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Viscosity-Dependent Conformational Relaxation of Ribonuclease A in the Thermal Unfolding Zone†

Tian Yow Tsong

ABSTRACT: Previous studies monitoring tyrosine absorption changes have failed to detect rapid unfolding reaction of ribonuclease A at pH 7 by the temperature jump method, although these reactions (in the 50-ms time range, or τ_2 reaction) have been observed by the stopped-flow pH jump in both unfolding (pH 7 → 2) and refolding (pH 2 → 7) directions. When the unfolding reaction is coupled to the color change of a colorimetric indicator, phenol red, in an unbuffered protein solution, at least three relaxations ranging from submilliseconds to seconds have been resolved with a 4 °C temperature jump over the range 10–80 °C, at pH 7. The fast reaction (τ_f = 0.9 ms at 25 °C), which detects a proton uptake with a pK_a of about 6.0, was reported earlier by French and Hammes [French, T. C., & Hammes, G. G. (1965) *J. Am. Chem. Soc. 87*, 4669–4673]. This reaction, observable in the entire folding–unfolding transition zone, has been found to depend strongly on viscosity additives to the solution such as ethylene glycol, glucose, and sucrose. The reaction was obscured by a substrate-induced conformational relaxation in the same time range, when cytidine 2′-monophosphate was present, but it could be separated from the latter in a viscous medium because of its stronger dependence on solvent viscosity. Thus, it is unlikely that it is a part of the substrate binding reaction. The other two slower relaxations also detect a proton uptake, but with a pK_a of about 8.0, are independent of the solvent viscosity, and have similar relaxation times for the folding–unfolding of ribonuclease A monitored by the tyrosine absorption, under similar experimental conditions ($\tau_2 \sim 80$ ms, $\tau_1 \sim 10$ s at pH 7, 25 °C). As is true for the τ_f reaction, both the τ_2 and τ_1 reactions were present even at 25 °C, i.e., a temperature more than 35 °C below the thermal melting temperature (T_m = 61.5 °C), or at a temperature near 80 °C, i.e., after the thermal transition is presumably complete. However, all three reactions were abolished in a 5 M guanidine hydrochloride solution. These observations are consistent with earlier results that thermally unfolded ribonuclease A retains residual structures which can be removed by guanidine hydrochloride. Two possible sources of the viscosity-dependent relaxation are suggested. If all of the protein molecules are in the folded state at 25 °C, the τ_f reaction could reflect solvent permeation into the protein structure. Conversely, if fractions of protein molecules are unfolded, or are in a dynamic equilibrium between the folded and the unfolded states, the τ_f reaction could have detected an early step in the protein chain folding. In both cases, the reaction would be expected to depend on the microscopic viscosity of the solution.

Different approaches have been used to investigate the mechanisms of protein chain folding (Baldwin & Creighton, 1980; Privalov, 1979; Anfinsen & Scheraga, 1975). Among these methods, kinetic measurement seems uniquely suitable for obaining dynamic information about the chain folding process (Baldwin & Creighton, 1980). For example, it is now clear that the recovery, from the unfolded form, of the active, native conformation of small proteins, whether they are disulfide containing or not, does not take more than seconds (Schechter et al., 1970; Tsong et al., 1971; Ikai & Tanford, 1971; Garel & Baldwin, 1973; Leutzinger & Beychok, 1981). Recent kinetic analyses of ribonuclease A (RNase A)[1] (Garel & Baldwin, 1973; Schmid & Baldwin, 1978; Kim & Baldwin, 1980), carp parvalbumin (Brandts et al., 1977), and a derivative of pancreatic trypsin inhibitor (Jullien & Baldwin, 1981) have shown that the unfolded state of these proteins exists in different isomeric forms, and transformation among these forms is slow compared to a major folding reaction (Baldwin & Creighton, 1980; Brandts et al., 1975). As a result, the overall kinetics of folding reflect these isomerizations in addition to the chain folding process. Identification and characterization of each kinetic phase are, thus, important steps in the kinetic study of protein chain folding.

In the case of RNase A, it has now become clear that proline isomerization in the unfolded state of the protein results in the separation of the population into two kinetic species, the fast-folding and the slow-folding species (Garel & Baldwin, 1973; Brandts et al., 1975). However, the kinetics of the chain folding from neither of the two species seem rate limited by the proline isomerization reaction (Nall et al., 1978; Cook et al., 1979; Kim & Baldwin, 1980). In an earlier study (Tsong & Baldwin, 1978), we have attempted to identify reactions

† From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received July 29, 1981.* This work was supported by National Science Foundation Grant PCM81-09630.

[1] Abbreviations: 2′-CMP, cytidine 2′-monophosphate; Gdn·HCl, guanidine hydrochloride; RNase A, bovine pancreatic ribonuclease A.

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that might be associated with chain motions of the peptide chain; such reactions, if existent, would be expected to depend on the external friction and thus the viscosity of the medium in which chain folding takes place. None has been found. This failure in finding a viscosity-dependent reaction has led us to conclude that the rate of the chain folding is limited by the accumulation of certain intermediates in the folding process and that stepwise folding involving stable intermediates is favored for the case of RNase A (Tsong & Baldwin, 1978). These observations, however, do not rule out the possibility that friction-limited chain motions (Karplus & Weaver, 1976, 1979) may occur on a faster time scale than that examined previously (>10 ms) (Tsong et al., 1972; Hagerman & Baldwin, 1976). Another result of an earlier study (Tsong & Baldwin, 1978) is that no reaction in a 50-ms time range (τ_2) could be detected by the temperature jump at pH 7, although by a pH jump or a Gdn·HCl concentration jump the τ_2 reaction was clearly seen at this pH (Tsong et al., 1972; Garel et al., 1976).

In this paper, we report an experiment where we have employed a sensitive means for detecting protein conformational change, namely, by monitoring the pK change of ionizable groups, coupled to the colorimetric indicator phenol red, in an unbuffered protein solution upon a rapid temperature jump. The method was used by French & Hammes (1965) for their studies of the protein–substrate interaction of RNase A, at room temperatures. By this method, we have now detected a rapid reaction in the submillisecond time range, which is strongly dependent on the microscopic viscosity of the solution. This reaction, along with two slower reactions (50-ms and 10-s time ranges), could be detected in the entire thermal transition zone at neutral pH, but they all diminished in a 5 M Gdn·HCl solution.

Materials and Methods

Materials. RNase A, type XIIA, was obtained from Sigma. The protein was further purified by chromatography on IRC-50 followed by gel filtration through a Sephadex G-25 column. Untreated and purified samples gave identical results, and in some experiments, the commercial sample was used directly without purification. Phenol red was supplied by Eastman Kodak. Spectroscopic grades of glycerol and ethylene glycol were purchased from Baker. Ficoll, with an average molecular weight of 1×10^2, was purchased from Pharmacia. Other chemicals were of the purest grade commercially available. All of the protein solutions used for kinetic and viscosity measurements were filtered through a 0.4-μm pore size Millipore membrane, except for the solutions containing ficoll.

Temperature Jump. A 4 °C temperature jump was done in the temperature range of 20–80 °C. A Messenlagen temperature jump apparatus was used. Two capacitors, one with a capacitance of 0.02 μF and the other 0.05 μF, were used. All of the signals detected by the color change of the phenol red containing RNase A solutions depended only on the magnitude of the temperature jump, and not on the initial charging voltage of the capacitors, indicating that no electric field induced reactions were present (Dourlent et al., 1974; Eigen & de Maeyer, 1963). All solutions contained 0.1 N NaCl, and the heating time was less than 5 μs. The signals were recorded with a Biomation Model 805 transient recorder, and manual analyses were performed to obtain the relaxation time. Dye solution alone was found to give no signals under all conditions studied.

Other Measurements. pH titration was performed with a Radiometer System TRS-622, in a water-jacketed cell, connected to a Lauda K-2/RD refrigerated water circulator. Heating was controlled by a Neslab TP-2 temperature programmer, and a heating rate of 1 °C/min was used. RNase A (100 mg) was dissolved in 5 mL of a well-degassed 0.1 N NaCl solution and was titrated with 0.010 N NaOH under nitrogen gas at pH 7.3, while the solution was being heated. This pH-stat titration eliminated most problems encountered in the pH titration experiment, e.g., CO_2 interference.

Viscosity measurements were done with a Cannon-Manning semimicroviscometer which was installed to a Wescan viscometer timer. The viscometer constant was 0.001294 cP/s. Absorption spectroscopy and melting transition measurements were performed with a McPherson 707K spectrophotometer. Heating rates of 0.5–1 °C/min were used for the equilibrium melting experiment.

Results

Proton Absorption upon Thermal Unfolding. Thermal unfolding of RNase A at neutral pH is an endothermic process; the ΔH of the transition at the midpoint (T_m = 61.3 °C) is 702 kJ/mol (Tsong et al., 1970). When changes in the ionization state of the protein were monitored, there were at least two opposing events accompanying the heating process. The first one was the temperature dependence of the ionization of titratable groups. The second one was the shift in the pK_a of titratable groups upon thermal unfolding of the protein (Tanford, 1962). The first event is predictable from the enthalpy of ionization of a titratable group according to the relation $\Delta K = -(\Delta H_i/R)K(1/T)$ where K is the equilibrium constant of ionization and ΔH_i is the enthalpy of ionization. If the ionization is endothermic, an increase in temperature will favor release of protons, and vice versa. However, the ΔH_i of the individual ionization group is not available for analysis. The second type of thermal effect is less predictable, and the group may either release or absorb a proton, depending on a shift to a higher or to a lower intrinsic pK_a upon thermal unfolding. Since the pK_a of a buried group can be either higher or lower than its intrinsic value (Tanford, 1968), there is no rule for predicting whether a group will release or absorb a proton upon the exposure of the group.

Because of these difficulties in estimating thermally induced ionization, we have done a pH-stat titration, and the result at pH 7.3 is given in Figure 1a. At temperatures below 10 °C, heating of the protein solution was accompanied by a release of 0.033 proton/(mol·°C). This value began to diminish at higher temperatures due to a proton absorption process associated with the unfolding. The rate of proton release increases again beyond 70 °C, and eventually reached a limiting value of 0.085 proton/(mol·°C) above 80 °C.

The number of protons absorbed can be estimated by the graphic method illustrated in the same figure, and it has a value of 0.95 per mol at T_m. After suitable base lines are drawn (dashed lines, the assigment of the base line on the low-temperature side was difficult and may be subject to a larger uncertainty), the degree of unfolding was estimated and indicated by α, and the transition curve so obtained is given in the solid curve of Figure 1b. The dashed curve is taken from the calorimetric result of Tsong et al. (1970) which reflects a highly cooperative two-state-like transition (Privalov, 1979). Although the proton absorption curve is broader, it is interesting to see that both curves exhibit, within experimental error, an identical midpoint of the transition (61.3 °C). This suggests that whereas the reaction detected by the proton absorption curve may reflect a less cooperative process, the process nevertheless is intimately associated with the chain folding–unfolding transition.

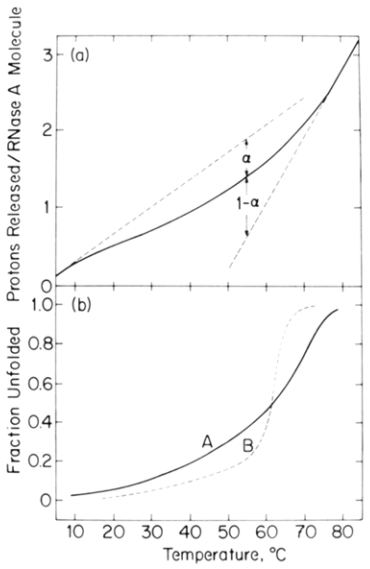

FIGURE 1: pH-stat titration of RNase A at pH 7.3. (a) RNase A (100 mg) dissolved in 5 mL of 0.1 N NaCl was kept at 2 °C and adjusted to pH 7.3. As the temperature was being raised (at a heating rate of 1 °C/min), the pH of the solution began to drop. This change in pH was titrated back by 0.010 N NaOH, and the volume of NaOH solution consumed was recorded and plotted as the number of protons released per protein molecules. Two base lines in dashed curves were arbitrarily drawn for the low- and the high-temperature states. α, then, denotes the fraction of protein that has undergone a transition. See *text* for details. (b) The fraction of protein that had undergone a transition was plotted against temperature in curve A. Curve B was taken from the calorimetric data of Tsong et al. (1970). The base line of the posttransition state has been corrected. Although the proton absorption curve shows a much broader transition, the midpoints of the transitions are identical, at 61.3 °C.

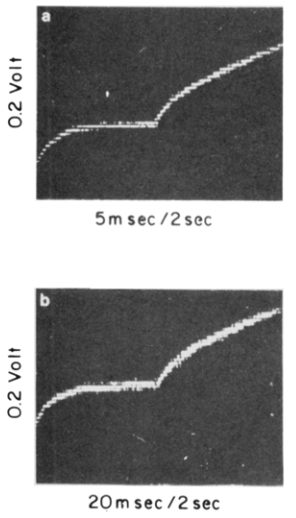

FIGURE 2: Oscilloscope records of the temperature jump kinetics of the proton absorption process shown in Figure 1. (a) Temperature jump, from 21.0 to 25.0 °C, of RNase A in 0.1 N NaCl, containing no buffer but 0.04 mM phenol red as a colorimetric indicator. The signal detected a color change at 560 nm (1 V = 0.15ΔA). The first half records kinetics in the first 5 ms, and the second half records the event of the next 2 s. Enzyme concentration was 0.29 mM, and the initial pH was 7.3. (b) Similar to (a) except that the solution contained 30% D-glucose, and the first half records events occurring in 20 ms. The viscosity of the glucose solution relative to that of the aqueous solution in (a) was 4.6. Although the fast event in (a) (τ_f = 0.95 ms) was significantly slowed down (to 3.0 ms), the slow reactions were little affected.

Three Relaxations Associated with the Proton Uptake. If the proton release was due to a temperature dependence of the ionization constant of titratable groups, the rate of dissociation of the proton would be very fast and should occur within the few microseconds heating time of the temperature jump apparatus. This was indeed the case. No reaction of proton release could be resolved when the release was coupled to the color change of phenol red, in an aqueous solution. Instead, there was an instantaneous drop of the absorption at 560 nm that measured a sudden change to acidity of the solution. (In more viscous media, it was less certain whether the dissociation of protons took place within the heating time. In any case, the dissociation should have a relaxation time of less than 10 μs.) In contrast, the absorption of protons was easily resolved by the color change of phenol red, as shown in Figure 2a.

In Figure 2a, two sampling rates were used in one measurement to record the proton absorption reaction. The first half of the oscillograph records the rapid event that occurred in the first 5 ms, and the second half records reactions that followed in the next 2 s. Beyond 2 s, the temperature of the solution began to return, and the reactions could not be recorded with confidence.

The rapid reaction has a relaxation time of 0.9 ± 0.1 ms (τ_f) and has been reported earlier by French & Hammes (1965). However, they did not pursue the slower reactions that followed. As can be seen, there are at least two or possibly three more relaxations, ranging from 55 ms to several seconds, after the τ_f reaction. In fact, a comparison of the previously published relaxations of the RNase A folding–unfolding transition (Tsong et al., 1972) suggests that the slower reactions in the second half of the oscillograph are the τ_2 (50-ms) and τ_1 (10-s) reactions of the unfolding process. This was further supported by the fact that all three reactions detected here disappeared in a 5 M Gdn·HCl solution.

The three relaxation times were independent of the protein concentration, in the range of 0.45–0.06 mM, and the dye concentration, in the range of 50–7.9 μM. The signals completely diminished when the solution contained 10 mM sodium cacodylate buffer. These observations indicate that the three reactions detected here reflect a proton absorption upon the unfolding of protein and were, thus, rate limited by the folding–unfolding transition of the protein molecule. Since we could not resolve the τ_1 reaction (10 s) accurately, we will focus only on the τ_f and τ_2 reactions (50 ± 5 ms) in our further discussion.

Only the Fast Relaxation, τ_f, Depended on Solvent Viscosity. The question of whether the τ_f reaction detects only a conformational isomerization of the native state (French & Hammes, 1965) or whether it also occurs in the folding–unfolding transition can be answered by studying the reaction in the entire thermal transition zone. As was mentioned, the reaction, as well as the two slower reactions (τ_1 and τ_2), has been detected in the temperature range of 20–80 °C. The result for the τ_f reaction is shown in Figure 3. The temperature dependence of the τ_2 reaction resembled that reported by Hagerman & Baldwin (1976) and was not pursued further. The activation energy of the τ_f reaction in the aqueous medium is 27.2 kJ/mol (closed circles), and it is 55.6 kJ/mol in a 62% sucrose solution (opened circles). The activation energy in the aqueous medium is comparable to that for a diffusion process, and as expected, it is twice as high in the viscous sucrose solution. In both media, the relaxation time shows a slightly peaked value around 62 and 65 °C, which are close to the T_m values of RNase A unfolding in these media (61.3 and 65 °C). The appearance of a maximal relaxation at the midpoint is generally observed for the cooperative transition of biopolymers (Schwarz, 1965). In the present case, τ_f seems to depend more strongly on the solvent viscosity and only slightly on the

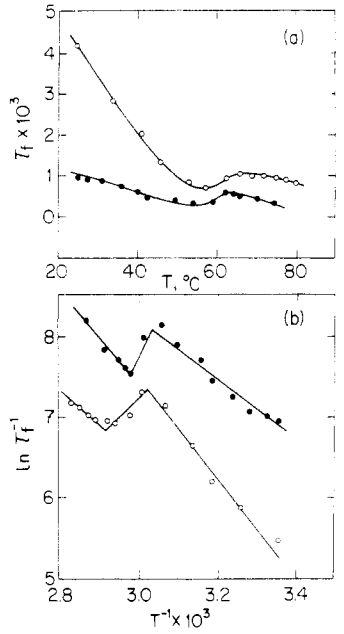

FIGURE 3: Temperature variation of τ_f in 0.1 N NaCl (●) and in 0.1 N NaCl containing 62% sucrose (○) (a); a plot equivalent to the Arrhenius plot (b). At low temperatures where the equilibrium constant of the τ_f reaction is much smaller than unity, τ_f^{-1} is roughly equal to the rate of the rapid folding reaction. This rate constant has an activation energy of 27.2 kJ/mol in the aqueous solution and 55.6 kJ/mol in the 62% sucrose solution.

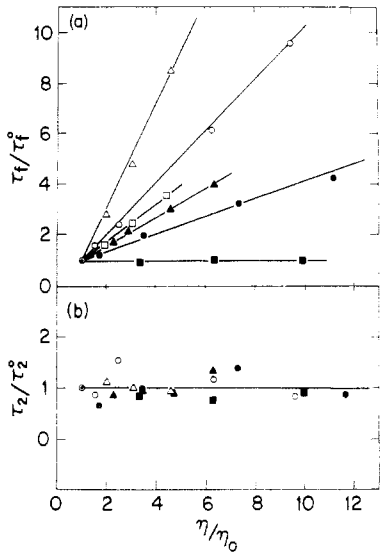

FIGURE 4: Viscosity dependence of τ_f and τ_2 reactions. (a) τ_f was found linearly dependent on the solution viscosity, although the effectiveness of the viscosity additives was very different. (△) Ethylene glycol as viscosity additive; (○) glycerol; (□) erythritol; (▲) glucose; (●) sucrose; (■) ficoll with a mean molecular weight of 1×10^5. All additives are from the polyalcohol family. The temperature was 25 °C, and the pH was 7.3. τ^0 and η_0 are respectively the relaxation time and viscosity of a 0.29 mM RNase A solution containing 0.1 N NaCl and 0.04 mM phenol red. $\tau_f^0 = 0.95$ ms was used. (b) The τ_2 reaction was found independent of the solution viscosity as previously reported (Tsong & Baldwin, 1978). The symbols used were identical with those in (a). $\tau_2^0 = 55$ ms was used.

transition. The viscosity dependence of the relaxation was systematically examined for all three reactions. The results for the τ_f and τ_2 reactions are given in Figure 4. It was observed that only the τ_f reaction depended on the viscosity of the solvent but not the other two slower reactions. Most interestingly, τ_f was found sensitive only to solvent viscosity which was produced by small-sized molecules, e.g., ethylene

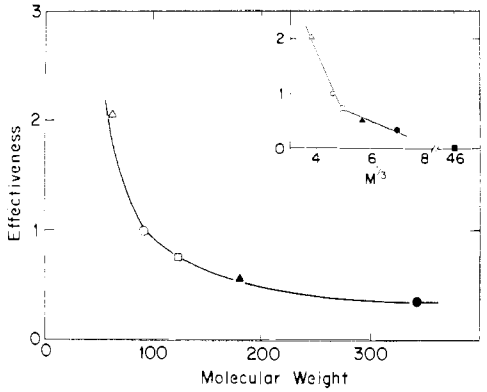

FIGURE 5: Size dependence of the effectiveness of viscosity additives as a retardant to the τ_f reaction. The slope of the curves in Figure 4a is plotted against molecular weight and the cubic root of molecular weight (inset). $M^{1/3}$ is proportional to the particle size of additive molecules. The result suggests that only the microscopic viscosity of the solvent is effective in slowing the τ_f reaction, and there seems to be a break point around the size of erythritol, which has a molecular weight of 122.

glycol, glycerol, glucose, etc. When ficoll (average molecular weight 1×10^5) was used, no effect of viscosity was found. Even for small-sized molecules, the effect of viscosity was most pronounced for ethylene glycol but was less effective for sucrose solutions. For a specific compound used to increase the viscosity, the relaxation time was found to be proportional to the solvent viscosity (Figure 4a).

The effect of solvent viscosity was felt at all temperatures studied (see, e.g., Figure 3). Figure 4 gives results obtained at 25 °C. This choice of temperature for a detailed study was based on two considerations. (1) If the τ_f reaction reflects an early step in the protein chain folding, then at low temperature τ_f^{-1} would measure the rate constant of this early chain folding reaction [see, e.g., Eigen & de Maeyer (1963) for a relaxation analysis]. Study of the viscosity dependence of this rate constant would be most relevant for the elucidation of chain folding mechanisms. (2) At 25 °C, RNase A was in the early part of the thermal transition, i.e., at least 25 °C below the T_m for the solvent conditions used here, precluding the folding transition from having an effect on τ_f.

Addition of different chemicals might substantially alter solvent properties, e.g., polarity, dielectric constant, etc., and this alteration may be responsible for the slowing down of τ_f. This is judged unlikely for the following reasons. (1) If a change in the solvent properties affected the τ_f reaction, it would likely have the same effect on the slower reactions, τ_2 and τ_1. This was found not to be the case (Figures 2b and 4b). (2) The amplitude of all three reactions was found not to depend on viscosity additives at 25 °C. The combined signal of the three reactions showed a maximum around the T_m, although the amplitude of the τ_f reaction exhibited a minimum around the T_m, as would be expected if it is part of the cooperative transition (Kanehisa & Tsong, 1978). (3) The molecules chosen as viscosity additives were all alcohol derivatives. Their molecular properties are expected to be similar, but their effect on τ_f was very different: the smaller the molecule, the more effective it was in retarding the rate (Figure 5). There seems to be a break around the size of erythritol. Molecules larger than erythritol were less effective in slowing down the reaction. (4) An interesting fact is that although all three reactions persisted at a temperature near 80 °C, they all diminished in a 5 M Gdn·HCl solution. Thus, they are part of the structure unfolding transition. The τ_f reaction was also found to be insensitive to the ionic strength of the solution when NaCl was added up to 1 M. Thus, the reaction is not rate

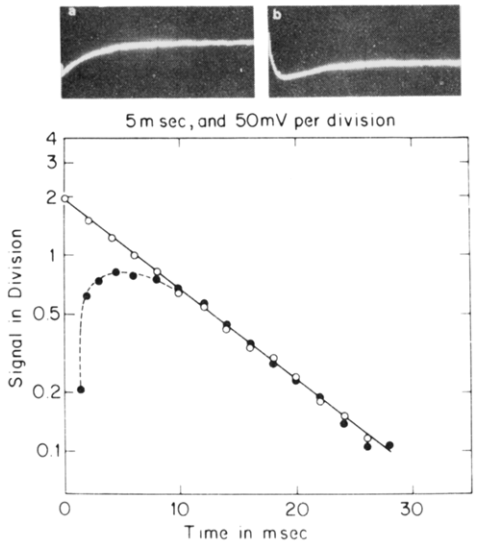

FIGURE 6: Separation of the τ_f reaction from a conformational change associated with 2′-CMP binding. In the presence of 0.3 mM 2′-CMP, the τ_f reaction monitored by the color change (increase at 560 nm) of phenol red was obscured by a new reaction with a relaxation time of 0.45 ms, but with a decrease in optical signals. Because of this shielding of the τ_f reaction by 2′-CMP, it was believed the τ_f reaction detected a hinge bending near the active site of RNase A. The two reactions can be separated in a highly viscous medium (60% ethylene glycol, relative viscosity 4.6 in this case), because of a stronger dependence of τ_f on the solvent viscosity (oscillograph b). In (b), the 0.45-ms reaction was slowed down to 1.5 ms, but the τ_f reaction was slowed down from 0.9 to 8.6 ms. This τ_f reaction in the presence of 2′-CMP is compared with the same reaction in the absence of 2′-CMP, but with identical solvent conditions in oscillograph a. Solutions contained 0.1 N NaCl, 0.04 mM phenol red, and 0.29 mM RNase A at initial pH 7.3. Temperature jump was from 21.0 to 25.0 °C. Open circles are for solution without 2′-CMP and filled circles for solution with 2′-CMP. See text for details.

limited by charge interaction.

Origin of the Proton Absorption Reactions. French and Hammes have done a careful study of the τ_f reaction, at low temperatures, as related to the enzyme's ability to bind substrates, or substrate analogues (French & Hammes, 1965). On the basis of their study, they have concluded that the proton absorption involves the normalization of an ionizable group, with an intrinsic pK_a of 6.1. The pH dependence of the relaxation time fits an ionizable group of this pK value. They have suggested that this probably is His-48 which sits at the top of the hinge region, and the motion involved is the opening and the closing of the hinge which alters the groove containing the active site.

We have checked the magnitude of the signal in the pH range of 5.8–9.0. When correction was made for the size of the color change of phenol red at different pH values, the signal associated with the τ_f reaction exhibited a transition around pH 6.0, confirming the observation of French and Hammes. In contrast, the other two slower reactions of the folding–unfolding transition, the τ_1 and τ_2 reactions, showed higher pK_as around pH 8. In the presence of 2′-CMP, either the τ_f reaction was obscured by a proton-release reaction, with a relaxation time of 0.45 ms, or it disappeared altogether [see also French & Hammes (1965)], but the two slower reactions were unaffected. Interestingly, we have found that the 0.45-ms proton-release reaction associated with substrate binding can be separated from the τ_f reaction in a viscous medium because of the stronger dependence of τ_f on the solvent viscosity. This is shown in Figure 6. In oscillograph b, the τ_f reaction in the presence of 2′-CMP is compared with oscillograph a in which no 2′-CMP was present. In both cases, 60% ethylene glycol was present to slow down the τ_f to 8.6 ms. The fast signal in (b) is due to the substrate-induced relaxation of the protein. In a buffer without viscosity additives, the τ_f reaction is overshadowed by the fast signal in (b) because of a similar relaxation time and opposite optical signals. This result suggests that the τ_f reaction may not be restricted to chain motions in the groove region near the active site, but rather it reflects a class of reactions in the overall protein conformational transition.

Discussion

Two observations reported here are especially noteworthy. (1) The τ_f reaction appeared to reflect a class of reactions, not merely the hinge bending near the active site, and it strongly depended on the microscopic viscosity, not on the macroscopic viscosity, of the solution (Figure 5). This conclusion is drawn from the following consideration. If the τ_f reaction detected the specific hinge bending involving opening and closing of the groove containing the active site, the reaction should either disappear or be greatly affected by the presence of 2′-CMP. This was found not to be the case (Figure 6). Neither the relaxation time nor the amplitude of the relaxation signal was altered by the addition of 2′-CMP when the kinetics were measured in a highly viscous medium. Hinge bending involving motion of a short peptide chain is unlikely to depend on solvent viscosity unless the peptide chain is longer than several peptide units (Hass et al., 1978). (2) The three relaxations detected were seen in the entire thermal transition zone, even at 25 °C, a temperature 36 °C below the T_m, although they all diminished in a 5 M Gdn·HCl solution. The τ_1 and τ_2 reactions resembled the folding–unfolding reactions monitored by tyrosine absorption and the recovery of 2′-CMP binding ability (Garel & Baldwin, 1973).

The molecular origin of the viscosity-dependent τ_f reaction is not yet clear. Two possible sources are offered. First, if all of the protein molecules are in the folded state at 25 °C, the τ_f reaction could reflect peptide chain motions that facilitate solvent permeation into the protein structure. This type of conformational "breathing" has been suggested from the hydrogen-exchange experiments (Englander et al., 1972; Woodward & Hilton, 1979) and from the quenching of the intrinsic fluorescence of the protein by molecular oxygen (Lakowicz & Weber, 1973).

The second possible interpretation of the τ_f reaction assumes that even at 25 °C fractional unfolding of the protein may occur (Kanehisa & Tsong, 1978, 1979a,b). In such a case, τ_f would measure an early step in the protein chain folding [see, e.g., Eigen & de Maeyer (1963) for an analysis of relaxation kinetics]. Karplus & Weaver (1976, 1979) have formulated a diffusion–collision model of protein folding and predict that the relaxation time of the merging of structure embryos depends on the solvent viscosity. In the present study, we have shown that of the three relaxations detected in the thermal unfolding zone, only the fastest submillisecond relaxation (τ_f) depends on the solvent viscosity. This could mean that in the chain folding process external friction-limited chain motions (Hass et al., 1978) subside in the submillisecond time range. Following this initial reaction, the chain folding is dominated by internal frictions and proceeds through discrete intermediate states (Tsong & Kanehisa, 1980). Candidates for the basic assembly unit of proteins have been discussed (Lim, 1974a,b; Ptitsyn & Finkelstein, 1980; Tanaka & Scheraga, 1977; Matheson & Scheraga, 1978; Rose & Roy, 1980; Kanehisa & Tsong, 1978, 1980). The molecular nature of the τ_1 and τ_2 reactions has been discussed by Baldwin and his co-workers [see, e.g., Baldwin (1980)].

Acknowledgments

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