Ferricytochrome *c* Chain Folding Measured by the Energy Transfer of Tryptophan 59 to the Heme Group†

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ABSTRACT: Recent kinetic studies of protein chain folding have relied largely upon the absorption changes of tyrosine, tryptophan, and/or other prosthetic groups associated with the chain folding. The absorptions usually reflect the polarity in the immediate vicinity of chromophores. They depend also on the state of ligand interaction of the chromophores. However, in certain cases, chromophoric absorptions have been found to be less sensitive to the configurational change of polypeptide chains. In cytochrome *c* (horse heart) the fluorescence of Trp-59 is 98% quenched by the heme group. The efficiency of this energy transfer depends on the orientation and the relative distance of the two groups, and is reduced to 40% upon the unfolding of the molecule by urea or guanidine hydrochloride at pH 7.0. It has been found that a close correlation exists between the intensity of Trp-59 fluorescence and the intrinsic viscosity of the protein. The time course of the fluorescence change in the chain folding of ferricytochrome *c* thus allows us to monitor directly the gross conformation of the molecule in the reaction pathway. By stopped-flow fluorescence measurements of the chain folding from guanidine hydrochloride and urea unfolded forms we have detected at least two relaxation times, one in the decasecond time range (τ_1) and the other in the decisecond time range (τ_2). In all conditions studied τ_2 constitutes the major part (60%) of the total fluorescence change, and τ_1 accounts for 15–20% of the reaction. The remaining 20% of the fluorescence change occurs within the mixing time of the apparatus, which is about 10 ms in this case. The missing reactions in the above experiment are resolved by the temperature jump of the protein in the urea and guanidine hydrochloride solutions. By following the heme absorption changes two relaxation times are found: one in the millisecond time range (τ_3) and the other in the submillisecond time range (τ_4). In guanidine hydrochloride solution the amplitude of τ_4 reaction is small. τ_2 shows a maximum at the midpoint of the transition and may represent a nucleation-dependent chain folding of the molecule. Since four reactions have been resolved, a three-step mechanism suggested by Ikai et al. (Ikai, A., Fish, W. W., and Tanford, C. (1973), *J. Mol. Biol. 73,* 165) for their analysis of the cytochrome *c* chain folding cannot be applied here. The results may be explained by an extended form of the nucleation-dependent simple sequential model (Tsong, T. Y. (1973), *Biochemistry 12,* 2209).

One of the important questions in the study of kinetics of protein chain folding is whether the starting material can exist in a truly random coil state. It has been suggested that by a purely statistical process an incredibly long period of time would be required for a protein molecule to acquire its unique three-dimensional structure (Levinthal, 1968; Wetlaufer, 1973). Yet, from most of the simple proteins studied the functional state of the molecules can be formed in the second or even in the millisecond time ranges (Schechter et al., 1970; Tsong et al., 1971; Ikai et al., 1973; Garel and Baldwin, 1973; Brandts et al., 1975). Different theories have been postulated to explain this phenomenon (Levinthal, 1968; Anfinsen and Scheraga, 1975; Tsong et al., 1972b; Karplus and Weaver, 1976; Wetlaufer, 1973). An experimental approach to this problem is to characterize peptide chain conformation under conditions where protein molecule is grossly unfolded (Tanford, 1968, 1970).

Several such studies have been reported. Sachs et al. (1972a,b) have employed antigen–antibody binding techniques to detect the formation of local native structures in the unfolded form of *Staphylococcus* nuclease. Deduced from their kinetic data, Baldwin et al. (Garel and Baldwin, 1975; Hagerman and Baldwin, 1976; Garel et al., 1976) and Brandts et al. (1975) have suggested that urea, Gdn·HCl,[1] and thermally unfolded ribonuclease do not behave like a homogeneous random coil; instead it is a mixture of at least two unfolded forms in a slow equilibrium.

In these kinetic studies the authors have employed a conventional approach to monitor the chain folding, namely, by the use of the absorption changes of tyrosine or tryptophan residues. These absorption changes reflect changes of the polarity in the immediate vicinity of the chromophores and may be less sensitive to the configurational change of peptide chain at a distance. Indeed, Summer and McPhie (1972) have earlier proposed that chromophoric changes may occur in the first step of protein unfolding and that the subsequent kinetic steps being invisible by optical measurement can only be inferred by their coupling with the first step. When such reactions occur, the kinetic pattern of the invisible portion of the entire chain-folding process would manifest in the slowest reaction.

Recently we have observed that in cytochrome *c* a much more sensitive way of monitoring the gross conformation of a molecule exists. This is to take advantage of the fact that in cytochrome *c* there is an energy transfer between the single tryptophan (Trp-59) and the heme group. The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between the two groups (Stryer, 1968), and hence it is also inversely proportional to the square of the effective volume of the molecule. Although, because of the continuing variation of heme absorptions in the chain-folding process, a precise value of the overlapping integral, J, in the energy-transfer equation (Stryer, 1968) is difficult to obtain, experimentally it has been found that a close correlation exists between the transfer efficiency and the effective volume of the

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

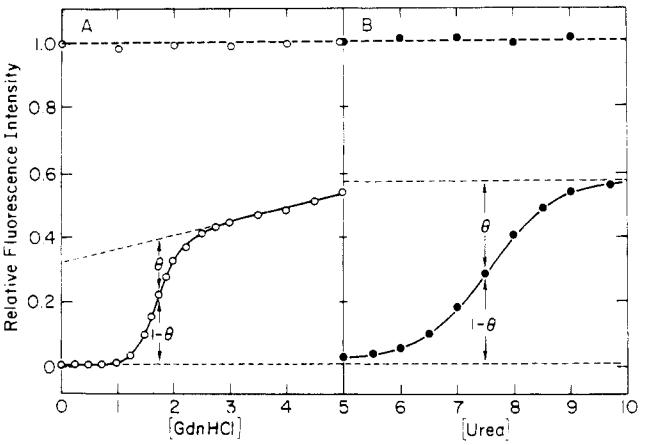

FIGURE 1: Equilibrium unfolding of ferricytochrome *c* at pH 7.0, 25.0 °C, measured by the Trp-59 fluorescence. Fluorescence intensities of ferricytochrome *c* in Gdn·HCl and urea solutions were measured relative to the intensities of a solution of tryptophan in the same solvents. Excitation wavelength was set at 280 nm and the emission at 340 nm was followed. The concentrations of the protein and tryptophan were the same, i.e., 2×10^{-6} M. The transition curve in Gdn·HCl solutions resembles that of the thermal unfolding curves of several simple proteins. Thus, the graphic method as outlined in Tsong et al. (1970) is adapted for the analysis of the transition curves. In the figure θ denotes the extent of the chain folding and $1 - \theta$ represents the fraction of the structures unfolded. The baselines for the native and the unfolded forms are indicated by the dashed lines. Because of the limited solubility of urea, determination of the post-transition baseline is arbitrary. In contrast, the posttransition baseline for the Gdn·HCl unfolding is easily determined. As shown in the upper dashed lines, the fluorescence intensity of tryptophan does not depend on the concentration of Gdn·HCl or urea solutions. This also indicates that the fluorescence of tryptophan is independent of the solvent viscosity. See text for the details. All solutions contain 0.1 M Tris buffer at pH 7.0.

molecule (Tsong, 1975). Tryptophan fluorescence measurement has enabled us to detect certain structural elements of the protein in concentrated solutions of Gdn·HCl and urea (Tsong, 1974, 1975). In this communication we report a kinetic study of the major chain-folding reaction of the protein in Gdn·HCl and urea. The result differs substantially from the earlier studies, using the heme absorptions, by Ikai et al. (1973) and Henkens and Turner (1973).

We have also extended our kinetic measurement to microsecond time range by the temperature-jump method (Eigen and de Maeyer, 1963). A preliminary kinetic study of the residual structures of cytochrome *c* has been reported (Tsong, 1976). Detailed studies of the effect of these structures on the overall kinetics of protein chain folding will be presented elsewhere (Tsong et al., 1976).

Experimental Procedure

Materials. Horse heart ferricytochrome *c* (type IV) was purchased from Sigma Chemical Co. In some experiments the protein was further purified by salt gradient chromatography through an Amberlite IRC 50 column, as described by Margoliash and Schejter (1966), before use. In other experiments no purification was done, except that the protein was quantitatively oxidized with ferricyanide and dialyzed against deionized-distilled water. Samples obtained by both methods gave identical results. The protein concentration was estimated spectrophotometrically by using ϵ_{410nm} 1.06×10^5 in aqueous solution at pH 7 (Babul and Stellwagen, 1972). Ultrapure Gdn·HCl and urea were supplied by Mann. The Gdn·HCl and urea solutions were freshly prepared before use; their concentrations were determined by measuring the refractive index. All other reagents are of analytical grade.

Kinetic Measurements. Stopped-flow experiments were performed with a Durrum D-110 spectrophotometer with a fluorescent attachment. Excitation wavelength was set at 280 nm and a 5-nm band width was used. An optical filter No. 0-54 supplied by the manufacturer was placed before the photodetector to eliminate wavelengths below 300 nm. The kinetics was recorded in a Tektronix 5103 N storage oscilloscope with a split-beam dual-time base which [illegible] recordin [illegible] both fast and slow reactions in the same exp [illegible]t. This [illegible]nts many precise measurements of the relat [illegible]plitude [illegible]e two reactions. Ratio mixing syringes [illegible] to 1:5 [illegible] used in Gdn·HCl and urea dilution experir [illegible]s. The r [illegible]g time of the machine was found to be sligh [illegible]igher, [illegible] ms, when concentrated solutions of urea or [illegible]n·HCl [illegible] involved. Since no reactions faster than 70 ms have been reported in our stopped-flow experiment (Table I), the higher mixing time observed here should have no significant effect on the analysis of our experimental result.

The temperature-jump experiments were done with an Eigen–de Maeyer temperature–jump kinetic system. When urea solutions were used, NaCl was added to 0.1 N to provide ionic strength. No addition of salt was necessary for Gdn·HCl solutions. In the experiment with partially or fully unfolded forms of cytochrome *c*, capacitors of different capacitances were used to ensure that the signals did not come from the electric dichroic effect (Dourlent et al., 1974).

Other Measurements. Equilibrium fluorescence measurements were done in an Aminco-Bowman spectrofluorometer as described elsewhere (Tsong, 1975). Kinematic reduced viscosity and intrinsic viscosity measurements were performed with a Wescan-Cannon-Manning semimicroviscometer assembly, using a Wescan Model 220 automatic timer. All other experimental details are given elsewhere (Tsong, 1975).

Result

Equilibrium Unfolding. Gdn·HCl-induced equilibrium transition curve of ferricytochrome *c*, monitored by Trp-59 fluorescence changes, resembles thermal unfolding curves of several simple proteins obtained by the microcalorimetric measurements (Privalov and Khechinashvili, 1974; Tsong et al., 1970; Jackson and Brandts, 1970). This is shown in Figure 1A. In the figure the relative fluorescence intensity of a tryptophan solution in the same solvent is given. As can be seen at 4 M Gdn·HCl where the major conformational transition is over the Trp-59 fluorescence is still half-quenched by the heme group. Continued increases in the Gdn·HCl concentration further uncouple the energy transfer between the two groups and, hence, increase the fluorescence intensity of the solution. This behavior has been discussed previously (Tsong, 1974, 1975) and will be given more consideration in the following paper. We shall now focus our attention only on the major conformational change which has been the subject of several recent studies (Ikai et al., 1973; Babul and Stellwagen, 1972; Henkens and Turner, 1973).

Since the kinetics of this conformational transition has been found to be complex, it must involve intermediate states in the unfolding. The apparent equilibrium constant of the transition may be defined by $K = (1 - \theta)/\theta$, where θ is the degree of folding. θ can be expressed as $\theta = (1/n)\Sigma_{i=0}^{n} i\bar{c}_i/\Sigma_{i=0}^{n} \bar{c}_i$, in which $\bar{c}_i$ denotes the equilibrium concentration of ith species and n denotes the number of species (Tsong et al., 1972a,b). As illustrated in the figure this analysis considers only the major transition (see, e.g., Tsong et al., 1970) and does not include reaction(s) which are represented by the slope of the posttransition curve. Fortunately all of the four relaxation

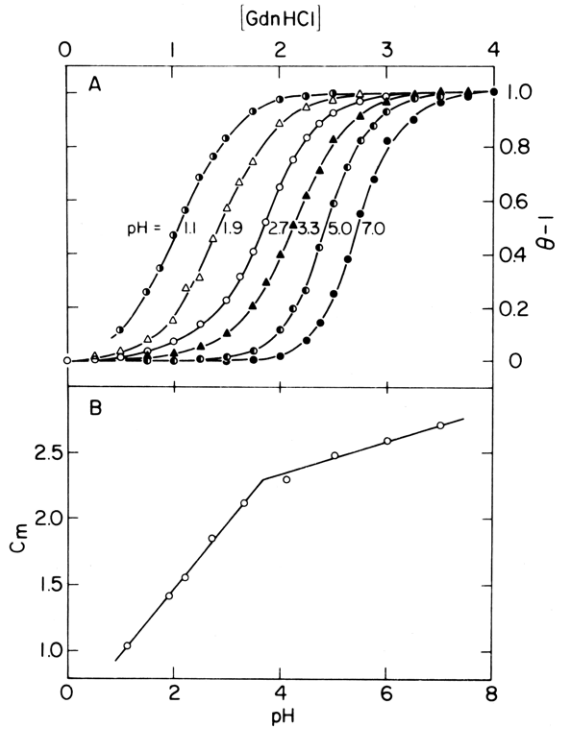

FIGURE 2: pH dependence of the Gdn·HCl induced unfolding of ferricytochrome c at 25.0 °C. The quantity $1 - \theta$ shown in A is plotted against Gdn·HCl concentration at different pH values in A. In B the midpoint of the transition, C_m, is plotted against pH. There is a clear break of the plot at pH 3.7. Experimental details are given in Figure 1, except that all solutions below pH 5.0 contain 0.05 M acetate and 0.05 M glycine.

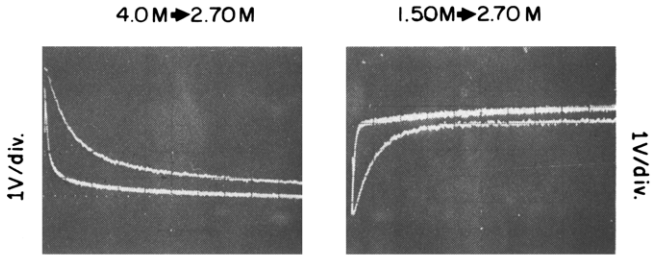

FIGURE 3: Stopped-flow kinetic records of ferricytochrome c chain folding in Gdn·HCl solution, at pH 7.0, 25.0 °C. The time course of the intensity changes of Trp-59 fluorescence associated with the chain folding and unfolding was followed to the same final conditions. The initial and final concentrations of Gdn·HCl are given in the figure. The oscillograph in the left records a chain-folding reaction and the oscillograph in the right records an unfolding reaction. The fast and the slow components of the reactions are shown in different time scales. The final protein concentration was 1.6×10^{-5} M.

times reported in this communication were detected only inside the major transition zone. Thus the slope of the posttransition curve must correspond to a very rapid reaction that is not within the resolving capabilities of the stopped-flow and the temperature-jump apparatuses.

Effect of pH. Changes in pH do not alter the shape of the transition curve. However, the midpoint of the transition (C_m) is reduced. A selection of transition curves monitored by the fluorescence change is given in Figure 2A. In Figure 2B the variation of C_m is plotted against pH. A clear break in the slope of the curve occurs at pH 3.8. As was reported in a previous study (Tsong, 1975), although Gdn·HCl is an effective reagent in promoting the major conformational transition described here, nevertheless, at acidic pH it is an inhibitor of a second conformational change. The acid-induced transition is accompanied by a spin-state transformation of the heme group, which is believed to involve dissociation of His-18 ligand (Tsong, 1975; Tsong et al., 1976). The protective effect of Cl^- on the His-18 ligand allows us to selectively study the major transition without the interference of the second transition.

Stopped-Flow Measurements of the Major Chain Folding. The unfolding and refolding of ferricytochrome c, in Gdn·HCl solutions, to the same final conditions at different θ values have been done by monitoring the tryptophan fluorescence changes associated with these reactions. The signal to noise ratio was very good. Two typical oscillographs, one for the refolding from 4 to 2.70 M and the other for the unfolding from 1.5 to 2.70 M are given in Figure 3. In both unfolding and refolding the signal resolved in the stopped-flow measurement is less than the equilibrium fluorescence intensity change of the reactions. Precise values of the rapid signal are difficult to obtain; however, it is estimated that about 15 to 20% of the total intensity change appears within the mixing time of the apparatus. These rapid signals will be shown to be resolvable by the temperature-jump method.

At least two time constants can be separated (to be designated as τ_1 and τ_2) both for the unfolding and refolding reactions (Figure 3). The standard method of resolving two exponentials (peeling back exponentials) was used to obtain the time constants and the amplitudes of the two kinetic phases. The result is given in Table I. In the table $\alpha_1 + \alpha_2 = 1$; i.e. only the reactions resolved by the stopped-flow apparatus are considered here.

In Figure 4 ln τ is plotted against θ, the extent of chain folding for τ_1 and τ_2. It is clear that the faster reaction τ_2 is a marked function of θ and exhibits a maximum at $\theta = 0.5$. On the other hand, τ_1 seems to depend more on the concentration of Gdn·HCl. At higher denaturant concentration τ_1 reaction becomes faster.

Experiments with urea solutions gave similar results. However, owing to a greater dilution required in the experiment (because of the broader transition curve as shown in Figure 1B), the thermal artefact was difficult to eliminate and the result has not been as satisfactory.

Temperature-Jump Measurements in Urea and Gdn·HCl Solutions. The missing reactions in the stopped-flow experiment were picked up by the rapid temperature-jump method (Eigen and de Maeyer, 1963). Because of the small signal available, the heme absorption at 395 nm instead of the tryptophan fluorescence was used to follow the rapid reactions. In Gdn·HCl solution the rapid kinetic phase is dominated by a relaxation time in a few millisecond time range (τ_3). A faster reaction in the submillisecond time range (τ_4) was also detected; however, the amplitude was too small to be precisely measured.

In urea solutions the situation is somewhat different. Here the rapid phase is dominated by the submillisecond reaction (τ_4), and the millisecond reaction (τ_3) has relatively small amplitude. Figure 5A gives oscilloscope records of τ_3 reaction in the Gdn·HCl, and τ_3 and τ_4 reactions in the urea solutions.

The signals due to these reactions show a maximum at the midpoint of the transitions and disappear outside of the transition zones. This indicates that the two reactions detected by the temperature-jump measurements are indeed part of the major chain-folding process. This is shown in Figure 5B.

A study of the temperature dependence of τ_3 and τ_4 in 8 M

TABLE I: Stopped-Flow Fluorescence Measurements of Ferricytochrome *c* Chain Folding in Gdn·HCl Solutions (at pH 7.0 and 25.0 °C).[a]

$[Gdn·HCl]_i$ (M)	$[Gdn·HCl]_f$ (M)	θ_i	θ_f	τ_1 (s)	τ_2 (ms)	α_1 (%)	α_2 (%)
			Refolding				
2.75	1.50	0.47	1.0	22	160	14	86
3.00	2.50	0.19	0.74	13	440	23	77
4.00	2.70	0	0.53	6.7	520	33	67
4.00	3.00	0	0.19	2.9	370	32	68
			Unfolding				
1.50	2.50	1.0	0.74	14	320	9	91
1.50	2.70	1.0	0.53	6.9	450	17	83
2.50	3.00	0.74	0.19	3.0	300	22	78
3.00	4.00	0.19	0	1.1	74	23	77

[a] The subscripts i and f denote, respectively, the initial and the final conditions. All solutions contain 0.1 M Tris buffer at pH 7.0. The final protein concentration was 1.6×10^{-5} M. The total fluorescence change can be described by $(I(\infty) - I(t))/(I(\infty) - I(0)) = \beta + \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}$ in which $I(0)$, $I(t)$, and $I(\infty)$ represent, respectively, the initial fluorescence intensity, the intensities at time t, and at infinitive time. β denotes signals too fast to be resolved by the stopped-flow apparatus. β constitutes 15–20% of the total signal. In this tabulation, however, the contribution of β is ignored. We use $\alpha_1 + \alpha_2 = 100\%$.

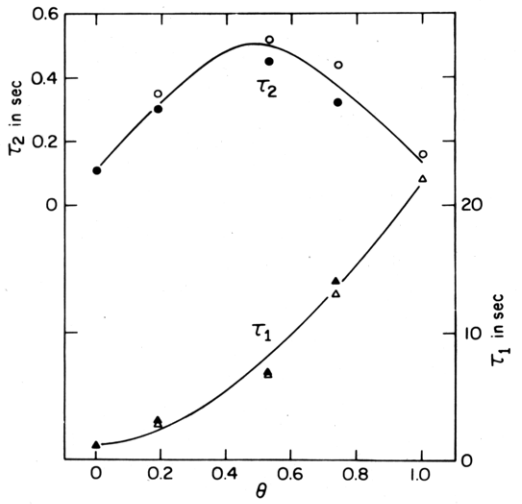

FIGURE 4: Dependence of the two reactions detected by the stopped-flow measurements on θ, the extent of the chain folding. The rapid (τ_2) and the slow (τ_1) components of the reactions shown in Figure 3 are plotted against θ, the extent of the chain folding. Filled circles and triangles: data from the unfolding experiment. Opened circles and triangles: data from the refolding experiment. A maximum of τ_2 is seen at $\theta = 0.5$.

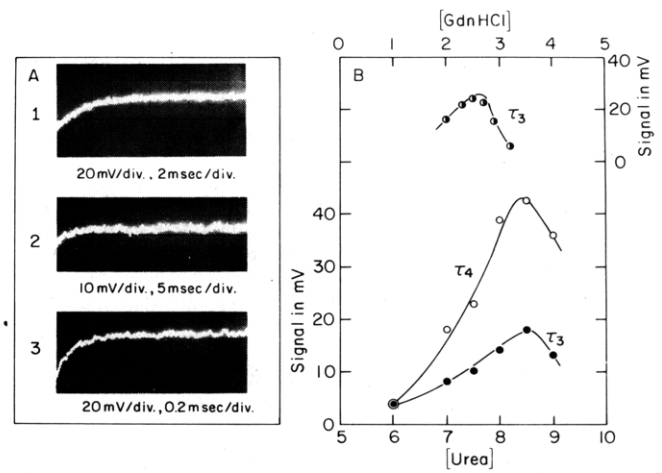

FIGURE 5: Temperature-jump relaxations of ferricytochrome *c* in Gdn·HCl and urea solutions. Temperature-jump measurements (from 22.5 to 25.0 °C) were done for ferricytochrome *c* in urea and Gdn·HCl solutions at pH 7.0. (A) Oscillograph 1 records the slower relaxation (τ_3) of the protein in 2.5 M Gdn·HCl; oscillographs 2 and 3 record, respectively, the slower relaxation (τ_3) and the faster relaxation (τ_4) in 8 M urea. (B) The signals of the three relaxations are plotted against the urea and Gdn·HCl concentrations. These curves indicate that the relaxations are part of the chain-folding process. In Gdn·HCl solutions, signals for the faster relaxation (τ_4) were small. All reactions were followed by the heme absorptions at 395 nm. Protein concentration was 3×10^{-5} M in each case.

urea indicates that the apparent activation energies of τ_3 and τ_4 reactions are small, only 5.6 kcal/mol for the former and 9.7 kcal/mol for the latter. The small activation energies could mean that these rapid reactions reflect small conformational changes in a local level. The results of the temperature-jump experiment are summarized in Table II.

Discussion

Effect of Gdn·HCl. Aune and Tanford (1969a,b) have reported that the denaturing effect of Gdn·HCl on lysozyme can be explained by the preferential "binding" of the denaturant to the unfolded form of protein. They have suggested that the free energy of the unfolding by Gdn·HCl $\Delta G = \Delta G_0 + \Delta G_1$, in which ΔG_0 is the free energy of protein conformational change, excluding the free energy of the preferential binding ΔG_1. And $\Delta G_1 = -RT \ln (1 + ka)^{\Delta n}$ in which k, a, and Δn represent, respectively, the binding constant of the protein, the activity of the denaturant, and the excess number of binding sites in the unfolded form. The same type of analysis has been successfully applied to the myoglobin system by Puett (1973).

We have employed the nonlinear regression analysis to calculate ΔG_0, Δn and k by fitting the equilibrium unfolding data of Gdn·HCl (part of which is given in Figure 2). The procedure is as follows: Given ΔG values from the experimental data, a Δn is assigned to calculate the best fit of k and ΔG_0. The values of k and ΔG_0 so collected are then plotted against Δn to see whether at certain Δn values k and ΔG_0 can reach minima. In the regression analysis a, the activity of the denaturant, was calculated by using eq 2 of Aune and Tanford (1969b).

The result was rather disappointing in that there are no clear minima for either k or ΔG_0 within the Δn values tested (Figure 6). Other calculations not shown here indicate that the values of Δn and k can be varied without significantly changing ΔG_0. This means that a unique solution of the binding equation cannot be found for the ferricytochrome *c* unfolding data. Two explanations may be given for this inadequacy of the binding

TABLE II: Temperature-Jump Relaxations of Ferricytochrome *c* Unfolding in Gdn·HCl and Urea Solutions at pH 7.0.[a]

Urea Solution				Gdn·HCl Solution		
[Urea] (M)	Temp (°C)	τ_3 (ms)	τ_4 (ms)	[Gdn·HCl] (M)	Temp (°C)	τ_3 (ms)
7.0	26.4	3.5	0.40	2.0	25.0	5.2
7.5	26.4	3.0	0.40	2.3	25.0	6.0
8.0	26.4	2.8	0.45	2.5	25.0	8.0
8.5	26.4	2.0	0.50	2.7	25.0	6.5
9.0	26.4	1.8	0.43	2.9	25.0	4.0
8.0	12.4	4.5	1.20	3.2	25.0	2.5
8.0	17.4	3.2	0.90			
8.0	20.4	3.0	0.80			
8.0	23.9	2.5	0.60			
8.0	29.9	2.5	0.45			
8.0	33.8	2.0	0.35			
8.0	37.9	1.8	0.30			

[a] In the urea solutions, 0.1 N NaCl was added. All solutions contain 0.05 M phosphate buffer at pH 7.0. Temperature-jump size was 2.5 °C. Protein concentration was 3×10^{-5} M. The apparent activation energies for τ_3 and τ_4 reactions in 8 M urea are, respectively, 5.6 and 9.7 kcal/mol. Owing to the very small signals available data for τ_4 reaction in the Gdn·HCl solutions are not given here.

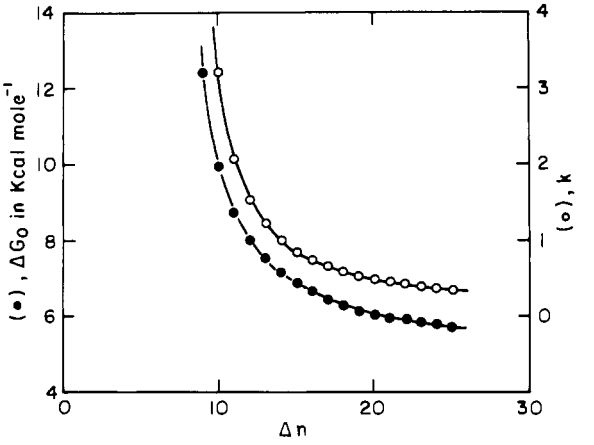

FIGURE 6: Regression analysis of the equilibrium unfolding of ferricytochrome *c* by Gdn·HCl, at pH 7.0. The nonlinear regression analysis was applied to the equilibrium unfolding data of Figure 2A by the preferential binding of Gdn·HCl (Aune and Tanford, 1969a,b). The curve in the opened circles indicates a change in the Gdn·HCl binding constant, k, as a function of Δn. The curve in the filled circles shows a change in the free energy of the protein unfolding as a function of Δn. As is discussed, in detail, in the text, if the mechanism of the preferential binding is applicable to the cytochrome *c* data, clear minima should appear in the plots. There are no minima for the two curves up to $\Delta n = 25$.

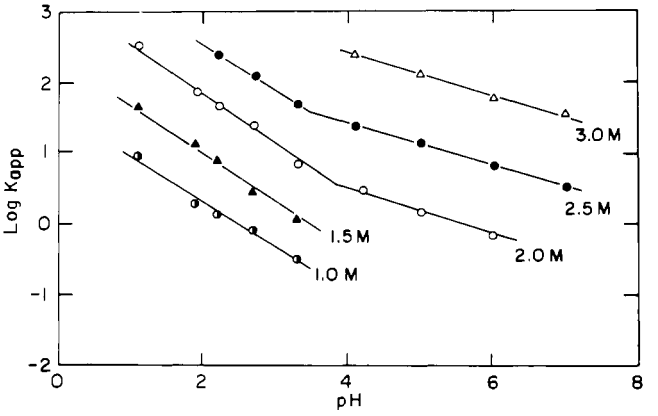

FIGURE 7: pH dependence of ferricytochrome *c* unfolding at different Gdn·HCl concentrations, at 25.0 °C. The variation of the apparent equilibrium constant of the protein unfolding ($K_{app} = (1 - \theta)/\theta$) is plotted against pH. The slope is equal to Δr, the excess number of protons absorbed upon the unfolding of the protein molecule. The plots give Δr of 0.6 to 0.7 below pH 4, and 0.3 to 0.4 above pH 4. See text for the details.

equation. First, the Gdn·HCl-induced conformational transition of cytochrome *c* is a complex process which cannot be described by an all-or-none transition that is implicitly assumed in the derivation of the binding equation (Aune and Tanford, 1969b). Second, if the guanidium ions do bind to the protein, the binding constant k for each binding site may be different. A general binding equation such as one given in eq 10 of Aune and Tanford (1969b) may have to be used. However, such equations contain a large number of undetermined parameters and cannot be meaningfully solved by the regression analysis.

Effect of pH. Changes in pH reduce the midpoint of the transition (Figure 2B). This indicates that acidic pH favors the unfolded form of the protein. For the analysis of pH-dependent transitions, many authors have used the relation (Wyman, 1964), $\partial(\log K)/\partial(\mathrm{pH}) = \Delta r$, where Δr is the excess number of proton absorbed upon the unfolding.

A plot of log K vs. pH is given in Figure 7. The slope below pH 4 appears to be unchanged by the difference in the concentration of Gdn·HCl and gives a value of 0.6 to 0.7. The slope above pH 4 is smaller, with a value of 0.3 to 0.4. A clear break of slope around pH 4 is also seen when C_m, the midpoint of the Gdn·HCl-induced unfolding, is plotted against pH (Figure 2B).

An accurate description of the titration data requires much more elaborate analysis of the titration curve (Aune and Tanford, 1969a,b). However, the quality of the experimental data does not permit information on the individual titration group to be extracted.

Comparison of Kinetic Data. Ikai et al. (1973) have reported a detailed stopped-flow kinetic study of the cytochrome *c* chain folding in Gdn·HCl by monitoring the heme absorption changes. Comparison of our result in the stopped-flow fluorescence measurements with their data indicates that, although the two kinetic constants in these studies fall in the similar time ranges, the amplitudes of the two reactions are quantitatively different. The most noticeable among these differences is that, in the data of Ikai et al., the amplitude of the fast kinetic phase is always greater for the chain folding than for the unfolding reaction. The opposite is found to be true in our result (Table I). The following explanations may be offered to account for this discrepancy. First, the two optical parameters chosen, namely, the heme absorption and tryptophan fluorescence (which intensity measures the energy transfer between Trp-59 and the heme group), report basically different physical properties of the molecule. Second, in the refolding experiment pH control is absolutely essential. It has been found that the Gdn·HCl unfolded cytochrome *c* exhibits a heme spin-state transformation at pH 5.2 which has a several times greater optical change than that associated with the conformational change of interest here (Tsong, 1975). A slight variation in the pH of the starting material may introduce large signals from the spin-state change in refolding reaction. Since the spin-state transformation is fast (Tsong, 1976), this would contribute to a larger amplitude for the fast reaction in the chain folding direction. The interference of the spin-state transformation was carefully avoided in our experiment by using adequately

buffered solutions. The importance of this difference in the two sets of data will become apparent in the following kinetic analysis.

Ikai and Tanford (1973) have formulated a set of criteria for a number of simple kinetic models. Application of these criteria to our stopped-flow data alone (τ_1 and τ_2; notice that the subscripts used here are different from their assignments) allows us to exclude the following equations:

$$D \rightleftharpoons I \rightleftharpoons N \quad (1)$$

$$X \rightleftharpoons D \rightleftharpoons N \quad (2)$$

$$X \rightleftharpoons D \rightleftharpoons I \rightleftharpoons N \quad (3)$$

In these equations, N, D, I, and X represent, respectively, the native state, normal unfolding intermediate, the denatured form, and the abortive, or incorrectly folded, intermediate.

Equation 3 has been found to be consistent with the result of Ikai et al. (1973); however, as noted above because of the difference in the amplitude of the fast kinetic phase in the stopped-flow measurements, the equation does not fit our data. In addition, we have detected two rapid reactions (τ_3 and τ_4) by the temperature-jump method. The simplest model consistent with our observation inevitably involves five species. Since the number of kinetic intermediates may exceed, and very likely does exceed, the number of the relaxation times resolved, we would like to discuss an extended simple sequential model adapted for the interpretation of the thermal unfolding study of ferricytochrome *c* at acidic pH (Tsong et al., 1972a,b).

An Extended Simple Sequential Model. The kinetics of the thermally induced and the Gdn·HCl- and urea-induced unfolding of ribonuclease A have been found to be very similar (Garel et al., 1976). Comparison of the data in Table I with that of the thermal unfolding of ferricytochrome *c* at pH 2 (Tsong, 1973) indicates that the two slower relaxation times (τ_1 and τ_2) fall in the same time ranges. These results suggest that, in the Gdn·HCl and urea unfolding experiments, the kinetics are not limited by the rate of binding of denaturants to the protein molecule. This is further substantiated by the fact that all the relaxation times reported here are unimolecular with respect to the protein concentration.

Because of the similarity in the protein chain folding under these solvent conditions, understanding of the kinetic data presented here via an extended form of a simple sequential model, which was proposed for the interpretation of the thermal unfolding data (Tsong, 1973), is possible. The main features of the model as given in eq 4

$$A_0 \underset{\rho_b}{\overset{\mu_f}{\rightleftharpoons}} A_1 \underset{\rho_b}{\overset{\rho_f}{\rightleftharpoons}} \cdots\cdots \underset{\rho_b}{\overset{\rho_f}{\rightleftharpoons}} A_m \underset{\sigma_b}{\overset{\sigma_f}{\rightleftharpoons}} B_1 \underset{k_b}{\overset{k_f}{\rightleftharpoons}} B_2 \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \cdots\cdots \underset{k_b}{\overset{k_f}{\rightleftharpoons}} B_n \quad (4)$$

are the following: That in the process of protein chain folding from its unfolded form (A_0) there is rapid formation of local structures ($A_0 \rightleftharpoons A_1 \rightleftharpoons \cdots\cdots \rightleftharpoons A_m$) before arriving at a rate-limiting step ($A_m \rightleftharpoons B_1$). After the rate-limiting, the unique globular structure of the native state (B_n) is formed through a series of propagation steps ($B_1 \rightleftharpoons B_2 \rightleftharpoons \cdots\cdots \rightleftharpoons B_n$).

The model makes no mention of the nature of the rate-limiting step or the number of the intermediate states. However, it does suggest that the kinetics of the local structure formation must be rapid, and the activation energy of these reactions must be small. Thus τ_3 and τ_4 reactions detected by the temperature jump seem to fall into these categories.

Assignment of τ_1 and τ_2 reactions in the framework of eq 4 is not difficult either. According to a simple sequential model, in which only the second half of eq 4 is considered ($A_m \rightleftharpoons B_1 \rightleftharpoons \cdots\cdots \rightleftharpoons B_n$), the overall kinetics is biphasic (Tsong et al., 1972a; Elson, 1972). In such a case the terminal reaction (τ_1) usually comes from the rate-limiting step, or the nucleation event in a broad sense. The faster reaction (τ_2) is a composite of the propagation reactions.

Careful examination of the data in Table I does suggest that the two reactions detected by the stopped-flow method fulfill many predictions of the simple sequential model (Elson, 1972; Tsong et al., 1972a). They include: (1) a clear separation of the two kinetic phases; (2) the amplitude of the fast reaction, τ_2, is larger in the unfolding than in the refolding to the same θ values; (3) when only unfolding is considered, τ_2 increases in amplitude as the θ value decreases.

Other evidence which shows that τ_2 may represent the main chain folding reaction comes from its dependence on θ (Figure 4). According to Schwartz (1965) the mean relaxation time of a nucleation-dependent structure formation of biopolymers should exhibit a maximum at the midpoint of the transition. His prediction has, indeed, been confirmed in many macromolecular systems, including the thermal unfolding of ribonuclease A (Hagerman and Baldwin, 1976). Figure 4 shows that τ_2 reaches a maximum at $\theta = 0.5$.

It is not yet clear what is the nature of the rate-limiting step. Garel et al. (1976) have suggested that peptide chain configurational change may be the limiting reaction. Brandts et al. (1975) more specifically suggested that it is the cis-trans isomerization of proline residues that limits the protein chain folding. Our result does not permit us to make any definite statement on the nature of the nucleation event.

It has been suggested that τ_3 and τ_4 reactions may be attributed to the abortive intermediates of Ikai et al. (1973). Reactions of such a nature would indeed be difficult to separate from the normal chain folding process, at the local level, by the kinetic analysis alone. On the other hand, our experiment shows that the Gdn·HCl and urea unfolded form of cytochrome *c* retains considerable structures at neutral pH (Tsong, 1975, 1976). It seems highly unlikely that the chain folding from such a partially unfolded form should involve the abortive reactions discussed by Ikai et al. (1973).

Conclusion

Not every kinetic step in protein chain folding is visible by the absorption change of chromophoric groups. In the case of the Gdn·HCl and the thermal unfoldings of ribonuclease A, it has been shown that the tyrosine absorption changes occur at the first step of the unfolding process (Garel et al., 1976; Brandts et al., 1975). Subsequent step(s), although not visible, may be deduced from careful analysis of the kinetic data; however, such an inference is not always possible. This is particularly true for minor structure alterations after the major unfolding process. The presence of an energy transfer system in cytochrome *c* provides us with a very sensitive way of monitoring the kinetics of the protein conformational change.

Cytochrome *c* is an atypical protein in the sense that the presence of a heme group greatly perturbs the intrapolypeptide chain interactions. On the other hand, looking at the x-ray structure of Dickerson et al. (1971), we know that all the molecular interactions found in other proteins are distinctively present here. Comparison of the cytochrome *c* chain folding with those of other simple proteins also shows strong resemblance in the kinetic pattern. The difference may attribute

more to the choice of the physical parameters monitoring the reactions rather than to the mechanisms of protein chain foldings.

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