An Acid Induced Conformational Transition of Denatured Cytochrome *c* in Urea and Guanidine Hydrochloride Solutions†

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ABSTRACT: Previous work has shown that at neutral pH ferricytochrome *c* (horse heart) retains certain residual structures in concentrated solutions of urea or guanidine hydrochloride (Tsong, T. Y. (1974), *J. Biol. Chem. 249*, 1988). Present studies reveal that cooperative unfolding of these residual structures can be achieved by acidification of the protein to pH 4 in 9 *M* urea but can only be partially achieved in a 6 *M* guanidine hydrochloride solution. The evidence that the residual structures unfold in 9 *M* urea upon acidification is twofold. (1) Further uncoupling of the Trp-59–heme interaction occurs; this is reflected in the intensification of the tryptophan fluorescence from 55 to 90% relative to that of free tryptophan in the same solvent. (2) The intrinsic viscosity of the protein solution increases from 15.0 to 21 ml/g. The acidification also induces a spin-state transformation of the heme group at pH 5 both in urea and in guanidine hydrochloride. Acidic titration of the protein in urea and guanidine hydrochloride indicates that the unfolding involves the absorption of a single proton. However, the kinetics of the spin-state transformation are triphasic. These results suggest that the displacement of the ligand His-18 by a solvent molecule and the subsequent disintegration of the residual structures are complex processes and involve at least three kinetic steps. The ineffectiveness of guanidine hydrochloride as a denaturant for ferricytochrome *c* is shown to be due to the presence of the high concentration of Cl^- which can stabilize certain elements of the protein structure.

The heme iron of native ferricytochrome *c* is coordinated with an imidazole nitrogen of histidyl-18 and the sulfur of methionyl-80 (Dickerson et al., 1971). The absorption spectrum shows that the heme group is in a low-spin coordination state. The heme Soret band exhibits a maximum at 408 nm and no absorption maximum is visible at 620 nm (Margoliash and Schejter, 1966). Since no disulfide bridge is present in cytochrome *c* it had originally been suggested that the molecule unfolds in concentrated urea or Gdn·HCl[1] solutions at neutral pH and the molecule would assume a truly random coil structure (Stellwagen, 1968; Ikai et al., 1973). However, Babul and Stellwagen (1971) have recently reported that at neutral pH the heme prosthetic group in ferricytochrome *c* is still occupied by strong field ligands of the protein molecule even in the presence of 9 *M* urea. These authors have demonstrated that only by acidification of the protein solution can one replace the strong field ligands of the molecule with weak field ligands of the solvent. Under these conditions the protein exhibits an absorption spectrum characteristic of a high-spin complex (Babul and Stellwagen, 1971; Nanzyo and Sano, 1968): a Soret maximum at 395 nm and a new absorption band at 620 nm. The strong field ligands that are persistently coordinated to the heme moiety of the protein in 9 *M* urea solution have been tentatively identified as the histidyl groups of the protein molecule.

As part of the investigation of the kinetics of unfolding and refolding of protein peptide chains we have reported a study of the thermal unfolding of horse heart ferricytochrome *c* at acidic pH, in the absence of denaturants (Tsong, 1973). We observed that three kinetic phases could easily be resolved: the fastest phase has a mean relaxation time (Schwarz, 1965) of about 20 μsec; a second phase in the decisecond time range; and the slowest phase in the decasecond time range. Although the two slower phases fall into time ranges previously observed in studies of the thermal unfolding of ribonuclease A (Tsong et al., 1971, 1972) and chymotrypsinogen A (Tsong and Baldwin, 1972) and of the guanidine-induced unfolding of ferricytochrome *c* at neutral pH (Ikai et al., 1973), no significant microsecond reactions had been reported in these cases. Moreover, the magnitude of the heme absorbance change observed in the thermal unfolding of ferricytochrome *c* at acidic pH (Tsong, 1973) was substantially larger than that observed in the guanidine-induced unfolding process.

In an attempt to clarify these differences in observations we have been investigating the Trp-59 fluorescence of ferricytochrome *c* under various denaturing conditions. It was found that the tryptophan fluorescence of cytochrome *c* after the major cooperative unfolding induced by Gdn·HCl represents less than 50% of the intensity level of free tryptophan in the same solvent conditions (Tsong, 1974). Moreover, it was found that the Trp-59 fluorescence continues to approach that of free tryptophan as the Gdn·HCl concentration is further increased. These observations indicate that certain residual structures of the protein molecule exist in the Gdn·HCl unfolded cytochrome *c*. However, it has been suggested that local charge effects such as the spin-state transformation of the heme group may have a strong influence on the fluorescence intensity of Gdn·HCl unfolded cytochrome *c*.

In light of the findings by Babul and Stellwagen (1971), it is logical and important for us to investigate the effects of

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[1] Abbreviation used is: Gdn·HCl, guanidine hydrochloride.

the spin-state transformation of the heme group and the intramolecular ligand binding of ferricytochrome *c* upon the overall protein conformation, and also to carefully examine its implication to the kinetics of the in vitro protein folding.

Experimental Procedure

Materials. Type IV horse heart ferricytochrome *c* was purchased from Sigma Chemical Co. The protein was quantitatively oxidized with ferricyanide and then exhaustively dialyzed against glass-distilled water before use. The protein concentration was estimated spectrophotometrically by using $\epsilon_{410nm} = 1.06 \times 10^5$, at pH 7 (Babul and Stellwagen, 1972). Ultrapure Gdn·HCl and urea were obtained from Mann. The Gdn·HCl and urea solutions were freshly prepared before use; their concentrations were determined by measuring the refractive index. Tryptophan was purchased from Sigma. $\epsilon_{279.8nm} = 5.60 \times 10^3$ was used for the concentration determination. All other reagents are of analytical grade.

Fluorescence measurements were done in an Aminco-Bowman spectrophotofluorometer. A 3-mm slit-width setting was used throughout the experiment. Under these conditions the fluorescence intensity of Gdn·HCl unfolded ferricytochrome *c* is linearly proportional to the protein concentration up to 2 μM. Experiments were confined to this range of concentration. Aged urea, Gdn·HCl solutions, and distilled water stored in a vinyl chloride bottle all exhibit strong background fluorescence for unknown reasons. Caution was taken and correction was made for the background fluorescence for every measurement. The fluorescence intensities reported here are relative to tryptophan fluorescence in the same solvent conditions. To avoid interference due to Rayleigh light scattering in the fluorescence measurements all the solutions were passed through a Millipore membrane of 1.2-μm pore size before use.

Kinetic Measurements. The stopped-flow pH jump experiments were carried out in a Durrum D-110 stopped-flow spectrophotometer. In the fluorescence kinetic measurements the excitation wavelength was set at 280 nm and an optical filter No. 0-54 supplied by the manufacturer was placed before the photodetector to eliminate wavelengths below 300 nm. The kinetics were recorded in a Tektronix 5103N storage oscilloscope.

Viscosity measurements. Kinematic reduced viscosity and intrinsic viscosity measurements were done either with a Cannon-Manning Semi-Micro viscometer or with a Cannon-Ubbelohde Semi-Micro viscometer. The viscometer constant is 0.006544 cSt/sec for the Cannon-Manning and is 0.004315 cSt/sec for the Cannon-Ubbelohde. The viscometers were kept in a temperature regulated water bath. The temperature regulation is good to ±0.05°. The timing was done manually with a Precision Scientific timer which can be read to 0.1 sec. The intrinsic kinematic viscosity $[\eta]$, is obtained by extrapolation according to the equation $[\eta] = \lim_{c \to 0}[(t - t^0)/t^0 c]$ in which t^0 and t denote respectively the times required for the solvent and the protein solution to pass through the viscometer. The concentration term, c, is expressed in gram per milliliter.

Results

Urea and Gdn·HCl Unfolded Cytochrome c. The effect of urea and Gdn·HCl on the structural properties of proteins has interested protein chemists for many years. Schellman (1955) has suggested that the competition for hydrogen bond formation of the urea molecule with the peptide amide groups in proteins greatly reduces the stability of the proteins. Currently the effect has been interpreted as due to the disruption of the hydrophobic interactions in the protein structures (Tanford, 1968, 1970). Although the urea and Gdn·HCl effect has not yet been fully understood the general observation has been that, in the presence of a high concentration of these two reagents, protein molecules assume a fully random coil state. In the case of cytochrome *c* it has been suggested that the protein unfolds in 9 *M* urea or 6 *M* Gdn·HCl and all portions of the molecule appear to be in contact with the solvent (Stellwagen, 1968; Ikai et al., 1973; Fisher et al., 1974). The absorption spectrum of cytochrome *c* in these solutions indicates, however, that the heme group is still in a low-spin coordination state, with a Soret maximum at 407 nm and with no absorption maximum at 620 nm (Table I). This is shown in curve c of Figure 1 (only spectrum in 9 *M* urea is given) to be compared with the absorption spectrum of the protein at neutral pH, in the absence of denaturants, in curve a. It appears that the heme moiety of the Gdn·HCl or urea unfolded cytochrome *c* is still coordinated to strong field ligands of the protein molecule.

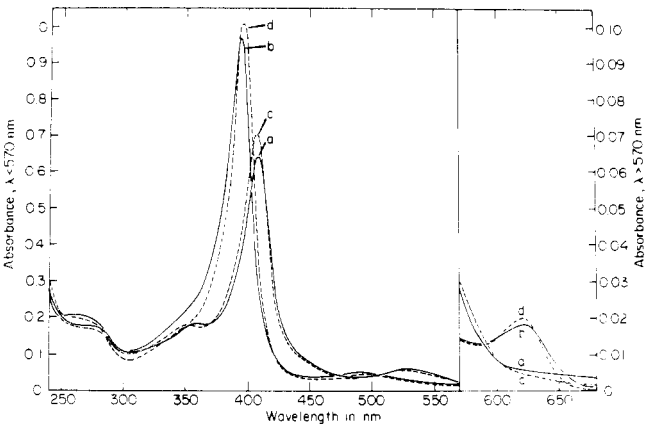

FIGURE 1: Absorption spectra of ferricytochrome *c* in various solvent conditions: (a) in aqueous solution, pH 7.0, 25°; (b) in aqueous solution, pH 2.0, 60°; (c) in 9 *M* urea, pH 7.0, 25°; (d) in 9 *M* urea, pH 2.0, 25°. The Soret maxima are 408, 394, 407, and 395 nm, respectively, for curves a, b, c, and d. Spectra a and c are characteristic of low spin heme complex, and b and d are characteristic of high spin complex, see text. Protein concentration, 6.0 μM in all cases.

In addition to the retaining of the heme coordinations under these denaturing conditions in an earlier communication (Tsong, 1974) we suggested that the Trp-59 fluorescence of cytochrome *c* is still half-quenched by the heme moiety in 4 *M* Gdn·HCl solution after a major conformational transition is over. The same phenomenon has also been observed for cytochrome *c* in 9 *M* urea. This is shown in Table I and Figure 2. In Figure 2 the continuous increase of the tryptophan fluorescence at high concentrations of Gdn·HCl is taken as the indication of the noncooperative disintegration of the residual structures of the protein molecule. The transition curves shown in Figure 2 agree with those obtained by the heme absorption changes. They also indicate that Gdn·HCl is a much effective denaturant for the first conformational transition. However, evidence will be given that Gdn·HCl inhibits the second gross conformational change of the protein, which is associated with the spin-state transformation of the heme group.

An Acid Induced Spin-State Transformation of the Heme Group. Acidification to pH 2.0 of the protein in 9 *M*

Table I: Some Numerical Values for Horse Heart Ferricytochrome *c* in Different Solvent Conditions.

Conditions	Soret Maximum (nm)	ϵ_γ Band $\times 10^{-5}$	620-nm Band	695- and 655-nm Bands	Intrinsic Viscosity (ml/g)	Relative[a] Fluorescence (%)
50 m*M* Tris-HCl, pH 7.0, 25°	409	1.05		+	2.5	2.0
HCl to pH 2.0, 60°	394	1.58	+		*b*	*b*
9 *M* urea, pH 7.0, 25°	407	1.15			15.0	55
6 *M* Gdn·HCl, pH 7.0, 25°	408	1.16			14.5	56
Buffer plus 50 m*M* CN^-, pH 7.0, 25°	408	1.07	*b*		2.7	3.0
6 *M* Gdn·HCl, 50 m*M* CN^-, pH 7.0, 25°	407	1.18			15.3	64
9 *M* urea, pH 4.0, 25°	395	1.65	+		22.0	91
6 *M* Gdn·HCl, pH 4.0, 25°	398	1.40	+		17.0	65

[a] The concentration of cytochrome *c* for the fluorescence measurement was 2 μM in each case. 2 μM of tryptophan in the same solvent conditions as 100%. Excitation at 280 nm, fluorescence measured at 350 nm. [b] Data not available.

Table II: An Acid Induced Spin-State Transformation of Ferricytochrome *c*.

	p*K*	*n*	pK_{int}[a]	*wZ*[b]
9 *M* urea, 25°	5.0	0.88	6.0	1.21
6 *M* Gdn·HCl, 25°	5.1	1.02	6.0	1.09
No denaturant, 75°	3.3	0.95	6.0	3.17
No denaturant, 25°	2.1	1.00	6.0	4.55

[a] The intrinsic p*K* of histidine imidazole at normal solvent condition (Christen et al., 1969). [b] pK = pK_{int} − 0.868 *w*Z.

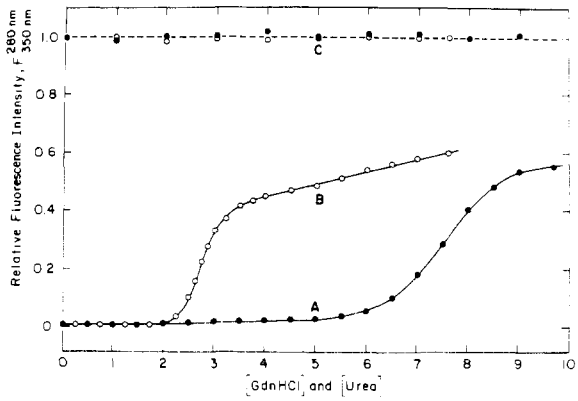

FIGURE 2: Equilibrium unfolding of ferricytochrome *c* in urea and Gdn·HCl solutions, as monitored by the intensification of Trp-59 fluorescence: curve A, unfolding by urea; curve B, unfolding by Gdn·HCl; curve C, equal concentration of tryptophan in urea in the filled circles and in Gdn·HCl in the open circles. Conditions: protein concentration was 2 μM in each case; 50 m*M* of phosphate buffer at pH 7.0; 25°. The fluorescence was measured at 350 nm, excitation wavelength was at 280 nm.

urea results in a shift of the Soret maximum to 396 nm and the appearance of a new absorption maximum at 620 nm (Babul and Stellwagen, 1971; curve d of Figure 1). These changes indicate that the low-spin form of the heme complex is now transformed into a high-spin state. The titration curves of the spin-state transformation under different solvent conditions are illustrated in Figure 3. According to the Henderson–Hasselbalch formulation

$$\log [\alpha/(1-\alpha)] = pK - n\text{pH}$$

the p*K* of the ionizing group(s) and number of protons (*n*) released upon alkalization of the solutions can be estimated

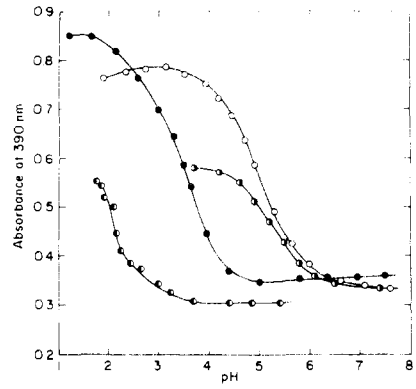

FIGURE 3: Proton titration curves of the spin-state transformation of ferricytochrome *c*: (○) titration in 9 *M* urea at 25°; (◑) in 6 *M* Gdn·HCl at 25°; (●) in aqueous solution at 75°; (◐) in aqueous solution at 25°. Protein concentration, 6.0 μM in all cases.

by plotting log $[\alpha/(1-\alpha)]$ vs. pH, in which α is the extent of protonation. The p*K* and *n* values so obtained are given in Table II. As can be seen although the p*K* values range from 5.1 to 2.1 under different solvent conditions *n* values stay close to unity. These observations suggest that the spin-state transformation of the heme group under these solvent conditions involves a single and perhaps the same ionization group.

The change in the p*K* values are due to different electrostatic interactions of the ionized group with its protein environment. The relationship between the p*K* and the intrinsic p*K* (pK_{int}) of the group can be approximated by the Linderstrøm–Lang equation

$$pK = pK_{int} - 0.868wZ$$

in which *Z* denotes the net charge on the protein and *w* is the Linderstrøm–electrostatic parameter.

The ligand which is responsible for the spin-state transformation has been identified as the imidazole group of His-18 (Babul and Stellwagen, 1971, 1972; Aviram and Krauss, 1974; Cohen et al., 1974). The pK_{int} of histidine under normal solvent conditions is 6.0 (Christensen et al., 1969). The fact that all the p*K* values reported here deviate considerably from the pK_{int} of histidine suggests that the electrostatic effects operate even in the urea and Gdn·HCl unfolded cytochrome *c*. However, precise values of *w* cannot be obtained, since no potentiometric titration data are

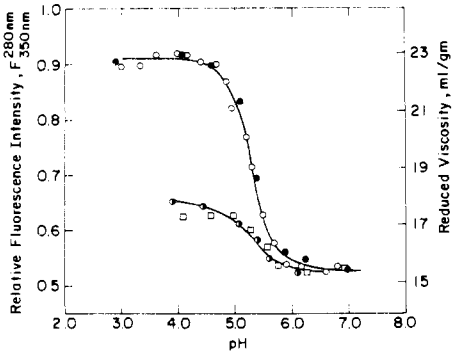

FIGURE 4: An acid-induced gross conformational transition of ferricytochrome *c* in urea and Gdn·HCl solutions. The transition is monitored by changes in the reduced viscosity of protein solutions in 9 *M* urea (●) and in 6 *M* Gdn·HCl (◑). The same transition is also detected by the Trp-59 fluorescence in the protein in 9 *M* urea (○) and in 6 *M* Gdn·HCl (□). Conditions: protein concentrations, 2 μM for the fluorescence measurements, 8 mg/ml for the reduced viscosity measurements; 25.0°.

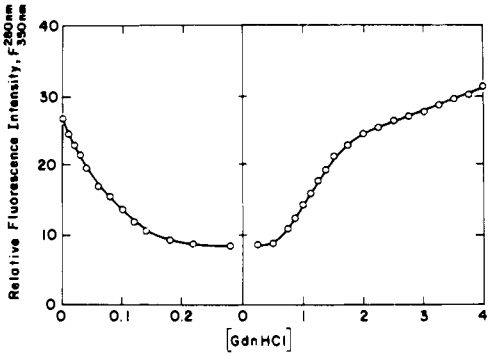

FIGURE 5: Effects of Gdn·HCl on the overall conformation of ferricytochrome *c*. At pH 1.1, 25°, low ionic strength the protein is partially unfolded. When Gdn·HCl is added the protein regains its structure up to 0.2 *M* and starts to unfold again at 0.5 *M*. This peculiar effect is interpreted in the text. Protein concentration, 2 μM. The fluorescence intensity is expressed in an arbitrary scale.

available for *Z* values (Table II). Our primary concern here is to investigate whether the spin-state transformation is accompanied by the gross protein conformational transition.

One interesting feature of the acid titration curves shown in Figure 3 is the different magnitudes of the absorbance changes that are associated with the spin-state transformation. Since the number of protons absorbed upon the acidification is the same in each case the different absorbance changes must also reflect different extent of protein conformational changes. We will focus our attention only on the urea and Gdn·HCl unfolded protein.

Further Unfolding of the Residual Structures. If the Gdn·HCl or urea unfolded protein is in a random coil state no further structural transition should parallel the spin-state transformation of the protein. This is in contrast to our observations shown in Figure 4. Not only is the Trp-59 fluorescence of the protein in 9 *M* urea shown to increase from 55% at neutral pH to 91% at pH 4, but the reduced viscosity of the protein solution also increases from 15.2 to 22.5 ml/g. This conformational transition exhibits a midpoint at pH 5.1 in accord with the midpoint of the spin-state transformation.

In the Gdn·HCl solution the situation is somewhat different. Although the protein residual structure does unfold further in the Gdn·HCl solution upon acidification changes in the tryptophan fluorescence and the intrinsic viscosity of the protein solution are much less pronounced as compared to the protein in urea solution. These observations suggest that whereas the complete unfolding of the protein may be accomplished in urea solution at an acidic pH the same extent of unfolding cannot be achieved in Gdn·HCl solution. This is rather surprising in view of the fact that Gdn·HCl is generally considered to be a more potent denaturant than urea.

The strange effect of Gdn·HCl on cytochrome *c* denaturation will be appreciated through an experiment shown in Figure 5. Cytochrome *c* unfolds at extreme pH in solution of very low ionic strength even at 25° (Babul and Stellwagen, 1972; Cohen et al., 1974). Partial refolding of the protein can occur by merely increasing anion concentration (unpublished results; see also Cohen et al., 1974). Indeed, Gdn·HCl can supply Cl^- necessary for the stabilization of certain structural elements in the cytochrome *c* molecule. In Figure 5 the Trp-59 fluorescence is monitored as the function of Gdn·HCl concentration at pH 1.1. At low Gdn·HCl concentrations the effect of Cl^- dominates and the protein regains its structure by increasing the denaturant concentration. On the other hand, at higher concentration the disruptive effect of $Gdn{\cdot}H^+$ dominates, and the reformed structure starts to unfold again. The protective effect of Cl^- on the cytochrome *c* structures makes Gdn·HCl a peculiar denaturant for studying the unfolding reaction of cytochrome *c*. This is dramatically demonstrated in Figure 5.

Comparison with Thermal Unfolding at Acidic pH. Ferricytochrome *c* undergoes reversible thermal unfolding at acidic pH's, in the absence of denaturant. At 25°, acidic titration shows a transition around pH 2.1 (Figure 3). The absorbance change measured at the Soret band is approximately one-half that of the change observed for the same titration in 9 *M* urea solution. However, if the titration is done at 75° (Figure 3) the change is comparable to that observed in a 9 *M* urea solution. The absorption spectrum at pH 2, 60° (Figure 1, curve b) shows that the heme group is in a high-spin state. The spectrum resembles curve d for the protein in 9 *M* urea solution at pH 2.0, 25°. These observations suggest that a conformation resembling that of the protein in 9 *M* urea (pH 2) can also be obtained at high temperature at acidic conditions. From these data and from other data on the reduced viscosity and the optical activity of the protein (Stellwagen, 1968; Babul and Stellwagen, 1972) we conclude that the thermal transition of ferricytochrome *c* at acidic conditions is a complete unfolding of the native globular conformation to a random coil configuration.

Met-80 Ligand to the Heme. Schechter and Saludjian (1967) have clearly demonstrated that the 695- and 655-nm absorption bands of cytochrome *c* come from the coordination of Met-80 to the heme group: the 695-nm absorption band, absent in dinitrogenous base iron porphyrin complexes of the hemopeptide (Schejter and George, 1964), is present in the *N*-acetyl-DL-methionine complex. Subsequently Stellwagen (1968) has shown that the carboxymethylation of the methionyl residue completely eliminates these two absorption bands. He has also shown that the Met-80 coordination to the heme is readily replaced either by CN^- or other nitrogenous base such as imidazole. Under such conditions the 695- and 655-nm absorption bands of cytochrome *c* are absent.

Table III: Stopped-Flow Kinetic Measurement of Proton-Induced Spin-State Transition of Ferricytochrome *c* in 9 *M* Urea Solution at pH 5.2 (Kinetics Followed at 390 nm).

Temp (°C)	τ_1 (sec)	α_1 (%)	τ_2 (msec)	α_2 (%)	τ_3 (msec)	α_3 (%)
		pH 10.5 → pH 5.2, Low Spin → High Spin[a]				
12.0	3.9	7.4	820	4.6	36	88
15.0	2.4	9.0	560	6.0	21	85
18.2	2.0	8.5	450	9.5	14.5	82
21.4	1.8	8.0	400	13	11.0	79
25.0	0.80	9.5	180	14	6.9	75
28.9	0.45	12	98	15	5.1	73
33.2	0.24	13	50	11	3.0	76
37.3	0.13	10	27	10	2.1	80
		pH 2.5 → pH 5.2, High Spin → Low Spin[a,b]				
15.0		small		small	28	>90
19.9	1.9	small		small	16.0	>90
22.7	1.5	small		small	12.1	>90
25.0	0.95	3		small	7.8	>90
27.5	0.75	4		small	6.4	>90
30.8	0.38	small		small	4.6	>90
36.0		small		small	2.7	>90

[a] The time course of the spin-state transformation can be expressed by the equation $(A - A_{eq})/(A_0 - A_{eq}) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) + \alpha_3 \exp(-t/\tau_3)$ in which A_0, A, A_{eq} represents respectively the initial absorbance, absorbance at time t, and the final value of absorbance. α_i denotes fraction of the reaction which occurs in each kinetic phase. In calculating α_3, 2-msec mixing time was corrected. Initial conditions: ferricytochrome *c* 11.2 μM in 9 *M* urea, unbuffered, at pH 10.5 and pH 2.5. Final conditions: 9 *M* urea, .05 *M* NaOAc, protein concentrations, 5.6 μM. [b] Owing to smaller amplitudes for the two slower reactions in these measurements, τ_2 is not resolved here.

In the earlier literatures the 695-nm absorption band has been widely used as a sensitive measure of the gross protein conformation (Schejter and George, 1964; Stellwagen, 1968; Kaminsky et al., 1973). This is so because any major change in the protein conformation has been found to be also accompanied by the disappearance of this absorption band. Screenathan and Taylor (1971), however, suggested that the 695-nm band reflects the state of the heme coordination, and much less of the overall protein conformation. They have shown that in the presence of CN^- the 695-nm band disappears but not more than 2% exposure of the tryptophan and tyrosine residues occurs as monitored by the difference spectrum of these chromophores. Our data (Table I) confirm the observation of Screenathan and Taylor. Although in the presence of 50 m*M* CN^- at pH 7.0 the 695- and 655-nm absorption bands are entirely missing, the Trp-59 fluorescence increases only from 2.5% that of free tryptophan fluorescence for the intact protein to 3% for the cyanogen protein. The intrinsic.viscosity only increases from 2.5 to 2.7 ml/g. It appears that the displacement of the Met-80 coordination to the heme by CN^- causes only a minor conformational change of the heme crevice but has very little effect on the overall protein conformation.

Kinetics of the Spin-State Transformation. Kinetics of the acid-induced spin-state transformation of ferricytochrome *c* in 9 *M* urea and 6 *M* Gdn·HCl solutions were measured in a Durrum D-110 stopped-flow apparatus by following the absorbance changes at the Soret band. After correction for the 2-msec mixing time, all the acid-induced absorption changes appear to be within the resolution capability of this instrument, indicating that there are no significant reactions faster than those reported here. At least three rate constants can be resolved in both directions: one in the millisecond time range (τ_3), one in the decisecond time range (τ_2), and a third in the second time range (τ_1). The results obtained at different temperatures are given in Table III.

In the Gdn·HCl solutions the kinetics appear to be dominated by the millisecond reaction (τ_3). Although the two slower reactions observed in the urea solution were also seen in the Gdn·HCl solutions their amplitudes were too small to be precisely measured. It is clear from these measurements that the reaction is complex and involves at least three kinetic steps or four molecular species in the transition process. It appears that the τ_3 reaction may be the protonation reaction and the τ_1 and τ_2 reactions detect the conformational transition accompanying the spin-state change of the heme group. However, detailed kinetic studies remain to be done.

Discussion

According to Tanford et al. (1966) the intrinsic viscosity, $[\eta]$, of a random protein chain obeys the empirical relation $[\eta] = 0.684n^{0.67}$ in which n is the number of amino acid residues in the protein. When $n = 104$ is substituted into the equation for cytochrome *c* a value of 15.4 ml/g is obtained. Although this value is close to the experimentally determined intrinsic viscosity for the cytochrome *c* in 6 *M* Gdn·HCl and 9 *M* urea solutions (Table I), the agreement could be coincidental. There are several reasons for believing that the empirical equation of Tanford et al. may not be strictly applicable to cytochrome *c*. First, the bulky heme group is still covalently attached to the protein chain and its contribution to the intrinsic viscosity of the protein is not yet clear. Secondly, in addition to the two covalent linkages between the heme group and the peptide chain, the heme group is still coordinated to a strong field ligand of the protein under these denaturation conditions. These intramolecular linkages may contribute to the abnormal behavior of the peptide chain in cytochrome *c*.

Whereas there are an abundance of cases where protein peptide chains do behave as random coils in Gdn·HCl and urea solutions, there are cases which show contrasting data (Tiffany and Krimm, 1973). Recent success in the crystallization of lysozyme in 9 *M* urea by Snape et al. (1974) strongly suggests that there must be a unique, thermody-

namically stable state for the protein in this denaturing solvent. It is inconceivable that molecules with an undefined random coil configuration should form stable crystalline structures. Our detection of stable structural elements for cytochrome *c* in 9 *M* urea and 6 *M* Gdn·HCl at neutral pH tends to support the notion that the protein possesses a unique conformational state in these solvents.

The effects of Cl^- on the cytochrome *c* conformation changes have been carefully examined by Boeri et al. (1953). More recently Cohen et al. (1974) have demonstrated that at pH 1.9, the unfolded cytochrome *c* starts to refold when the concentration of NaCl approaches 0.1 *N*. We have observed that the effect is due to general anion effects rather than to a specific interaction of Cl^- with the protein (unpublished results). Boeri et al. suggested that the effect of Cl^- ion is to form a salt bridge between the protonated His-18 ligand and the ferri ion of the heme. This salt bridge stabilizes part of the protein structure which is associated with the His-18–heme interaction. Our experiment tends to support their propositions.

Further evidence of residual structures for proteins in thermally unfolded state have been reported in several cases (Garel and Baldwin, 1973, 1975a,b; Sachs et al., 1972a,b; Aune et al., 1967). The effect of these residual structures on the kinetics of protein chain folding would be of particular interest, and is now under study.

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