Thermodynamic Studies of the Opening of the Heme Crevice of Ferricytochrome c†

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ABSTRACT: The thermodynamic changes associated with the opening of the heme crevice of ferricytochrome *c* have been determined using absorbance changes of the 695-nm band of the protein. The influence of the denaturants 1-propanol, methanol, and urea and of variations in pH and ionic strength in facilitating the reversible thermal denaturation of ferricytochrome *c* has been investigated. The 695-nm absorbance band of cytochrome *c* is a measure of the integrity of the Met-80–heme iron bond and disruptions or weakening of this bond produces a quenching of the absorbance band and an opening of the heme crevice. In all cases van't Hoff plots were biphasic, indicating that denaturation proceeds *via* different mechanisms at high and low temperatures. Enthalpy changes in the upper temperature range were independent of denaturant concentration but were dependent on pH with a maximum positive value of pH 6. Enthalpy changes in the lower temperature range were profoundly influenced by the type and concentration of denaturant and the pH and ionic strength. Conditions which increasingly favored denaturation, as evidenced by a lowering of the transition temperature, were associated with increasingly more unfavorable enthalpy changes and increasingly more favorable entropy changes. Enthalpy changes obtained by extrapolation to zero denaturant concentration were 42 kcal/mol at pH 8, 32 kcal/mol at pH 7, 5 kcal/mol at pH 6, and 16 kcal/mol at pH 5. The major energetic consideration involved with the opening and closing of the heme crevice of ferricytochrome *c* thus appears to be the delicate balance between large opposing enthalpy and entropy changes of approximately equal magnitude.

It is well established that the heme iron ligands in horse heart ferricytochrome *c* are Met-80 and His-18 (Dickerson *et al.*, 1971). The iron–Met-80 bond has been shown to give rise to an absorbance band at 695 nm in the visible spectrum of ferricytochrome *c* (Schechter and Saludjian, 1967; Sreenathan and Taylor, 1971) and this absorbance maximum can be used as a measure of the integrity of this bond.

It has become apparent from X-ray crystallographic studies (Dickerson *et al.*, 1971; Takano *et al.*, 1971) and from a wealth of studies in solution (*e.g.*, Kaminsky *et al.*, 1972; Ulmer and Kagi, 1968) that although ferricytochrome *c* has a closed crevice structure containing the heme, the heme crevice of ferrocytochrome *c* is very much more tightly closed and compact. This difference in the strengths of the heme crevice is clearly relevant for the biological function of electron transport of cytochrome *c* (Takano *et al.*, 1971). Furthermore, disruptions of this heme crevice lead to dramatically altered properties of the protein (Kaminsky *et al.*, 1971; Wilson and Greenwood, 1971). It is thus apparent that a knowledge of the thermodynamic changes associated with the opening of the heme crevice of cytochrome *c* is an important prerequisite for the elucidation of the mechanism of action of cytochrome *c*.

In view of the important role of the iron–Met-80 bond in maintaining the closed crevice structure of cytochrome *c* (Takano *et al.*, 1971; Kaminsky *et al.*, 1972b), the 695-nm band can be used as a probe for the conformational state of this crevice. Thus evaluation of the effects of temperature on the absorbance at 695 nm should provide data on the thermodynamic changes for the opening of the heme crevice of cytochrome *c* in solution. Attempts to obtain such data have not yet been completely successful (Schejter and George, 1964) since the high concentrations of cytochrome *c*, necessitated by the very weak extinction coefficient of the 695-nm band, induce the protein to precipitate at temperatures too low to produce complete quenching of the absorbance. We have previously shown (Kaminsky *et al.*, 1971, 1972a,b; Kaminsky and Davison, 1969) that alcohols and urea interact with cytochrome *c* and produce a weakening and eventual opening of the crevice at higher solute concentrations. We speculated that the presence of these denaturants would lower the range of temperatures required to completely bleach the 695-nm band and that the data obtained from such a study would provide thermodynamic parameters for the opening of the heme crevice under differing denaturing conditions and could also be extrapolated to zero denaturant concentration. We report here our studies on the effects of temperature on the 695-nm absorbance of ferricytochrome *c* in solutions of varying concentrations of methanol, 1-propanol, and urea at pH 5–8 and at varying ionic strengths.

Experimental Procedure

Materials. Horse heart cytochrome *c*, grade I, 95% pure, 0.425% Fe, was purchased from Miles-Seravac, Maidenhead, England, and was oxidized with a small quantity of potassium ferricyanide in the cuvet just prior to commencing an experiment. The alcohols were the purest grade obtainable from British Drug Houses and were used without further purification. The urea was extra pure crystallized grade from Merck. Water was glass distilled and deionized with an Elgastat mixed-bed deionizer.

Methods. Solutions of the alcohols and urea were prepared as previously described (Kaminsky *et al.*, 1971) and were corrected for volume shrinkage and pH shifts. All solutions were

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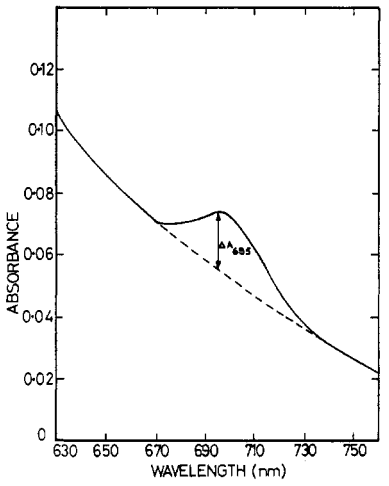

FIGURE 1: Method used to correct for the contribution of lower wavelength absorbance bands to the absorbance band at 695 nm of ferricytochrome *c*.

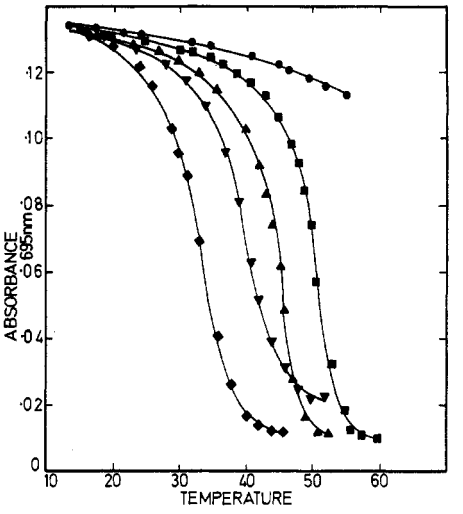

FIGURE 2: The 695-nm absorbance of ferricytochrome *c*, in varying concentrations of methanol, as a function of temperature. Methanol concentrations: (●) 0 mol %; (■) 12.2 mol %; (▲) 15.1 mol %; (▼) 18.2 mol %; (◆) 21.6 mol %; cytochrome *c* concentration 30 μM, path length 20 mm, 0.1 M sodium acetate buffer, pH 5.

stored in the frozen state when not in use. The concentration of ferricytochrome *c* was 30 μM and was determined using the molar extinction coefficient of Margalit and Schejter (1970).

Absorbance changes were measured using a Beckman DBGT spectrophotometer fitted with a W + W Model 1100 25-cm strip chart recorder adjusted to give a full scale deflection for 0.1 absorbance unit. The temperature in the cuvet was monitored with a Yellow Springs Instrument thermistor and Model 43 Telethermometer and was recorded using a W + W 25-cm strip chart recorder. Temperatures were varied by using the water-jacketed cell holder of the spectrophotometer and a water bath fitted with heating and refrigeration devices. Temperature was continuously and linearly varied from approximately 10 to 50° over a period of 85 min. The absorbance at 695 nm of the cytochrome *c* solution was recorded over this period and plots were made of absorbance against temperature. We verified that this method of monitoring the influence of temperature on changes of absorbance at 695 nm involved negligible contributions from alterations of background absorbance. In a number of experiments the temperature was varied in steps and a spectrum of the cytochrome *c* solution was scanned in the 695-nm region after the solution had reached thermal equilibrium. The contribution of background absorption was subtracted by extrapolation of the lower wavelength absorbance bands in the manner shown in Figure 1. The influence of temperature on the 695-nm absorbance changes calculated in this manner was the same as that determined by the previously described method. This precludes the possibility of changes in other absorbance bands from interfering with the continuous readings of 695-nm absorbance changes and thus the continuous method was used for the majority of the readings. pH values were determined in the cuvet using a Radiometer Model 22 pH meter.

The calculation of the equilibrium constant for displacement of Met-80 from the heme iron was based on the following assumptions: the protein was in the fully closed crevice form when the 695-nm band was fully developed and was in the open crevice form when this absorbance maximum was completely bleached. The equilibrium constant for this reaction was calculated as follows

$$K = \frac{A_{cc} - A_{obsd}}{A_{obsd} - A_{oc}}$$

where A_{obsd} is the absorbance at 695 nm at a particular temperature under the conditions of the experiment, A_{cc} is the maximum absorbance for the closed crevice form at 695 nm which could not be increased by a decrease in temperature, and A_{oc} is the minimum absorbance for the open crevice form at 695 nm which could not be decreased by a further increase in temperature.

Results

Addition of the denaturants urea, methanol, or 1-propanol to aqueous buffered solutions of horse heart ferricytochrome *c* considerably increased the susceptibility of its 695-nm absorbance band to thermally induced changes. At certain concentrations of the denaturants the ferricytochrome *c* 695-nm absorbance band changed in a sigmoid relationship with temperature (Figure 2). All thermally induced absorbance changes were completely reversible on lowering the temperature. At zero or low concentrations of denaturant the 695-nm absorbance peak was only partially quenched by 50° and at high concentrations of denaturants the 695-nm absorbance peak did not attain its maximum possible value even at the minimum temperature of 10°. This temperature range (10–50°), which was limited by practical considerations, thus also determined the range of denaturant concentrations which could be usefully considered.

A representative set of results demonstrating the influence of temperature on the 695-nm absorbance band of ferricytochrome *c* in a series of solutions of increasing concentration of methanol is shown in Figure 2. Similar results were obtained with 1-propanol, where a lower concentration of the denaturant was required to produce complete quenching of the 695-nm absorbance at similar temperatures, and with urea. The values of temperature corresponding to a 695-nm absorbance midway between the maximum and minimum values obtainable ($t_{1/2}$) are given in Table I. The influence of temperature on the 695-nm band in the presence of denaturants is profoundly influenced by changes of pH which alter the $t_{1/2}$ values. A linear relationship between $t_{1/2}$ values and denaturant concentration was obtained for all three denaturants at pH 5–7 and also at pH 8 for 1-propanol. Ex-

TABLE I: Transition Temperatures for the Opening of the Heme Crevice of Ferricytochrome *c* as Monitored by 695-nm Absorbance Changes.

Denaturant	Denaturant Concn (mol %)	pH	$t_{1/2}$ (°C)
Methanol	12.20	5.0	50.8
	15.10	5.0	45.6
	18.20	5.0	40.2
	21.60	5.0	33.5
	15.10	6.0	52.5
	18.20	6.0	50.0
	21.60	6.0	45.9
	9.00	7.0	36.0
	15.10	7.0	31.0
	21.60	7.0	25.1
1-Propanol	3.15	5.0	49.0
	4.25	5.0	38.8
	5.30	5.0	30.5
	6.90	5.0	19.1
	5.25	6.0	39.0
	6.10	6.0	33.2
	6.90	6.0	27.6
	7.75	6.0	24.8
	8.65	6.0	20.2
	4.55	7.0	25.0
	5.25	7.0	23.5
	6.85	7.0	22.2
	8.60	7.0	21.6
	1.18	8.0	36.0
	2.45	8.0	26.5
	3.90	8.0	21.5
	5.11	8.0	18.5
Urea	4.5 M	5.0	43.3
	5.0 M	5.0	37.75
	5.5 M	5.0	32.35
	5.0 M	6.0	41.0
	6.0 M	6.0	34.0
	7.0 M	6.0	29.0
	4.0 M	7.0	37.75
	5.0 M	7.0	33.90
	6.0 M	7.0	28.75

trapolation of these plots to zero denaturant yielded the same values in the case of all three denaturants at any given pH. In Figure 3 the influence of the concentration of 1-propanol on $t_{1/2}$ values of cytochrome *c* is shown. Slightly lower values were obtained in acetate buffer at pH 7 than in phosphate buffer of the same pH and ionic strength. Similar results were obtained with methanol and urea. The denaturant concentration had only an insignificant effect on the $t_{1/2}$ values at pH 7 although it lowered the temperature required to obtain complete bleaching of the 695-nm absorbance band when compared to aqueous solution.

Plots of the logarithm of the equilibrium constant for the displacement of the methionine ligand of ferricytochrome *c*, as calculated from the thermal effects on the 695-nm band (see Experimental Procedure), against the inverse of the absolute temperature were biphasic for all the denaturants and at all the pH values studied. A representative set of these plots is shown in Figure 4 for various concentrations of methanol as de-

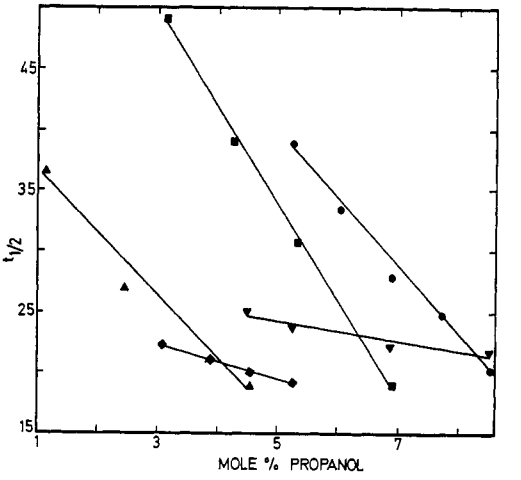

FIGURE 3: The influence of 1-propanol concentration on the transition temperature ($t_{1/2}$) of ferricytochrome *c*, as monitored by the 695-nm absorbance: (◆) pH 7, acetate buffer, μ = 0.1; (▼) pH 7, phosphate buffer, μ = 0.1; (▲) pH 8, phosphate buffer, μ = 0.1; (●) pH 6, acetate buffer, μ = 0.1; (■) pH 5 acetate buffer, μ = 0.1; cytochrome *c* concentration 30 μM.

naturant at pH 5. In all cases the slopes at differing denaturant concentrations were virtually constant at the higher temperature range and were variable for the lower temperature range. The standard enthalpy changes for the displacement of the Met-80 ligand and thus for the opening of the heme crevice of ferricytochrome *c* were calculated from the slopes of these plots and are given in Tables II and III. In all cases the plots of the standard enthalpy changes (calculated from the data in the lower temperature range) against denaturant concentration were curvilinear and yielded approximately the same values for the three denaturants at any given pH when extrapolated back to zero denaturant concentration. The mean values obtained from these extrapolations for the three denaturants are plotted in Figure 5, and exhibit a minimum value at between pH 6 and 7 which correlates with the results shown in Figure 3 where the $t_{1/2}$ values are lowest at about pH 7. These values are only approximate because of the difficulty of extrapolating the curved plots. The data at higher temperatures, on the other hand, yield enthalpy values which

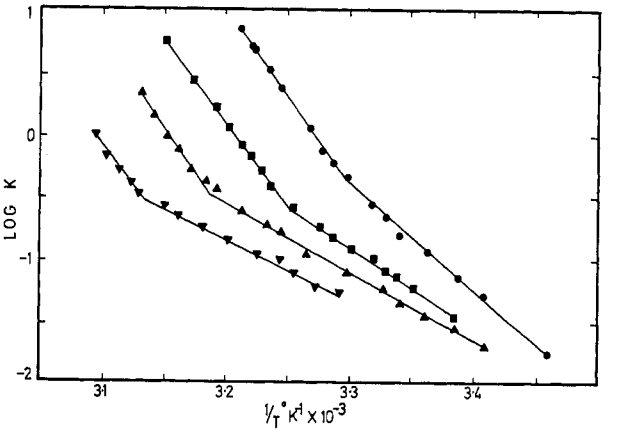

FIGURE 4: The effect of temperature on the equilibrium constants for the disruption of the heme crevice of ferricytochrome *c* in varying concentrations of methanol: (▼) 12.2 mol %; (▲) 15.1 mol %; (■) 18.2 mol %; (●) 21.6 mol %; cytochrome *c* concentration 30 μM, acetate buffer pH 5, μ = 0.1.

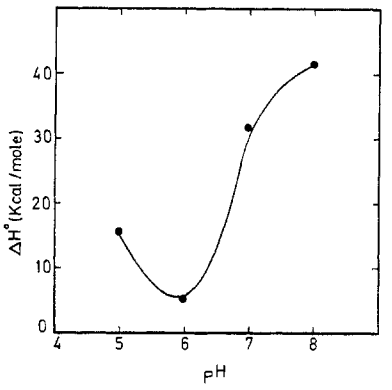

FIGURE 5: The standard enthalpy change for the disruption of the heme crevice of ferricytochrome *c* as a function of pH; acetate buffers pH 5 and 6, phosphate buffers pH 7 and 8, $\mu = 0.1$. Enthalpy values are calculated from equilibrium constants in the lower temperature range and are the mean values obtained by extrapolation of the enthalpy values in 1-propanol, methanol, and urea to zero denaturant concentrations.

exhibit a maximum value at about pH 6 (Table III), and which are apparently independent of denaturant concentration. Methanol and 1-propanol solutions of cytochrome *c* exhibit very similar enthalpy changes in this temperature region but the values in urea are at variance with the others.

Standard free-energy changes at 25° for the displacement of Met-80 in the various denaturants at pH 5 and 6 were calculated from the equation $\Delta G^\circ = -RT \ln K$, where the equilibrium constants at 25° were obtained from plots such as Figure 4 using only values of K lying on the slopes representing the lower temperature ranges. This precluded calculation of ΔG° at 25° and pH 7 and 8. These values (Table III) were used to calculate the standard entropy change from the equation $\Delta H^\circ = \Delta G^\circ - T\Delta S^\circ$. The entropy changes so calculated were also curvilinear with denaturant concentration at constant pH (Table IV) and extrapolation to zero denaturant concentration gave the same ΔS° value for methanolic and propanolic solutions. At pH 5 $\Delta S^\circ = 40$ eu and $\Delta G^\circ = 3.4$ kcal/mol, and at pH 6 $\Delta S^\circ = 4$ eu and $\Delta G^\circ = 3.8$ kcal/mol.

The effects of increasing ionic strength (addition of NaBr) on the thermal effects on the 695-nm absorbance of ferricytochrome *c* in 1-propanolic solution at pH 5 are shown in Figure 6. At higher ionic strengths the $t_{1/2}$ values are decreased and the absorbance difference between the open and closed crevice forms is diminished. The relationship between ionic strength and $t_{1/2}$ is linear and plots of the logarithm of the equilibrium constant for crevice opening against the inverse of the absolute temperature were biphasic over the range of ionic strengths tested. The standard enthalpy changes in the lower temperature range varied in a linear manner with ionic strength and inversely with $t_{1/2}$ values (Figure 7).

TABLE II: Standard Enthalpy Changes for the Opening of the Heme Crevice of Ferricytochrome *c*.[a]

Denaturant	Denaturant Concn (mol %)	pH	ΔH° (kcal/mol)
Methanol	12.2	5.0	22.4
	15.1	5.0	25.4
	18.2	5.0	30.5
	21.6	5.0	38.1
	15.0	6.0	22.9
	18.2	6.0	35.0
	21.6	6.0	49.6
	9.0	7.0	65.4
	15.1	7.0	82.4
	21.6	7.0	163.4
1-Propanol	3.15	5.0	22.9
	3.8	5.0	25.9
	4.25	5.0	41.2
	5.3	5.0	83.9
	6.9	5.0	205.9
	5.25	6.0	27.9
	6.1	6.0	37.4
	6.9	6.0	47.6
	7.75	6.0	60.8
	8.65	6.0	118.9
	4.55	7.0	51.0
	5.25	7.0	56.0
	6.85	7.0	65.4
	1.18	8.0	45.7
	2.45	8.0	55.9
	3.9	8.0	98.8
	5.1	8.0	119.0
Urea	4.5 M	5.0	27.9
	5.0 M	5.0	54.0
	5.5 M	5.0	54.9
	6.0 M	5.0	60.1
	5.0 M	6.0	37.1
	6.0 M	6.0	63.1
	7.0 M	6.0	91.2
	4.0 M	7.0	65.2
	5.0 M	7.0	91.5
	6.0 M	7.0	183.0

[a] Enthalpy values are calculated from equilibrium constants for the lower temperature range.

Discussion

The molecular origins of the 695-nm absorbance band of ferricytochrome *c* and the implications of absorbance changes of this band have been a frequent source of speculation and controversy (Schejter and George, 1964; Eaton and Hochstrasser, 1967). There is a large body of evidence, however, which indicates that the band is associated with the heme iron–Met-80 bond in native ferricytochrome *c* (Sreenathan and Taylor, 1971; Schechter and Saludjian, 1967) and that its presence is a measure of the integrity of this bond. The quenching of the 695-nm absorbance by denaturants has been reported to indicate a purely conformational origin for the band (Schejter and George, 1964) but it is now apparent that the overall conformation of the protein only determines the 695-nm absorbance insofar as it stabilizes the iron–methionine bond (Sreenathan and Taylor, 1971). On the other hand cleavage or weakening of the iron–methionine bond must produce conformational changes of the protein, in particular an opening of the heme crevice, in view of the role of this bond in maintaining the closed crevice structure (Dickerson *et al.*, 1971). Consequently we assume that the absorbance at 695 nm can be used as an indirect measure of the integrity of the closed crevice conformation of ferricytochrome *c*.

The influence of increasing concentration and increasing carbon chain length of alcohols in decreasing the transition

TABLE III: Mean Standard Enthalpy Changes for the Opening of the Heme Crevice of Ferricytochrome *c*.[a]

Denaturant	pH	$\Delta H°$ (kcal/mol)
Methanol	5.0	62.7
	6.0	71.6
	7.0	34.3
1-Propanol	5.0	63.9
	6.0	73.1
	7.0	39.8
	8.0	28.7
Urea	5.0	45.7
	6.0	52.2
	7.0	32.6

[a] Enthalpy values are calculated from equilibrium constants for the higher temperature range.

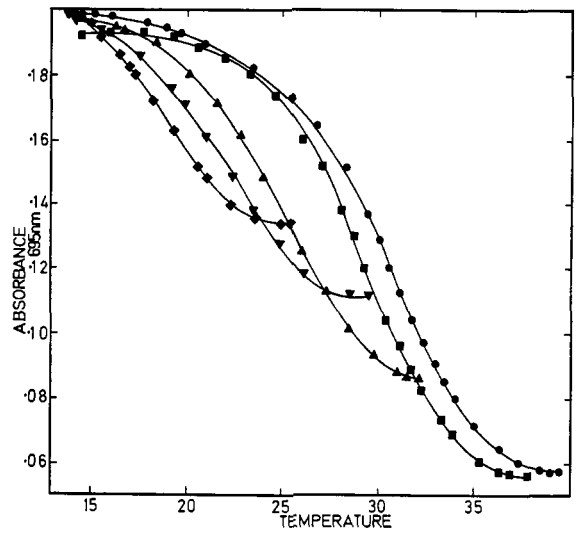

FIGURE 6: The 695-nm absorbance of ferricytochrome *c* in 5.3 mol % 1-propanolic solutions of varying ionic strength as a function of temperature. Ionic strength was varied by the addition of NaBr: (●) μ = 0.15; (■) μ = 0.20; (▲) μ = 0.22; (▼) μ = 0.29; (◆) μ = 0.34; cytochrome *c* concentration 30 μM, sodium acetate buffer pH 5.

temperatures ($t_{1/2}$) of ferricytochrome *c* as monitored by the changes in 695-nm absorbance (Table I) is further evidence for the very important role of hydrophobic interactions in maintaining the native conformation of cytochrome *c* in the region of the heme group.

To our knowledge the only previous attempt to extract thermodynamic data from a study of the effects of temperature on the 695-nm band of ferricytochrome *c* was that of Schejter and George (1964). These authors were compelled to estimate the absorbance values at high temperature since the protein precipitated at temperatures above 60° prior to the complete quenching of the 695-nm peak, which precluded their determination of a transition temperature. It is also evident that although these authors suggest a linear relationship between the logarithm of the equilibrium constant for the displacement of the methionine ligand and the inverse of absolute temperature, a biphasic plot would more accurately fit their data, and bring it into line with our results. The biphasic nature of such plots of all our data indicates that the thermally induced disruption of the Met-80–iron bond proceeds *via* different mechanisms at high and low temperatures.

The marked dependence of the thermodynamic data, in the lower temperature ranges, on denaturant concentration probably reflects control of the heme iron ligands by the conformation of the protein. Conversely, the independence in the higher temperature range of the thermodynamic parameters from denaturant concentration indicates that some factor other than conformational stability of the polypeptide chain plays a predominant role in the displacement of the methionine ligand in this range. A possible explanation for the change in the slope of the plots at higher temperatures is the onset of a simultaneous displacement of the His-18 ligand and the Met-80 ligand. A displacement of the His-18 ligand would probably influence the strength of the methionine–iron bond, and thus modify the 695-nm absorbance change. The displacement of the histidine would also produce a greatly enhanced open crevice structure for ferricytochrome *c* (Babul

TABLE IV: Thermodynamic Constants for the Opening of the Heme Crevice of Ferricytochrome *c*.[a]

Denaturant	Denaturant Concn (mol %)	pH	$\Delta G°$ (kcal/mol)	$\Delta S°$ (eu)
Methanol	12.2	5.0	2.19	67.8
	15.1	5.0	1.90	78.9
	18.2	5.0	1.70	96.6
	21.6	5.0	1.17	123.9
	15.1	6.0	1.16	72.9
	18.2	6.0	0.48	115.8
	21.6	6.0	0.12	166.0
1-Propanol	3.15	5.0	1.74	71.0
	4.25	5.0	1.58	133.0
	5.30	5.0	1.06	278.0
	5.25	6.0	1.37	89.0
	6.10	6.0	0.97	122.2
	6.90	6.0	0.52	158.0

[a] Calculations are based on equilibrium constants in the lower temperature range.

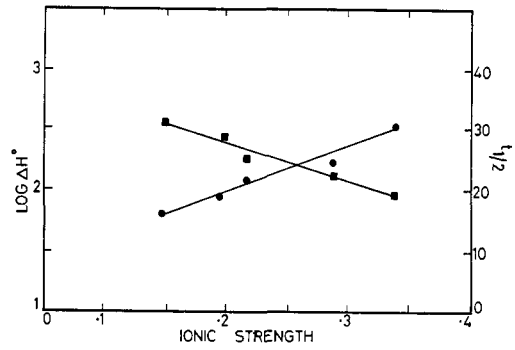

FIGURE 7: Effect of ionic strength on the transition temperature and standard enthalpy change for the opening of the heme crevice of ferricytochrome *c* in 5.3 mol % 1-propanolic solution: (■) $t_{1/2}$; (●) $\Delta H°$; cytochrome *c* concentration 30 μM, sodium acetate buffer pH 5. Enthalpy values were calculated from equilibrium constants in the lower temperature range.

and Stellwagen, 1972) which could possibly eliminate further conformation changes of the protein from being a factor in the displacement of the remaining methionine ligand from those protein molecules still retaining this ligand. An alternative explanation for the independence of ΔH° from denaturant concentration in the higher temperature range follows from the theoretical prediction of Nemethy and Sheraga (1962) that ΔH° for hydrophobic bond rupture is vanishingly small in the temperature range 45–65°. The contribution to ΔH° from conformational changes would thus be negligible in this temperature range and consequently denaturant concentration would not make a contribution to ΔH° for methionine displacement.

The pronounced influence of pH on the thermodynamics of the displacement of the methionine residue of ferricytochrome *c* in the upper temperature range, however, is consistant with this participation of the histidine ligand. Although pH changes also influence the data in the lower temperature range it is not possible in this temperature range to determine whether pH changes induce conformational changes which in turn lead to disruption of the methionine–iron bond or whether hydrogen ions directly influence the binding of ligand. In the higher temperature range, since conformational effects have been eliminated, the involvement of protons in the equilibrium reaction itself is indicated, which suggests the participation of the histidine ligand.

It is apparent from our results that there is a similarity in the conformational changes of ferricytochrome *c* induced by denaturants and by changes or pH, and that the two effects are cooperative. This is evident from the plots of transition temperatures ($t_{1/2}$) against denaturant concentration at various pH values (Figure 3). The intersection of the plots at different pH values implies that cytochrome *c* exhibits greater thermal stability at one pH value than another at a particular denaturant concentration and a reversed order of stability with respect to pH at another denaturant concentration.

The virtual independence of transition temperatures and denaturant concentration at pH 7 (Figure 3) is consistent with our earlier findings (Kaminsky *et al.*, 1972a) that ferricytochrome *c*, in a solution of denaturant of sufficient concentration to completely quench the 695-nm absorbance band, is in the low spin form at pH 7 and high spin at pH 5. This implies that at pH 7 the methionine ligand is displaced by a strong field ligand which requires a lesser contribution from protein conformational changes to displace the methionine; the change is thus relatively independent of denaturant concentration. At pH 5 the methionine is displaced by a weak field ligand (possible water); this change would require greater conformational changes of the protein and thus be much more dependent on denaturant concentration.

The influence of increased ionic strength in facilitating crevice opening implies the development of charge during crevice opening. The increase in the enthalpic resistance to denaturation concomitant with increasing ease of denaturation (Figure 7) implies an increasing entropic contribution to the denaturation energy at higher ionic strength. We can offer no explanation for the decreased change in extinction at 695 nm with increased ionic strength (Figure 6).

It it clear from our results that the opening of the heme crevice of ferricytochrome *c* is associated with a very unfavorable enthalpy change at all pH values studied and that this term becomes even more unfavorable as the thermal denaturation is facilitated by increasing concentrations of denaturants. This increasingly unfavorable enthalpy change, however, is overcome by an increasingly favorable entropy change of even greater magnitude: consequently the thermal denaturation is facilitated by the denaturants. It is interesting to compare these data with our previous results on the disruption of the His-18–heme iron bond of ferricytochrome *c*, under conditions of high concentrations of 1-propanol where the Met-80 ligand was already completely displaced (Kaminsky *et al.*, 1972a). The thermodynamic values obtained were $\Delta G^\circ = -6.07$ kcal/mol, $\Delta H^\circ = 4.75$ kcal/mol, and $\Delta S^\circ = 36.3$ eu, which can be compared to the present results of $\Delta G^\circ = 1.06$ kcal/mol, $\Delta H^\circ = 83.9$ kcal/mol, and $\Delta S^\circ = 278$ eu under identical conditions except that the concentrations of 1-propanol were much lower which permitted the methionine residue to remain liganded at lower temperatures. The more favorable free-energy change for the displacement of the histidine residue clearly is a consequence of the less unfavorable enthalpy term since the entropy term is much more favorable for the displacement of the methionine than for the histidine residue.

The values for the enthalpy changes for the disruption of the iron–methionine bond calculated from the data in the higher temperature range are similar when methanol and 1-propanol were used as denaturants but are different for urea solutions (Table III). This suggests that the mode of disruption of the heme crevice by urea, in the higher temperature range, is different from that induced by alcohols, since the recent work of Babul and Stellwagen (1971) excludes the other possibility that the urea can act as a ligand to the heme iron.

It has been proposed that the heme crevice of cytochrome *c* undergoes open–closed transitions when the protein performs its normal biological function in mitochondrial electron transport. Clearly the major energetic consideration in maintaining this elegantly poised bistable conformational ambiguity is the delicate balance between large opposing enthalpy and entropy impulses of approximately equal magnitude.

A more detailed knowledge of the energetic properties of cytochrome *c* in media approximating the mitochondrial environment is prerequisite to a full understanding of its function.

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Influence of Steroid Binding on the Tryptic Hydrolysis of Serum Albumin†

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ABSTRACT: The interactions of steroids, ethanol, and Ca^{2+} with serum albumin have been found to influence the rate of tryptic hydrolysis. This effect has been attributed to conformational change induced in the protein by the ligand. Testosterone, 3β-hydroxy-5-androsten-17-one, progesterone, and cortisol inhibit hydrolysis of *human* albumin in a manner suggestive of an involvement of a maximum of four sites in the case of the two androgens. However, inhibition only occurs above a minimum binding level of 0.4 mol of steroid/mol of protein. All neutral and charged derivatives of estrane and androstane inhibit hydrolysis of *bovine* albumin to a degree which varies with structure of the steroid, while all pregnane and cholestane derivatives stimulate hydrolysis. Unlike results published for dyes, steroid inhibition in the case of human albumin leads to only slight change in the relative amounts of the tryptic fragments observed by gel filtration on G-100 Sephadex, whereas Ca^{2+} produces a larger effect. The inhibitory effect of testosterone increases markedly during the course of the hydrolysis. Measurements of bound steroid have been made for the interaction of testosterone and 3β-hydroxy-5-androsten-17-one with human serum albumin over the entire binding range ($\rightarrow \bar{\nu} = 5.25$ for testosterone and 6.6 for 3β-hydroxy-5-androsten-17-one). Results of these indicate simple binding up to $\bar{\nu} = 2$–3 and departure from this in the higher binding range in a manner suggestive of cooperativity rather than heterogeneity.

The elucidation of the structure of hemoglobin and the recognition of the precise and sophisticated molecular engineering involved at every level of structure and function in the interaction of this molecule with small ligand molecules has focused attention on the possible general significance of this phenomenon in biochemistry. This is particularly true in the steroid field where several groups have discovered the existence of specific binding proteins, not only in plasma (Westphal, 1971a) but also in the target organs (Williams-Ashman and Reddi, 1971), thus providing hope for the elucidation of the mechanism of action in these tissues. The interaction of ligands and proteins has an intrinsic physicochemical interest, even where the physiological relevance may seem remote, and has been studied for many years (Steinhardt and Reynolds, 1969a). While the high-affinity (formation constants of 10^{8}–10^{14}) specific functional proteins of tissues and plasma must have properties not shared by other proteins which bind the same ligand, it seems likely that a full understanding of low-affinity interactions (10^{3}–10^{5}) of steroids and proteins such as serum albumins will prove relevant to ultimate understanding in the case of the high-affinity proteins. In at least some instances the steroid binding sites of albumin (Slaunwhite *et al.*, 1963; Plager, 1965) are heterogeneous in nature. We have been attempting to apply different approaches (Ryan, 1968; Ryan and Gibbs, 1970a,b) to the study of this problem in seeking to clarify its nature further. It is well known that interactions of some ligands with proteins tend to produce conformational changes which "stabilize" the protein (Markus *et al.*, 1967a; Steinhardt and Reynolds, 1969c). One of the most sensitive indicators of such conformational change is the altered susceptibility to enzyme-catalyzed hydrolysis. Thus, Markus and coworkers have been able to demonstrate such an effect in the combination of aspartyl transcarbamylase and ribonuclease with their substrates as well as of Methyl Orange and other dyes with human serum albumin. Such binding-induced conformational changes [or "ligand-stabilized" conformations (Markus *et al.*, 1967a,b)] have also been demonstrated by other means, as in the evidence of "cooperativity," or unmasking, in the binding of some ligands to serum albumins (Steinhardt and Reynolds, 1969d). In the work presented here we have attempted to apply this experimental approach to the steroid–albumin problem and to correlate our findings on the effects of steroids on the tryptic hydrolysis of bovine and human serum albumina with steroid structure and level of bound steroid. One report (Bellamy and Leonard, 1966) has appeared in the literature in which a less developed form of this approach has been used in studying the effect of steroids on bovine albumin. We have also had reason to study the effects of non-steroid ligands, Ca^{2+} and ethanol, on tryptic proteolysis. The former has been shown many years ago (Gorini and Audrain, 1952) to alter the susceptibility of albumin to hydrolysis and we have demonstrated its influence on steroid binding to human albumin (Ryan and Gibbs, 1970b). Its effect on trypsin activity is also well known (Sipos and Merkel, 1971). The low solubility of steroids in water has required that they be added to the protein solutions in small volumes of concentrated (10^{-1}–10^{-3} M) ethanol solutions, thus yielding much higher binding levels than is usually the case. It has been shown that alcohols become hydrogen bonded to the backbone of synthetic peptides (Strassmair *et al.*, 1969), so an effect of ethanol on tryptic hydrolysis of albumin might be anticipated. It has also been shown (Milar

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