 70 to 120 kcal mol^{-1}. The ionic strength dependence of the enthalpy is in contrast to our results where no change in enthalpy was observed upon increasing the ionic strength. There is precidence for ionic strength affecting the transition temperature but not the enthalpy. Yeast hexokinase, which contains two domains according to the results of X-ray crystallography, exhibits a double-peaked endotherm for the thermal unfolding at low ionic strength in the absence of glucose (Takahashi et al., 1981). Added NaCl decreases the midpoint temperature for the most stable transition but has no significant effect on the enthalpy.

Apocytochrome c Peroxidase. Removal of the heme from the holoenzyme has a profound effect upon the thermal denaturation of CcP (Figure 3 and Table I). ApoCcP shows only a single thermal transition which corresponds very closely to both t_{m2} and ΔH_2 for the holoenzyme. The apparent loss of the low-temperature transition in the apoenzyme could be used to argue that the less stable domain is completely unfolded by removal of the heme. There are seveal observations which indicate that this is not true. The sedimentation and diffusion coefficients of the apoprotein are only slightly smaller than those of the holoenzyme and correspond to an increase in the frictional ratio from 1.03 for the holoenzyme to 1.08 for the apoenzyme (Dowe & Erman, 1985). Analysis of the pH titration curves for both apoenzyme and native enzyme by means of the Linderstrom–Lang theory suggests the apoprotein has a slightly larger radius (2.8 nm) in comparison to the holoenzyme (2.4 nm) (Conroy & Erman, 1978). Finally, apoCcP can be crystallized (Yonetani, 1967). All of these properties suggest that apoCcP retains its globular form although it may be slightly expanded.

The very large effect of heme binding on the enthalpy of unfolding for CcP is very similar to the effect of glucose binding to yeast hexokinase (Takahashi et al., 1981). Binding of glucose to hexokinase nearly doubles the specific enthalpy from about 1.6 to 3.0 cal g^{-1}, an effect which cannot be attributed to the binding enthalpy of glucose. Heme binding to apoCcP increases the specific enthalpy from 3.1 ± 0.4 to 5.6 ± 1.1 cal g^{-1}. In spite of the similarities of small-molecule binding on the enthalpies of unfolding for CcP and yeast hexokinase, these small molecules have opposite effects on the transition temperatures. As noted above, apoCcP unfolds in a single transition centered at 60 °C (pH 7) while binding heme causes the protein to unfold in two transitions centered near 44 and 63 °C. On the other hand, yeast hexokinase unfolds in two thermal transition centered at 41 and 48 °C in the absence of glucose and unfolds in a single transition centered at 51 °C when glucose binds (Takahashi et al., 1981).

The ratio of the van't Hoff to calorimetric enthalpy for the apoprotein is near 2 at both pH 4.8 and pH 7.0, indicating the importance of intermolecular interactions during the unfolding of the apoprotein as well as the holoenzyme.

Heme Ligands. In view of the large effect of heme on the thermal unfolding of CcP, the influence of heme ligation was investigated by the addition of fluoride (a weak-field ligand which gives a high-spin ferric heme derivative) and cyanide (a strong-field ligand which gives a low-spin ferric heme derivative). The largest effect was observed by fluoride binding at pH 4.8. Fluoride binding increases the value of t_{m1} such that only a single thermal transition is observed at saturating concentrations of fluoride. The change in t_{m1} is about 8 °C. There is no significant change in the value of t_{m2} upon fluoride binding at pH 4.8. The effect of fluoride is less dramatic at pH 7; t_{m1} increases by about 2.6 °C, and again there is no change in t_{m2}. Cyanide ligation (pH 8) has the opposite effect on the transition temperatures. Cyanide has no effect on t_{m1} but increases t_{m2} by 4.8 °C.

Cytochrome c Binding. Ferricytochrome *c* and CcP form a strong one-to-one complex at low ionic strength (Erman & Vitello, 1980; Vitello & Erman, 1987), and DSC experments show significant effects on the thermal denaturation of both proteins in the presence of the other (Figure 4 and Table II).

At 8 mM ionic strength, pH 7, t_{m1} of the enzyme increases by 4.8 °C and t_{m2} decreases by 1.4 °C in the presence of equimolar or higher ratios of ferricytochrome *c*. There is no change in ΔH_1, but ΔH_2 increases by 56% in the presence of cytochrome *c*, and the transition becomes sharper (Figure 4), with the half-width decreasing by 30%. In addition, no endotherm due to cytochrome *c* unfolding is observed in the presence of CcP up to 90 °C (even with excess cytochrome *c*) even though cytochrome *c* alone begins to denaturate at about 80 °C with an apparent maximum at 87 °C before precipitation is observed. Both cytochrome *c* and CcP are precipitated at the end of the DSC experiments using mixtures of CcP and cytochrome *c* while the enzyme alone does not precipitate. These observations indicate that cytochrome *c* does denature in the presence of CcP and, since there is no separate endotherm for cytochrome *c* unfolding, it must unfold simultaneously with CcP. The increase in ΔH_2 is essentially equal to the enthalpy for cytochrome *c* unfolding alone.

These data suggest that the mechanism for thermal denaturation of CcP is not substantially altered upon complex formation with cytochrome *c*. The lower temperature enzyme transition is stabilized somewhat in the presence of cytochrome *c*, with t_{m1} increasing by 4.8 °C. The second domain of the enzyme and cytochrome *c* unfold simultaneously with a transition temperature only slightly less than that of the enzyme alone, but with a lowering of the cytochrome *c* transition by over 27 °C.

Very similar observations were made by Yu et al. (1983) on the interaction of cytochrome *c* and cytochrome c_1. Cytochrome c_1 unfolds in a single transition centered near 63 °C at low ionic strength, pH 7. In the presence of equimolar ferricytochrome c_1, both proteins simultaneously unfold in a single endotherm centered at 67 °C, about 15 °C lower than that observed for ferricytochrome *c* alone. The enthalpy change for the cytochrome *c*–cytochrome c_1 complex was approximately equal to the sum of the enthalpies for the individual proteins. The effect of both cytochrome c_1 and CcP on the thermal stability of ferricytochrome *c* is similar and

quite dramatic, lowering the t_m for cytochrome *c* unfolding by 15–27 °C. Both cytochrome c_1 and CcP bind to cytochrome *c* with high affinity at low ionic strength, and both unfold at significantly lower temperatures than cytochrome *c* alone. We suggest that a strong interaction between cytochrome *c* and its partner is maintained as the less stable protein unfolds and this induces the unfolding of cytochrome *c* to occur simultaneously with that of the protein to which it is complexed.

At high ionic strength, the interacton between cytochrome *c* and both cytochrome c_1 (Yu et al., 1983) and CcP (Vitello & Erman, 1987) is decreased. No complex formation is observed at 1.0 M ionic strength for cytochrome *c* and CcP. Under these ionic strength conditions, cytochrome *c* has little effect on the thermal denaturation of CcP (Table II) or of cytochrome c_1 (Yu et al., 1983). However, the unfolded CcP or cytochrome c_1 does interact with ferricytochrome *c*, reducing the t_m for cytochrome *c* unfolding by about 10 °C in both cases.

In contrast to the cytochrome *c*–CcP and cytochrome *c*–cytochrome c_1 systems, a calorimetric investigation of *Streptomyces* subtilisin inhibitor, subtilisin BPN′, and their complex by Takahashi and Sturtevant (1981) indicated that both proteins in the enzyme–inhibitor complex unfolded more or less independently. The reasons for the differences between the cytochrome *c* systems and the subtilisin–subtilisin inhibitor complex are unknown, but possibilities include bulk solvent effects and nonspecific interaction between folded and unfolded proteins.

Domain Structure. The crystallographic structure of CcP shows that about 50% of the 294 residues are in an α-helical conformation. There are 13 helical segments consisting of 6–19 residues each (Finzel et al., 1984). The protein is folded into two well-defined domains with the heme bound in a cleft between the two domains. Domain I contains both the N-terminal and C-terminal residues and is comprised of residues 1–145 and 266–294. Domain II contains residues 146–265. Domain I is more loosely organized than domain II with about half of the main-chain atoms of domain I having high values of the isotropic thermal parameter, indicating either greater static disorder or larger thermal motion (Finzel et al., 1984). Either property suggests that these regions of domain I are more flexible than the remainder of the molecule. The flexible portion of domain I is set apart from the rest of the molecule by a channel of solvent containing at least a dozen sites for water with varying degrees of occupancy and hydrogen bonding. We suggest that this flexible portion of domain I is unfolded in the low-temperature transition observed in the holoenzyme and that the more rigid section of domain I, that portion near the heme, and domain II unfold in the higher temperature transition. The strongest evidence for this is the spectroscopic study (Gross & Erman, 1985) which shows that solvent exposure of the heme does not increase as the low-temperature transition is traversed, and we believe the heme binding site remains essentially intact through this transition. There must be some interaction between the less stable portion of domain I and the heme binding site in light of the large effect of heme binding and fluoride on t_{m1}.

Conclusions. The most important findings in this study are that the thermal unfolding of CcP occurs in two well-resolved transitions at pH 7 and that neither transition can be described by a two-state unfolding process. Partial unfolding of the enzyme occurs near 44 °C, and evidence is presented to indicate that this transition involves intramolecular intermediates. The low-temperature transition is complete before the remainder of the protein unfolds with a transition temperature near 63 °C. Comparison of van't Hoff to calorimetric enthalpies indicates that intermolecular interactions are important in the unfolding process for both the low- and high-temperature transitions. Removal of the heme has an extremely large effect; the apoprotein unfolds in a single transition with a specific enthalpy of 3.1 cal g^{-1}, about 45% less than the value of 5.6 cal g^{-1} found for the holoenzyme. Heme ligands have small but significant effects on the thermal denaturation of the enzyme, with fluoride affecting the low-temperature transition and cyanide affecting the high-temperature transition. The unfolding mechanism for CcP bound to ferricytochrome *c* is essentially identical with that of the free enzyme with a small stabilization of the low-temperature transition. However, the transition temperature for cytochrome *c* decreases by over 27 °C through interaction with CcP at low ionic strength, pH 7. Even at high ionic strength, where no complex formation between the native proteins is observed at room tempeature, the presence of unfolded CcP decreases the transition temperature for cytochrome *c* denaturation by 10 °C.

Registry No. CcP, 9029-53-2; C^{3+}, 9007-43-6; CN^-, 57-12-5; F^-, 16984-48-8.

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Photodissociated Cytochrome *c* Oxidase: Cryotrapped Metastable Intermediates

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ABSTRACT: By freezing CO-bound cytochrome *c* oxidase at cryogenic temperatures, we have been able to cryotrap metastable intermediates of photodissociation. The differences in the resonance Raman spectrum between these intermediates and ligand-free reduced cytochrome oxidase at cryogenic temperatures are the same as those between the phototransient and the fully reduced preparation detected with 10-ns excitation at room temperature. The largest difference occurs in the iron–histidine stretching mode of cytochrome a_3, which shifts by up to 8 cm^{-1} to higher frequency in the photoproduct. At 4 K the iron–histidine mode displays two unrelaxed frequencies in the photoproduct, which we attribute to two different unrelaxed structures of the heme pocket. The frequencies and intensities of the lines in the resonance Raman spectrum are sensitive to the incident laser power density in both the ligand-free fully reduced preparation and the photoproduct even at 4 K. At 77 K the carbonyl stretching mode of the formyl group in cytochrome a_3^{2+} is especially sensitive to laser power, displaying two frequencies—1666 cm^{-1} at low-flux density and 1674 cm^{-1} at high-flux density. These frequencies may reflect a change in conformation of the formyl group or a change in its interaction with the protein such as in hydrogen bonding to the carbonyl of the formyl group. The absence of immediate relaxation of the CO photoproduct must be considered when one studies the structure and kinetics of the O_2 intermediates that are formed in triple trapping and flow-flash experiments following photodissociation of the CO-bound enzyme.

In order to determine the molecular basis for the function of heme proteins that bind oxygen, it is necessary to understand the interactions between the heme and the protein and also how these interactions change when oxygen is bound or released. The static interactions may be studied by using techniques that probe equilibrium states, but to follow the evolution of changes in interactions upon going from one state of ligand binding to another, techniques must be used in which metastable intermediates may be isolated. Ultimately, this should lead to an understanding of how protein structure controls and regulates active-site properties. Although oxygen is the physiological ligand of many heme proteins, it is often not a good candidate for studies of metastable intermediates because it is reduced by the active site. This reduction is slow in oxygen-transport and -storage proteins such as hemoglobin and myoglobin (Antonini & Brunori, 1971) but is very rapid in enzymes such as cytochrome oxidase (Hill & Greenwood, 1984). Carbon monoxide (CO) therefore has been used successfully as a model for oxygen since it is the same size, binds very strongly, and is relatively unreactive. Another advantage realized in studies that utilize CO is its very high photolability (Sawicki & Gibson, 1976). Thus, after the CO-bound protein is formed, generation of the ligand-free form of the protein may be initiated on the subpicosecond time scale by optical photodissociation (Martin et al., 1983). The ensuing relaxation may be followed by using time-resolved spectroscopy to study the structure and kinetics of the intermediates in the pathway from the ligand-bound to the ligand-free state. Resonance Raman scattering has been found to be a particularly useful spectroscopic technique with which to monitor the time evolution of heme changes following photodissociation and to map out the relaxation pathway (Friedman et al., 1982; Friedman, 1985; Rousseau & Friedman, 1987).

Cryogenic techniques have also been used successfully to cryotrap metastable intermediates by first freezing the CO-bound protein at cryogenic temperatures and then photodissociating the CO (Ondrias et al., 1983b; Rousseau & Argade, 1986; Sassaroli et al., 1986; Rousseau & Friedman, 1987). It was demonstrated that in hemoglobin the spectroscopic differences between the equilibrium deoxy preparation and the CO-photodissociated preparation were the same at cryogenic temperatures (80 K) as at room temperature on the nanosecond time scale (Ondrias et al., 1983a). This was a satisfying result because it suggested that spectroscopic studies at cryogenic temperatures could be used to infer properties of the protein under physiological conditions.

The comparison between room temperature time-resolved intermediates and cryotrapped intermediates of photodisso-

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