Detection of Three Kinetic Phases in the Thermal Unfolding of Ferricytochrome *c*†

Tian Y. Tsong*

ABSTRACT: The thermal unfolding of horse heart ferricytochrome *c* is readily reversible under acid conditions. This property, plus an abundance of heme-specific absorption bands, make this protein a useful choice for studying the mechanism of protein folding. In contrast to the biphasic kinetics reported by previous authors for guanidine-induced unfolding (Ikai, A., Fish, W., and Tanford, C. (1973), *J. Mol. Biol. 73*, 165), there are three well-resolved kinetic phases in thermal unfolding and refolding at acidic pH's. The very fast phase ($\bar{\tau}_3 \sim 20$ μsec) is kinetically complex, and shows different time constants at different heme absorptions. The terminal phase (τ_1 in seconds) and the second phase ($\bar{\tau}_2$ in milliseconds) fall in time ranges comparable both to the guanidine-induced unfolding of cytochrome *c* and to the thermal unfolding of ribonuclease A and chymotrypsinogen A. If the very fast phase is excluded from consideration, the relative amplitudes and the time separations of the τ's for the other two phases show a behavior comparable to that observed for the thermal unfolding of ribonuclease A and chymotrypsinogen A and different from the guanidine-induced unfolding of cytochrome *c*. It may be possible to understand the thermal unfolding of cytochrome *c* in terms of a sequential model discussed previously (Tsong, T. Y., Baldwin, R. L., McPhie, P., and Elson, E. L. (1972), *J. Mol. Biol. 63*, 453), in which an early slow step (a major nucleation event) limits the rate of refolding. The very fast phase shows a small apparent activation enthalpy and probably reflects local structural fluctuations in the vicinity of the heme group. It has a large amplitude in refolding, indicating that local folding reactions might precede the critical very slow step in refolding. The significance of these very fast reactions to the mechanism of protein folding is discussed.

The reversible thermal unfolding of a few selected simple proteins, ribonuclease A (Tsong *et al.*, 1971, 1972a), chymotrypsinogen A (Tsong and Baldwin, 1972a), and myoglobin (Summers and McPhie, 1972), has been reported to follow biphasic kinetics. These observations are consistent with a basic prediction of a simple, nucleation-dependent sequential model proposed by Tsong *et al.* (1972b). Since more than one chromophoric group was used to follow the kinetics in each case, the two reactions might simply reflect two independent unfolding reactions of the different chromophoric groups in the molecules. Experiments reported for the unfolding of N_2Ph-41-ribonuclease A (Tsong and Baldwin, 1972b) represented an attempt to solve this problem, since unfolding could be measured by the single N_2Ph group. It was observed that the unfolding of the N_2Ph substituent at the Lys-41 position showed the same biphasic kinetics as observed when the reactions were measured *via* the tyrosine chromophores. These observations lend strong support to the notion that the fast and slow reactions detected for the ribonuclease A unfolding belong to the two kinetic phases of an overall kinetic process.

Chromophoric labeling of specific groups can provide important information concerning the detailed mechanism of protein folding *in vitro*. However, modification of a protein molecule may seriously alter its stability, and perhaps change the overall reaction pathway of the molecule in unfolding and refolding processes. Moreover, the structures of modified proteins are not always available; thus it may be difficult to link the kinetic data to the structural features of the molecule.

Cytochrome *c* seems to possess many desirable features. It is a relatively small protein consisting of a single polypeptide chain and a heme moiety which is covalently attached through the cytsteine residues at positions 14 and 17 to the peptide chain (Margoliash and Schejter, 1966). The three-dimensional structure of the molecule (Dickerson *et al.*, 1971) shows that in the crystalline state the heme group is nearly uniformly surrounded by the peptide chain which has a hydrophobic interior and a hydrophilic exterior. Since the heme group exhibits a profusion of strong absorptions, and since its transition moments point at different directions along the porphyrin plane (Eaton and Hochstrasser, 1967), the heme group could be especially sensitive for detecting structural alterations in the molecule. The absence of disulfide linkages in the molecule also aids in making cytochrome *c* a useful model for studying the mechanism of polypeptide chain folding.

The equilibrium unfolding of ferricytochrome *c* has been extensively studied in many laboratories (Urry, 1965, 1967; Myer, 1968; Stellwagen, 1968; Babul and Stellwagen, 1971, 1972; Ikai *et al.*, 1973). The general observation from these studies is that the guanidine, pH, and thermally induced conformation changes follow qualitatively the same pattern, with a marked alteration in the heme environment, and disruption of the secondary structure of the protein backbone. The intrinsic viscosity in 6 M guanidine-HCl at neutral pH was reported to be 14.4 cm^3/g (Ikai *et al.*, 1973), close to the expected value for a linear random coil in the solvent (Tanford, 1968). Babul and Stellwagen (1972) reported that the reduced viscosity of ferricytochrome *c* was increased from 2.6

† From the Department of Physiological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and the Department of Biochemistry, Stanford University Medical School, Stanford, California 94305. *Received February 26, 1973.* This work was supported by National Institutes of Health (AM-04763) and National Science Foundation (GB 8016) research grants to Professor R. L. Baldwin at Stanford University. The work was continued, and was supported by a U. S. Public Health Service National Institutes of Health General Research Grant No. FR-5378 to the author at the Johns Hopkins University.

* Address correspondence to the Johns Hopkins University School of Medicine.

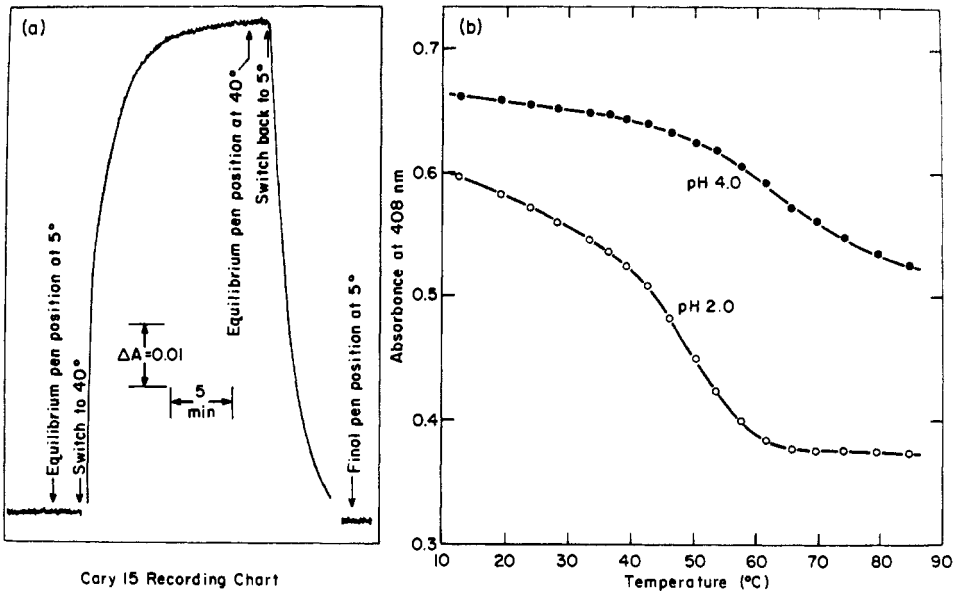


FIGURE 1: (a) Reversibility test of the thermal unfolding of ferricytochrome *c* at pH 2.0 (5° → 40° → 5°) as measured in a Cary 15 spectrophotometer at 408 nm. (b) Equilibrium thermal transition curves of ferricytochrome *c* at pH 4.0 (0.015 N $NaClO_4$, 0.01 N NaAc) in filled circles, and at pH 2.0 (0.015 N $NaClO_4$, small amount of $HClO_4$) in open circles. The protein concentrations are the same in both curves. The van't Hoff ΔH of the transition curves are around 35 kcal/mol at the midpoint of the transitions.

cm^3/g at neutral pH to 22.5 cm^3/g at pH 2, 25°, in the absence of other denaturants, indicating that the peptide chain undergoes a transition from a globular form to an extended random configuration in the latter conditions. All these observations suggest that the thermally denatured ferricytochrome *c* molecule is represented by an extended random configuration at acidic conditions.

Experimental Procedure

Materials. Horse heart cytochrome *c* type IV was purchased from Sigma Chemical Co. The protein was further purified by using an Amberlite IRC 50 column, as described by Margoliash and Schejter (1966). The absorption spectrum taken by a Cary 15 spectrophotometer indicated that the protein was in the Ferri form; its concentration and purity were checked spectrophotometrically. Amberlite IRC 50 was purchased from Mallinckrodt Chemical. Other chemicals are of analytical grade.

Kinetic Measurements. The temperature jump kinetic measurements were performed with an Eigen–deMaeyer temperature jump apparatus. The protein concentration was maintained at about 0.2 mg/ml. A new solution was used for every few temperature jumps. The stopped-flow pH jump experiments were carried out in a Durrum D-110 stopped-flow spectrophotometer. The terminal reaction at low temperature is sufficiently slow to be checked by using the "adder-mixer" method described elsewhere (Tsong *et al.* 1972b), and this was done occasionally with a Cary 15 spectrophotometer.

Experimental Results

Reversibility. When the pH is changed, at fixed temperature, from 4.0 to 2.0 the heme absorptions in the visible as well as the composite absorptions in the ultraviolet (uv) region of ferricytochrome *c* undergo a substantial change (Babul and Stellwagen, 1972). The difference spectrum so generated rapidly diminishes when the pH of the solution is adjusted back to its original value. Thermally induced conformational changes are highly reversible in the pH range from 2.0 to 4.0. All kinetic experiments reported here were thus confined to this pH range, and the reversibility of the test solutions was checked regularly as described elsewhere (Tsong and Baldwin, 1972a). Figure 1a shows a Cary 15 tracing of the reversibility experiment done at pH 2.0. As shown, the reversibility is complete within experimental uncertainty.

Equilibrium Measurements. In order to compute the fraction of the total absorbance change occurring in each kinetic process, thermal transition curves at equilibrium were taken at different heme absorptions where the kinetic measurements were done. In the stopped-flow pH jump experiments the equilibrium absorbance changes were taken from the difference between the two thermal transition curves for the initial and the final pH values. Figure 1b gives an example of the transition curves measured at the heme Soret band, at pH 2.0 and 4.0. Transition curves measured at the other heme absorptions may have an absorbance change of opposite sign (*e.g.*, at 375 nm), depending on the shape of the difference spectrum generated. In our kinetic analysis, only the absolute value of the change is important.

Temperature Jump Experiment. Temperature jump measurements at pH 2.0 , using five-degree jumps, have detected at least two relaxation processes, one in the microsecond time range (τ_3), the other in the millisecond time range (τ_2). Both of them were not observed in jumps beginning above the transition zone. However, both of them were observed in jumps ending at very low temperatures (<25°), indicating that the thermal transition at pH 2.0 starts at a very low temperature. The experiment was done at three different heme absorptions, 425, 408, and 375 nm, and also at 240 nm where complex chromophoric contributions are observed. The kinetics observed at temperatures lower than 30° and those measured at 240 nm are in general agreement with the conclusion drawn in this article, and are not presented here because of their poorer signal-to-noise ratio.

The temperature jump experiments failed to resolve the terminal reaction (τ_1) observed in our stopped-flow measure-

TABLE I: Fast Temperature Jump Kinetic Measurement of Ferricytochrome *c* Unfolding at pH 2.0 (Five-Degree Jumps).[a–c]

Final Temp (°C)	408 nm				425 nm				375 nm			
	$\bar{\tau}_3$ (μsec)	α_3 (%)	$\bar{\tau}_2$ (msec)	α_2 (%)	$\bar{\tau}_3$ (μsec)	α_3 (%)	$\bar{\tau}_2$ (msec)	α_2 (%)	$\bar{\tau}_3$ (μsec)	α_3 (%)	$\bar{\tau}_2$ (msec)	α_2 (%)
30	38	61	54	34	42	65	53	30	24	65	58	30
35	28	50	45	45	35	50	43	45	16	58	48	37
40	26	39	32	56	27	49	33	46	14	47	31	48
45	24	40	20	55	30	45	20	50	13	40	21	55
50	28	42	9.0	33	32	36	11	59	22	50	10	45
55	40	58	5.7	37	44	62	5.5	33	24	48	5.7	47
60	48	65	4.1	30	50	58	4.0	37	16	63	4.0	32
65	45	64	2.4	31	45	65	2.3	30	15	63	2.6	32

[a] Conditions, 0.03 M $NaClO_4$, adjusted to pH 2.0 by $HClO_4$; protein concentration, $A_{408\ nm} = 1.49$ at pH 2.0, 18.0°. [b] Differential mean relaxation times $\bar{\tau}$ as defined by Schwarz (1965) are given here. [c] α values are the fractions of absorbance changes which occur in the reactions divided by the total equilibrium change in absorbances. Estimation of α_3 and α_2 at 408 nm makes allowance for α_1 from the stopped-flow experiment, and at 425 and 375 nm assuming 5% contribution from α_1.

ment, owing purely to a technical difficulty; at low temperatures where the reaction has a sizable amplitude, it is too slow for measurement in the available time range (<1 sec); at high temperature the reaction is within the time range of the machine but the amplitude of the reaction is now rapidly diminishing.

Figure 2 shows oscilloscope records of a five-degree temperature jump experiment measured at 408 and 375 nm. Two relaxation processes ($\bar{\tau}_3$ and $\bar{\tau}_2$) are easily resolved. The noise level is sufficiently low for a precise analysis. The results of the experiment are given in Table I.

Stopped-Flow pH Jump Experiment. The stopped-flow pH jump experiment confirmed our observation of a reaction in the millisecond time range in the temperature jump experiment. In addition, the terminal reaction (τ_1) could be resolved. The stopped-flow method also allowed us to study the two slower reactions ($\bar{\tau}_2$ and τ_1) in refolding.

Two typical oscilloscope records, one (a) for unfolding at pH 2.0, 20.0°, and the other one (b) for refolding at pH 4.0, 45.0°, are given in Figure 3. In both oscillographs the two reactions are recorded in the same pictures.

Table II gives results of the stopped-flow pH jump experiments measured at the Soret band, 408 nm. Experiments were also done at other heme absorptions. Since the kinetics observed at the different wavelengths were quantitatively similar to those shown for 408 nm, they are not presented here. As can be seen, the terminal reaction in unfolding is important only at low temperatures. As the temperature goes above the T_m (about 45°) it diminishes rapidly and the second reaction becomes dominating.

In refolding the terminal reaction always has a larger amplitude than the second reaction ($\bar{\tau}_2$). It is also interesting to see that the amplitudes for the second reaction (α_2) obtained in the stopped-flow pH jump experiments agree well within the experimental uncertainty with those observed in the temperature jump experiments. The absorbance changes that are too fast to be resolved in the former again agree with the α_3 for the fastest reaction in the latter. These points of agreement are not evident if one looks only at the equilibrium transition curves (Figure 4). These observations may be interpreted by a sequential model to be discussed below.

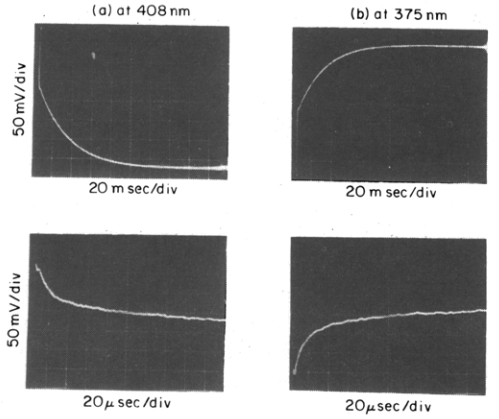


FIGURE 2: Oscilloscope records of a fast temperature jump experiment for the unfolding of ferricytochrome *c* at pH 2.0 (35.0° → 40.0°). The absorbance mode was used: (a) the kinetics followed at 408 nm and (b) the same reactions followed at 375 nm. The fast components in the upper oscillographs are resolved in the lower oscillographs.

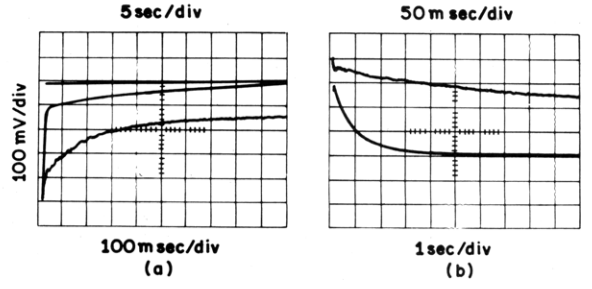


FIGURE 3: Oscilloscope records of a stopped-flow pH jump experiment. (a) The unfolding of ferricytochrome *c* at pH 2.0, 20.0°. The fast component of the upper kinetic curve is resolved in the lower curve. (b) The refolding at pH 4.0, 40.0°. The upper curve shows that there is little fast component in the lower curve. The transmittance mode was used in these records.

TABLE II: Stopped-Flow pH Jump Kinetics of Ferricytochrome *c* (Measured at 408 nm).

Temp (°C)	Unfolding (pH 4.0–2.0)[a] $\bar{\tau}_2$ (msec)	α_2[b] (%)	τ_1 (sec)	α_1 (%)	$\alpha_2/(\alpha_1 + \alpha_2)$	$\bar{\tau}_2/\tau_1$	Refolding (pH 2.0–4.0) $\bar{\tau}_2$ (msec)	α_2 (%)	τ_1 (sec)	α_1 (%)	$\alpha_2/(\alpha_1 + \alpha_2)$	$\bar{\tau}_2/\tau_1$
15.0	304	7.6	36	5.6	0.58	0.0086	45	3.4	19.1	4.2	0.45	0.0024
20.0	209	8.9	23	3.8	0.70	0.0090	30	2.7	11.3	4.1	0.40	0.0026
25.0	135	14	14	3.1	0.82	0.0096	20	1.9	8.0	5.0	0.28	0.0025
30.0	65	21	5.4	2.2	0.90	0.012	30	1.7	5.0	5.3	0.24	0.0060
35.0	50	31	3.6	1.5	0.95	0.014	45	0.9	3.0	6.5	0.12	0.015
40.0	30	47	1.5	1.1	0.98	0.020		Small	1.7	7.0	Small	
45.0	17	54	0.55	0.7	0.99	0.031		Small	0.94	6.3	Small	
50.0	8.9	46		Small	~1.0			Small	0.67	6.8	Small	
55.0	4.1	33		Small	~1.0		18	0.8	0.51	5.3	0.13	0.035
60.0	2.5	31		Small	~1.0		16	0.8	0.32	4.7	0.14	0.050
65.0							14	1.3	0.18	3.5	0.27	0.078

[a] Final conditions for unfolding: 0.015 N $NaClO_4$, 0.005 N NaAc, with suitable concentration of $HClO_4$; for refolding: 0.015 N $NaClO_4$, 0.01 N NaAc. Protein final concentration, $A_{408\ nm} = 1.24$ at pH 4.0, 25°. [b] Estimation of α_2 assumed the machine mixing time of 2 msec, and the absorbance changes were extrapolated to zero time.

Discussion

Ikai and Tanford (1971) and Ikai *et al.* (1973) have recently reported a kinetic study of ferricytochrome *c* unfolding and refolding, using guanidine hydrochloride as denaturant. They have observed that the kinetics of unfolding and refolding are biphasic, and that the time course of the optical changes accompanying the conformational transitions is adequately described by a rate equation containing two exponential decay terms. On the basis of measuring the two rate constants and the corresponding relative amplitudes, they concluded that a metastable, incorrectly folded molecular species exists which is in a rapid equilibrium with the denatured form of the molecule. We have reported here at least three well-separated relaxation processes in the thermal unfolding and refolding of ferricytochrome *c* at acidic pH values.

Many kinetic systems can give rise to three relaxation times (Eigen and deMaeyer, 1963; Ikai and Tanford, 1973; Hijazi and Laidler, 1972), among them the simplest being the one shown in Scheme I. A special example of Scheme I is

SCHEME I

$$A_0 \underset{k_{11}}{\overset{k_{01}}{\rightleftharpoons}} B_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} B_2 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} B_3$$

a scheme proposed by Ikai and Tanford (1971) to account for their observations of ferricytochrome *c* unfolding and refolding by guanidine-HCl (eq 1). Here X denotes a metastable intermediate, or incorrectly folded species, not on the normal pathway of the unfolding processes. D, I, and N are, respectively, the denatured form, an unfolding intermediate and the native form of the protein. Although eq 1 is compatible with our observation of three relaxation processes, we think it unlikely, under our experimental conditions, for the following reasons: (1) our kinetic curves cannot be adequately described by an equation including only three exponential decay terms, since the fastest kinetic phase appears to be a relaxation spectrum of a complex reaction system (see Figure 2), and the entire course of the reactions evidently involves more than two intermediates; (2) adaptation of eq 1 would assume the incorrectly folded species X has absorption properties that are more unfolded than D. For these reasons and others to be brought up later, we will consider a sequential model below.

$$D \rightleftharpoons I \rightleftharpoons N \qquad I \rightleftharpoons X \qquad (1)$$

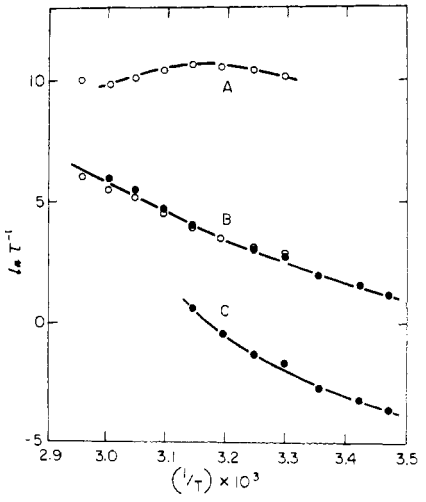
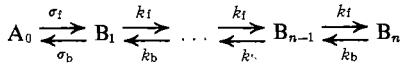
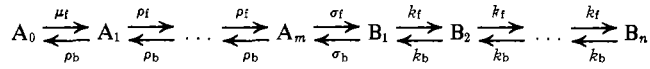


FIGURE 4: The variation of three relaxation processes as a function of temperatures, in the unfolding of ferricytochrome *c* at pH 2.0, $1/\bar{\tau}_3$, $1/\bar{\tau}_2$, and $1/\tau_1$ in curves A, B, and C, respectively. The rate constants obtained in the fast temperature jump are given in open circles, and those from the stopped-flow pH jump measurements are given in filled circles. The apparent activation enthalpy of the τ_1 reaction is 20 kcal/mol below T_m, increasing to 30 kcal/mol above T_m, and for the $\bar{\tau}_2$ reaction it is 16 kcal/mol below T_m, increasing to 24 kcal/mol above T_m. The apparent activation enthalpy of the $\bar{\tau}_3$ reaction is small.

SCHEME II



SCHEME III



A Simple Sequential Model. The fact that Scheme I fails to account for our experimental observations suggests that a scheme involving more than two reaction intermediates has to be considered. Careful examination of the stopped-flow experiments both in the unfolding and refolding directions (*cf.* Figure 3 and Table II) indicates that the most important features of the two slower kinetic phases ($\bar{\tau}_2$ and τ_1) fulfill many predicted properties of a simple sequential model proposed by Tsong *et al.* (1972b) and Elson (1972). The model is shown in Scheme II, in which the unfolded species A_0 has to cross an entropy-controlled nucleation event, with rate constants σ_f and σ_b, before it proceeds in a series of propagation steps, with rate constants k_f and k_b for each step, to reach the native form B_n.

The important features of the simple sequential model, which are observed in similar experiments with ribonuclease A (Tsong *et al.*, 1971, 1972a), and also in our stopped-flow experiments for cytochrome *c*, are the following: (1) there is a wide separation between the two kinetic phases, the fast phase being a transient phase and the slow phase being an almost steady-state phase (presumably $\bar{\tau}_2$ and τ_1 here), of the cooperative conformational transitions; (2) the amplitude of the terminal reaction (α_1) is larger for unfolding at the beginning of the transition zone, and becomes less significant at the upper end; the opposite is true for the fast phase (α_2) (*i.e.*, $\alpha_2/(\alpha_1 + \alpha_2)$ increases with the temperature); (3) in refolding, the terminal phase (α_1) is always largest; the amplitude of the fast phase (α_2) decreases to a very small value of $\alpha_2/(\alpha_1 + \alpha_2)$, near the T_m; (4) the ratio of the two time constants, $\bar{\tau}_2/\tau_1$, in unfolding increases with temperature; τ_1 approaches $\bar{\tau}_2$ in the upper part of the transition zone (see Figure 4).

Local Structural Formation Preceding a Major Nucleation Event. The idea that some structural nuclei have to form before a major folding reaction can take place has wide currency in studies of structural formation by biopolymers (Ross and Sturtevant, 1962; Epstein *et al.*, 1971; Craig *et al.*, 1971). Lewis *et al.* (1971) have, through theoretical considerations, suggested that some peptide residues in protein molecules have a much higher probability of α-helix formation, and that these local structures can then be stabilized by long-range interactions with a tetrapeptide β bend unit. Such a structural initiation is expected to exhibit a dominant effect in kinetics of refolding if a sensitive reporting group can be assigned in the molecule. In our study of the kinetics of ribonuclease A unfolding and refolding, no such effect was detected (Tsong *et al.*, 1971, 1972a). However, it has been suggested that thermal unfolding of ferricytochrome *c* might proceed in two major steps: the uncoupling of the heme–protein interactions followed by the disintegration of the α-helical structure (Urry, 1965; Myer, 1968). The very fast reactions, in this case, would mean the formation of secondary structure prior to a major nucleation reaction.

The other plausible explanation of these very fast reactions is that they detect the ligand binding reactions to the heme moiety. Babul and Stellwagen (1971, 1972) have demonstrated that at neutral pH the two strong-field ligands could be displaced by two weak-field ligands of the solvent at acidic pH values, and the substitution could induce an unfolding of the molecule. They have also demonstrated that modification of His-18 by carboxymethylation hinders the binding of this ligand to the heme iron, and stabilizes the unfolded form of the protein. On the basis of these observations, they concluded that the coordination of His-18 to the heme group is an important process in the folding of the ferricytochrome *c* molecule in solution.

One interesting observation of this kinetic phase is that the relaxation times measured at different heme absorptions are appreciably different (Table I). This is not seen in the two other relaxation processes ($\bar{\tau}_2$ and τ_1) reported here. Although direct measurements of $\bar{\tau}_3$ and hence α_3 in refolding are not yet accessible, it is possible to estimate α_3 by merely taking the equilibrium absorbance change less the change that occurs in the two slower phases. The α_3 so obtained is about 90% near T_m for refolding at pH 4.0, in comparison to the measured value of 40% for unfolding at pH 2.0. The very fast phase reported here also shows a small apparent activation enthalpy (Figure 4). These observations are consistent with the interpetation that the $\bar{\tau}_3$ reaction reflects local structural formations in the vicinity of the heme group prior to a slow rate-limiting step (a major nucleation step).

Consider a kinetic scheme, Scheme III, in which A_i denotes the unfolded forms with various amounts of local structures, μ_f is the rate constant for the initial step, and ρ_f and ρ_b are the rate constants for the rapid conversion among these species. If μ_f, ρ_f, and ρ_b are much faster than the k_f and k_b, one expects to observe a very fast kinetic phase in which the concentration of B_i rapidly adjusts, when the system is subject to a perturbation.[1]

Other kinds of minor structural alterations in highly refolded states, such as one reported by Teipel and Koshland (1971a,b), may occur at a much slower rate, however. There is no evidence, under our solvent conditions, for us to include such reactions in our kinetic analysis here.

Mechanisms of Ferricytochrome c Unfolding. As mentioned earlier, if the time course of conformational transition can be followed with a single chromophoric group, then complex kinetics must reflect different kinetic phases of an overall unfolding scheme. We have shown that the thermal and acidic unfolding of horse heart ferricytochrome *c* measured by the heme absorptions follows triphasic kinetics, and that the two

[1] Dr. E. L. Elson of the Chemistry Department of Cornell University has kindly done the kinetic analysis of Scheme III for this article by using the following set of parameters: $\mu_f = \rho_f = 10^3 s k_b$; $\mu_b = \rho_b = 10^3 k_b$; $\sigma_f = 10^{-3} s k_b$; $\sigma_b = 10^{-2} k_b$; $k_f = s k_b$; $m = 12$; $n = 7$; $\Delta H = -2$ kcal for each step except for the nucleation step value $\Delta H = 0$. He has found that the unfolding and refolding of Scheme III follow a triphasic kinetics, dominating by three modes, $|\lambda_1| \sim 10^{-3} k_b$ sec^{-1}, $|\lambda_2| \sim 0.2 k_b$ sec^{-1}, and $|\lambda_8| \sim 100 k_b$ sec^{-1} (*cf.* Elson, 1972). The first mode corresponding to the quasi-steady-state unfolding, or τ_1 in our experiment, has larger amplitude at the first part of the transition and becomes less important at the top of the transition. The second mode has relatively smaller amplitude than the corresponding $\bar{\tau}_2$ observed experimentally. The eighth mode corresponding to the $\bar{\tau}_3$ reported here has a larger amplitude at the top of the transition and in refolding. Although the experimental observations of the three kinetic phases are not entirely reproduced by the model, the close agreement between them is encouraging.

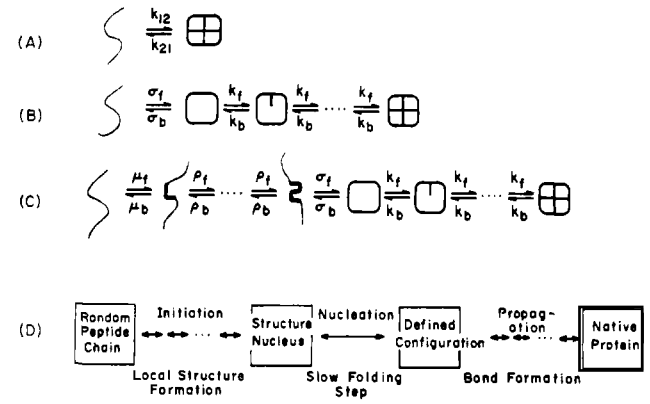


FIGURE 5: Schematic diagrams of protein *in vitro* folding. (A) A unique, two-state folding from the random peptide chain(s) to the native form. (B) A simple sequential model, assuming that the peptide chain(s) has to cross an entropy barrier to achieve a stable configuration before a series of bond formation reactions can take place. (C) An extended simple sequential model, assuming certain structural initiation steps prior to a major nucleation event exist.

slower reactions ($\bar{\tau}_2$ and τ_1) resemble in many respects those reported for the unfolding of ribonuclease A under the similar conditions. The unfolding and refolding of ribonuclease A have been shown to follow a sequential pathway, with a major nucleation event, that limits the rate of reaction in the first step of refolding.

Data reported for chymotrypsinogen A are more complex (Tsong and Baldwin, 1972a); an additional relaxation process in the microsecond time range was resolved at acidic pH. Although the microsecond reaction in chymotrypsinogen A unfolding has a much smaller amplitude as compared to the one reported here, nevertheless it suggets that the extent of cross-linkage in the molecules may explain the differences observed for the unfolding of the three proteins. For example, in the ribonuclease molecule, the longest chain, not cross-linked to the other part of the peptide chain through the disulfide bridges, is only 26 residues long; however, there are two long chains, one with 42 residues and one with 53 residues in the chymotrypsinogen molecule. In the case of cytochrome *c* the non-cross-linked peptide chain extends even longer to 87 residues. The cross-linkage of the peptide chain could impose constraints which limit the formation of local structures in the peptide chain. On the other hand, the lack of constraints in the unfolded cytochrome *c* molecule could result in a rapid formation and dissociation of local structures, which give rise to the microsecond reaction ($\bar{\tau}_3$) reported here.

Consider the schematic diagram C Figure 5. It is conceivable that for the *in vitro* folding of a randomly oriented peptide chain(s) some structural nuclei have to be formed before a major nucleation (a condensation of local structures into a well-defined, relatively stable configuration) could take place. Once the nucleation is achieved a series of propagation steps follows. If the molecular constraints do not allow the local structural formations to occur, or if the chromophoric groups designated to measure the reaction do not detect them, the scheme appears like scheme B. Scheme A represents an extremely simplified version, namely a two-state or all-or-none process. The kinetics of ferricytochrome *c* seem to be best explained by scheme C shown in Figure 5.

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