Comparison of Kinetics of Formation of Helices and Hydrophobic Core during the Folding of Staphylococcal Nuclease from Acid

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ABSTRACT Our previous kinetic study of the acid and base-induced folding/unfolding transitions of staphylococcal nuclease (SNase) has monitored Trp-140 fluorescence. Trp-140 is located near the flexible COOH terminus and whether or not its fluorescence reflects the overall conformation of the protein has yet to be established. Here we show that the fluorescence intensity of Try-140 correlated closely with the thermal stability (i.e., the calorimetric enthalpy, ΔH_{cal}, of unfolding) of the protein in the pH range 7 to 2.5, confirming that it is a good measure of the overall protein structure. Circular dichroism (CD) at 222 nm, which reflects the helical content of the protein molecule, was used to follow the same folding/unfolding transition in order to compare kinetics of the helix formation and of the appearance of the hydrophobic core. In addition to the three kinetic phases reported earlier with the fluorescence detection, there were a rapid reaction (completed within the 25 ms mixing time of the instrument), which comprised 15% of the signal, and a very slow reaction (time constant > 300 s), which comprised 19% of the signal. With the fluorescence detection for the folding from acid, only 5% of the signal occurred in the rapid phase and there was no reaction slower than 300 s. By comparing kinetics of folding at pH 7 by the CD and fluorescence detection methods, we concluded that: (*a*) Roughly 15% of the helix content of SNase accumulated before significant changes in the hydrophobic environment (<5%) of Trp-140 could be detected. The rapid appearance of CD signal reminiscent of helix formation within 25 ms would be consistent with the framework model of protein folding. Note, however, that, 15% of the 22% helix content of the protein amounts to an equivalent of fewer than 5 amino acid residues. (*b*) For the time-resolved signal between 2 ms and 300 s, kinetics measured by both properties are consistent with the sequential model, $D_4 = D_3 = D_2 = D_1 = N_0$ (the four Ds are the four substates of the denatured protein and N_0 is the native state). The major folding step by both signals is the D_1 to N_0 transition, which gave approximately a 50% change in fluorescence and CD and had a time constant of 160 ms at 25°C, pH 7.0. (*c*) The slow phase with the CD signal (>300 s), which is insensitive to Trp-140 fluorescence, has been assigned to be the cis/trans isomerization of Pro-117 by other studies. (*d*) Kinetics in the unfolding direction are consistent with the above interpretation.

INTRODUCTION

To understand the mechanisms of protein folding, it is crucial to characterize early intermediates in the chain-folding process. Does the secondary structure of a protein, especially helices, forms before the growth of the hydrophobic core, as was postulated by the framework model (Kim and Baldwin, 1982; Kim and Baldwim, 1990; Roder et al., 1988; Udgaonkar and Baldwin, 1988), or do the hydrophobic residues collapse to form a compact unfolded state (Dill, 1990; Kanehisa and Tsong, 1978, 1980), or a molten globule (Dolgikh et al., 1984; Kuwajima, 1989), on which the secondary structure grows, as was postulated by the hydrophobic collapse model? Both models have proponents and detractors, and there is experimental evidence from nuclear magnetic resonance (NMR) studies that supports either models (Neri et al., 1992; Roder et al., 1988; Udgaonkar and Baldwin, 1988). Other studies have considered long range electrostatic interactions to be an important determinant in the chain condensation process, and there is experimental evidence that supports this concept (Perry et al., 1989; Sharp and Honig, 1990). Our previous study of kinetics of the acid and base induced folding/unfolding of Staphylococcal nuclease (SNase) has found that the acid and base-denatured protein is a mixture of three unfolded species, D_1, D_2, and D_3, whereas the native protein at neutral pH is composed of a single species, N_0 (Chen et al., 1991, 1992a,b). The kinetics were monitored by the fluorescence intensity of Trp-140, which reflects the hydrophobic environment of the tryptophan. Our result was in conflict with the NMR studies that have shown that the native state is a mixture of three species, two monomers N and N* at low protein concentration, and an additional dimer N″ at higher protein concentrations (Alexandrescu et al., 1989; Evans et al., 1987; Evans et al., 1989; Fox et al., 1986). The unfolded state is shown to be composed of two isomers, U and U*, in slow equilibrium (Evans et al., 1987; Evans et al., 1989; Fox et al., 1986). The circular dichroism (CD) stopped-flow studies of folding from the urea-unfolded SNase have detected complex kinetics (final urea concentration 0.4 M), one of which (30% of the signal) was completed within the 15-ms mixing time of their instrument, and another was in the 500-s time range (Kuwajima et al., 1991; Sugawara et al., 1991). These CD kinetic studies were for the urea-induced folding/unfolding and cannot be compared with our previous studies of the acid-induced folding/unfolding with the fluorescence detection. Furthermore, since Trp-140 is located near the flexible

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COOH-terminus of the protein, questions have been raised whether or not its fluorescence is an adequate reflection of the overall conformation of the protein. In this communication, we show first that the intensity of Trp-140 fluorescence correlates with the overall stability of SNase as measured by DSC and, second, that kinetics of the time-resolved acid-induced folding/unfolding transition of SNase by the CD detection are significantly different from our early results, the main one being that a reaction much slower than 300 s was detected with the CD signal. This comparison allowed us to gain some insight on the nature of the early intermediates in the folding from acid.

MATERIALS AND METHODS

SNase was purified according to the procedure of Shortle and Meeker (1986). *Escherichia coli* AR120 carrying the plasmid pL9 containing the nuclease gene was provided by Dr. David Shortle of the Johns Hopkins University School of Medicine. The *E. coli* was grown in a 120-l fermentor. After protein purification, the purity of SNase was checked by SDS/page with Coomassie blue stain and was judged to be greater than 98%. Protein concentration was determined using the optical density at 280 nm, 0.93 at 1 mg/ml (Chen et al., 1991, 1992a,b). SNase aggregates at concentration above 70 μM (Chen et al., 1992a). All our experiments were done with a starting concentration of less than 70 μM. Differential scanning microcalorimetry (DSC) was performed with an MC2 microcalorimeter by MicroCal. A scanning rate of 1°C/min was used (Chen et al., 1991; Privalov, 1979; Privalov and Gill, 1988). Analysis of DSC data has been described elsewhere (Chen et al., 1991; Tsong et al., 1970). All DSC and kinetic experiments were done in the presence of 0.1 M NaCl, 50 mM potassium phosphate, 50 mM sodium acetate, 50 mM glycine, and 20 μM EDTA, at 25°C.

CD measurements of equilibrium unfolding were performed with a Jasco Model 700 spectropolarimeter. Acid-induced unfolding at different temperatures was done in order to obtain the number of protons absorbed upon unfolding. CD Kinetics were done with the stopped-flow attachment of the Jasco, which has a nominal dead time of 25 ms. Each kinetic record is an average of 10 measurements. As was explained in detail elsewhere, the time resolved signal from 25 ms to 5 s was fit into two relaxation processes, and that from 5 to 300 s was fit into one. A time constant of a signal slower than 300 s was not resolved because of the instability of the signal beyond 15 min, due to slow drifts of baseline. Both the amplitudes of signals faster than 25 ms and those slower than 300 s were obtained from the equilibrium values. Deconvolution of a kinetic record was done as described previously. The uncertainty for kinetic constants is ±15% and for amplitudes is ±5%. For fluorescence detection, the equilibrium unfolding was measured with an Aminco-Bowman spectrofluorometer and kinetics were measured with a Hi-Tech Model PQ/SF-53 stopped-flow apparatus, as was described in details elsewhere (Chen et al., 1991, 1992a,b).

RESULTS

Trp-140 fluorescence intensity correlates with protein stability

SNase unfolds at acidic pH and refolds at neutral pH, with a midpoint of transition at pH 3.8 (Chen et al., 1991, 1992a,b). The fluorescence intensity of the acid or base-denatured protein is approximately 20% that of the native SNase at pH 7.0. Thermal denaturation with DSC was used to measured the stability of the protein at different pH values. The enthalpy of denaturation, ΔH_{cal}, was 85.4 kcal/mol at pH 7.0, as was reported in the literature, but it diminished to zero at pH 3.0. At pH between 7 and 3, ΔH_{cal} fell between 85.4 kcal/mol and zero. Fig. 1 *A* shows three typical DSC runs, curve 1 at pH 7.0, curve 2 at pH 3.6 and curve 3 at pH 3.0. Fig. 1, *B* shows that the changes in the Trp-140 fluorescence intensity upon acidic unfolding of SNase paralleled changes in the protein thermal stability, or the ΔH_{cal} of unfolding, in the pH range 7.0 to 2.5.

Equilibrium unfolding of helices and hydrophobic core

The CD of SNase at pH 7.0, 25°C indicates that the native state has 22% helix, 38% β-sheet, 7.7% turn, and 32.4%

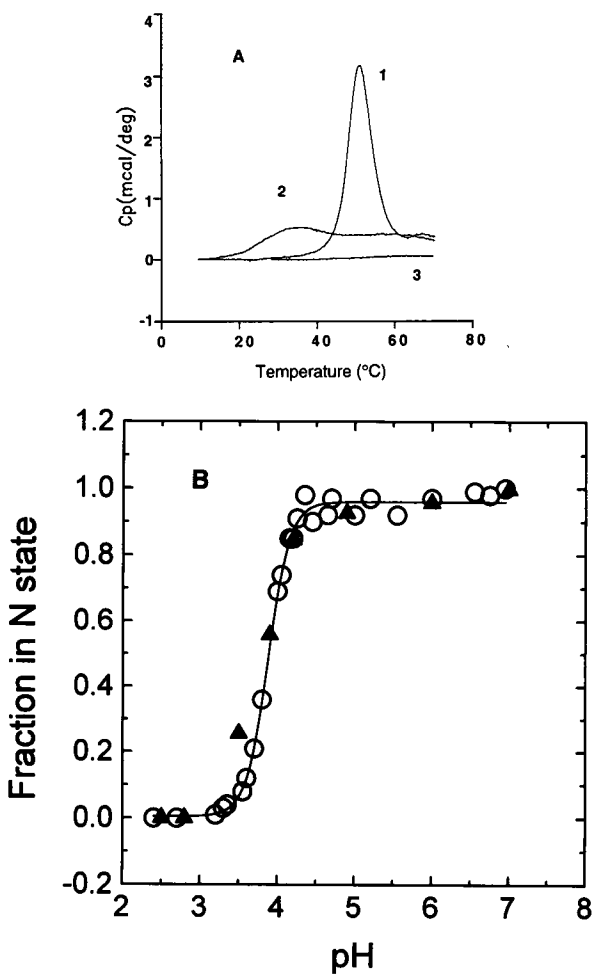

FIGURE 1 (*A*) Differential scanning microcalorimetry of staphylococcal nuclease at different pHs. DSC was performed with a MC2 microcalorimeter by Micro Cal (Amherst, MA), with digital data acquisition capability. Protein concentrations of 1.00 mg/ml were used. The scanning rate was 1°C/min. The buffer contained 0.1 M NaCl, 0.05 M phosphate, 0.05 M acetate, 0.05 M glycine, and 20 μM EDTA. Standard data analysis of Privalov was used to extract ΔH_{cal} of thermal unfolding.(*B*) Comparison of Trp-140 fluorescence intensity (25°C) with the thermal stability of SNase. The fluorescence intensity of Trp-140 (○) is scaled to compare with the ΔH_{cal} of thermal unfolding (▲) in the pH range 7.5 to 2.5. For the fluorescence measurement, the protein concentration was 8 μM. The excitation wavelength was 285 nm and the emission at 335 nm was monitored. In the ordinate, unity means 85.4 kcal/mol and zero means 0 kcal/mol for ΔH_{cal}. The fluorescence intensity of SNase at pH 2.5 is about 18% that of the protein at pH 7.0 (Chen et al., 1991).

random coil (Chen et al., 1991). At pH below 3, the helical content of the protein diminishes, although the CD at this pH resembles that of a protein where 50% is β-sheet. At 25°C, the equilibrium acid titration curves by both CD and fluorescence detection exhibit a sharp transition at pH 3.8, suggesting that protonation of certain titratable groups, such as glutamic acids, aspartic acids, or histidines, destabilizes the protein (Fig. 2, *A* and *B*). A series of equilibrium pH titration curves, such as those shown in Fig. 2, were obtained at different temperatures and the Henderson-Haselbalch plot for each titration curve was then constructed to obtain the number of protons absorbed upon unfolding at a specified temperature. The results are given in the insets of Fig. 2, *A* and *B*. Both CD data and fluorescence data agree within experimental uncertainty in terms of the numbers of protons absorbed upon unfolding at different temperatures. At 25°C, the unfolding of SNase leads to the absorption of an average of 2.5 protons. This number decreased steadily to less than one above 50°C. These proton absorption curves do not show a sharp transition around 53.3°C, which is the melting temperature of SNase (T_m) measured by differential scanning calorimetry (Fig. 1). The changes in the CD at 222 nm reflect melting of the helix (Fig. 2A) whereas the changes in Trp-140 fluorescence presumably reflect melting of the hydrophobic core (Fig. 2 *B*).

Kinetics of the formation/melting of helices

Fig. 3 *A* gives a typical CD stopped-flow kinetic record of the folding from pH 3.0 to 7.0 in the time range 5 to 300 s. The solid curve writing through the signal is the fitted curve for a single exponential decay. Signal that occurred faster than 5 s and slower than 25 ms is shown in curve 2, Fig. 3 *B*. Curve 1 of Fig. 3 *B* is the equilibrium position of signal at pH 3.0 and was obtained by mixing a protein solution at pH 3.0 with a buffer of the same pH. Curve 3 is the equilibrium position at pH 7 (concentration due to mixing corrected), which was obtained by mixing a protein solution of pH 7.0 with a buffer of the same pH. Curve 2 was fitted into two exponential decays and the fitted curve is represented by the solid curve. The amplitude for reactions faster than the 25 ms mixing time of the instrument was 15% and for reactions slower than 300 s was 19%. These data show that there are at least five kinetic phases in folding, of which three that occurred between 25 ms and 300 s can be studied with the CD stopped-flow with reasonable precision.

For unfolding from pH 7.0 to 3.1, only one kinetic phase was time-resolved (25 ms to 300 s), as was the case with the fluorescence detection. A typical kinetic record and the fitted curve are shown in Fig. 3 *C* (curve 2), along with two reference curves (curves 1 and 3). Here 14% of the signal occurred within the 25-ms deadtime of the instrument and 25% was too slow to be resolved. In other words, there are a total of three kinetic phases in the unfolding. A complete set of data with the CD detection is summarized in Table 1 to be compared with previous fluorescence kinetic results for the same folding/unfolding transition.

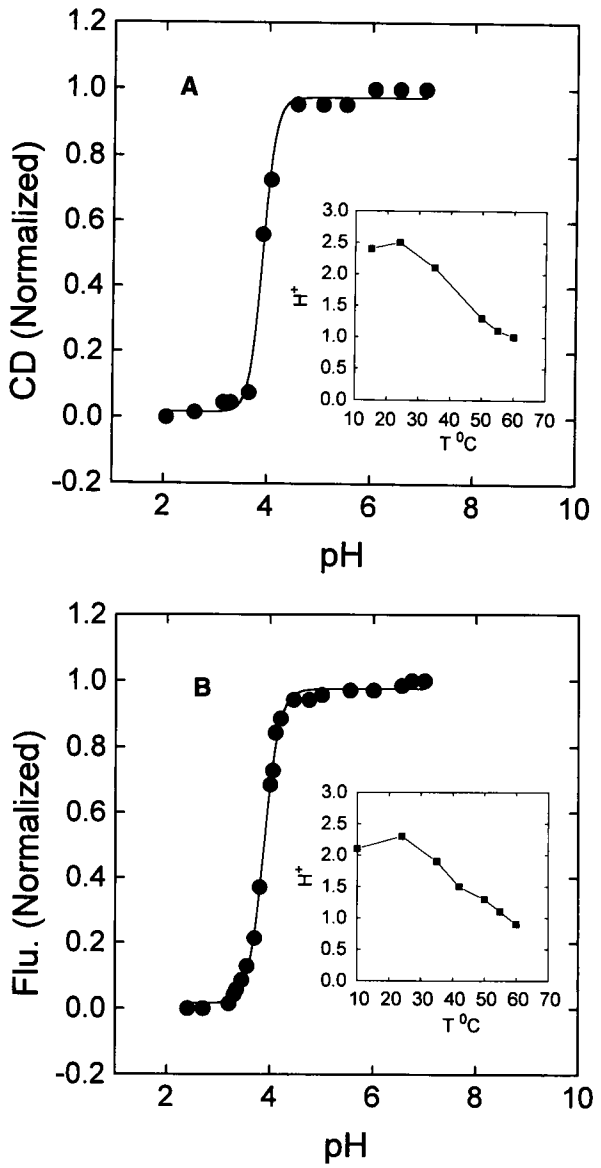

FIGURE 2 (*A*) pH titration of staphylococcal nuclease at 24°C, monitored by the CD at 222 nm (*B*) and the fluorescence of Trp-140. Similar pH titrations were done at different temperatures. The Henderson-Haselbalch plot was constructed to extract the number of proton absorbed upon unfolding of the protein, at different temperatures. These results are given in the insets. In (*A*), the normalized CD intensity of unity is equal to the ellipticity value of −9200°-cm²/decimol and of zero is equal to −2800°-cm²/decimol. The number of proton absorbed upon thermal unfolding at a given pH represents an average value.

DISCUSSION

The acid-induced unfolding of SNase is known to be highly cooperative (Anfinsen et al., 1971; Cuatrecasas et al., 1968). The pH titration curves (acidic unfolding) measured with different properties of the protein, such as tryptophan fluorescence, tyrosine absorption, intrinsic viscosity, molar ellipticity at 220 nm, chemical shifts of proton NMR, etc., all coincided (Anfinsen et al., 1971; Cuatrecasas et al., 1968). DSC data shown in Fig. 1 *B* agree with these results. In addition, analysis of DSC data based on the van't Hoff equa-

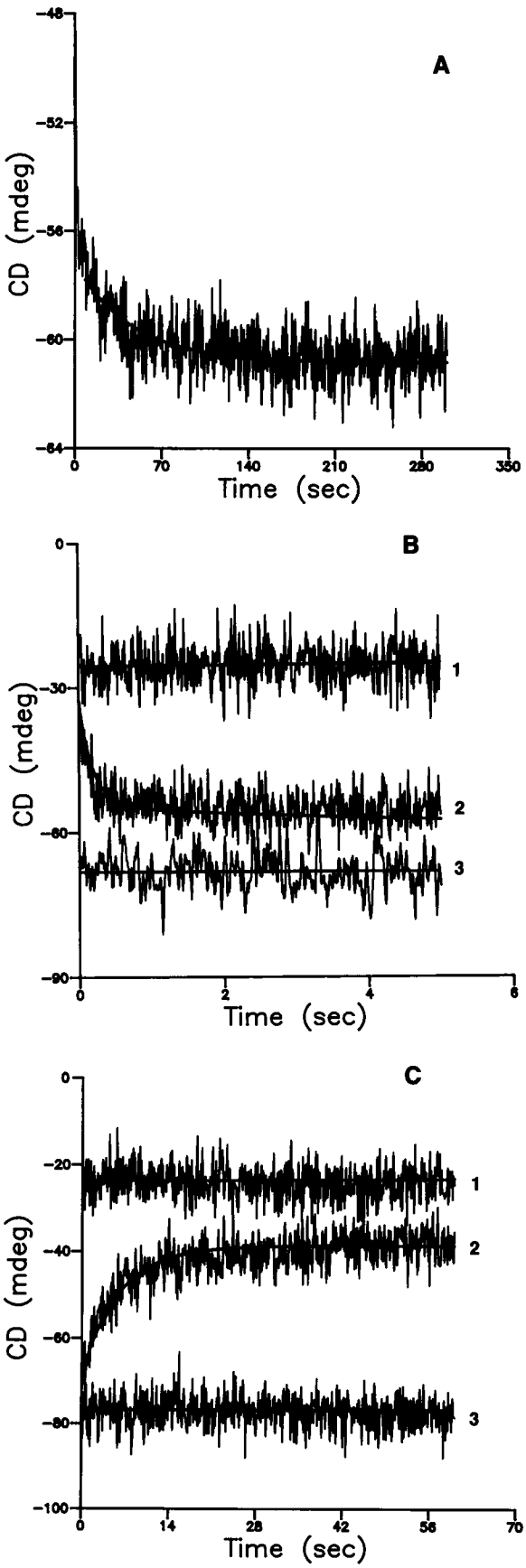

FIGURE 3 Kinetics of folding (*A*) and (*B*) and unfolding (*C*) of staphylococcal nuclease, monitored with changes of CD at 222 nm. The folding was from pH 3.0 to pH 7.0 and the unfolding was from pH 7.0 to pH 3.1. Each kinetic record is the average of 10 separate experiments. (*A*) shows the τ_3 reaction in folding (5–300 s). (*B*) Curve 1 is the baseline at pH 3.0 and curve 3 is the baseline at pH 7.0. Curve 2 records both the $\tau_1$and the τ_2' reactions. (*C*) shows the baselines at pH 3.1 (*curve 1*) and pH 7.0 (*curve 3*), and the kinetics of unfolding (*curve 2*). The time resolved folding reaction (*curve 2*) fits single relaxation time τ_2'. All the solid lines writing across the signal are least square fits of kinetic records. For the assignment of the relaxation times to each kinetic step, see Chen et al. (1991).

TABLE 1 pH-Induced folding/unfolding of staphylococcal nuclease

	Int. pH	Final pH	Time Constant	Amplitude (%)
Unfolding				
Trp 140 Fluorescence	7.0	3.1	<2 ms (τ_f)	<5
			1.15 s (τ_2)	95
			>300 s (τ_s)	0
CD (222 nm)	7.0	3.1	<25 ms (τ_f)	14
			6.0 s (τ_2')	61
			>300 s (τ_s)	25
Folding				
Trp 140 Fluorescence	3.0	7.0	<2 ms (τ_f)	<5
			151 ms (τ_1)	53
			856 ms (τ_2)	32
			30 s (τ_3)	10
			>300 s (τ_s)	0
CD (222 nm)	3.0	7.0	<25 s (τ_f)	15
			189 ms (τ_1)	47
			5.3 s (τ_2')	9
			46.3 s (τ_3)	10
			>300 s (τ_s)	19

Fluorescence kinetic data are from the previous work of Chen, H. M., V. S. Markin, and T. Y. Tsong. Biochemistry. 31:1483–1491 (1992). For the assignment of kinetic constants to different relaxation times, see Panel B of Fig. 2 in the reference.

The midrange reaction (τ_2 and τ_2') in both folding and unfolding show considerable differences by the CD and fluorescence detections. A dash sign is added to these relaxation times by the CD detection to indicate this fact.

The uncertainty in CD kinetics was ±15% for the time constant and ±5% for the amplitude.

tion shows that $\Delta H_{vH} = \Delta H_{cal}$. These observations taken together suggest that, from the energetic point of view and under equilibrium conditions, there are only two discreet states, the native (N) and the denatured (D) states in the pH range studied.

However, kinetics of the same acid-induced folding/unfolding transition monitoring the Trp-140 fluorescence are much more complex (Chen et al., 1991, 1992a,b). The unfolding of SNase from pH 7.0 to 3.0 followed a single exponential decay process, consistent with the two state transition, but folding from pH 3.0 to 7.0 followed a triphasic kinetics, incompatible with the two-state model. These conflicting results may be explained by Scheme 1.

$$D_3 \rightleftarrows D_2 \rightleftarrows D_1 \rightleftarrows N_0$$

SCHEME 1

In the scheme, the three Ds are substates of the denatured protein and N_0 is the native state. These three Ds have similar free energies (ΔG between any two Ds < 0.5 kcal/mol; see

Chen et al., 1991, 1992a,b) and enthalpies, and their existence cannot be inferred from the DSC studies. However, these three Ds are separated by considerable activation energies (6 to 14 kcal/mol; see Chen et al., 1991, 1992a) and are kinetically distinct. According to this scheme, during the unfolding, only one relaxation process will be detectable for the N_0 to D_1 step. No fluorescence signals would be available for the subsequent steps, the D_1 to D_2 to D_3 reactions. In the refolding from pH 3, however, the fluorescence signal would be more complex. Because of the small free energy differences among the three D states, all D states will be populated at pH 3.0. Folding from D_1 to N_0 would give one relaxation time. Folding from D_2 to N_0 would give another relaxation if the D_2 to D_1 reaction is slower than the D_1 to N_0 reaction. Likewise, folding of D_3 to N_0 would give a third relaxation if the rate for the D_3 to D_2 reaction is smaller than that for the D_2 to D_1 reaction. Kinetic experiments by the single-jump and the double-jump methods all support Scheme 1 (Chen et al., 1991, 1992a). In the subsequent kinetic experiments performed inside the transition zone, i.e., pH 3 to 4, it was shown that each of the four species in Scheme 1 is composed of microscopic states in rapid equilibrium, which are separated neither by large ΔGs nor by large activation energies (Chen et al., 1992b).

In these analyses, only the three time-resolved kinetic phases were considered (Chen et al., 1992b). The unresolved reaction faster than 2 ms mixing time of the stopped-flow instrument, which constituted only 5% of signal, was omitted. To include such a rapid reaction, one would have to add an intermediate species, I, between the D_1 and the N_0. The transition would no longer behave like a two-state transition in an equilibrium measurement (for example by DSC) because the existence of an I would make the thermal unfolding curve broader than that expected for a two state transition (Privalov, 1979; Tsong et al., 1970). Note that Scheme 1 is a quasi-two-state transition (Chen et al., 1991). However, if an I is structurally similar to either N_0 or D_1, an equilibrium transition curve would not be sensitive enough to detect the existence of such a state. The introduction of an I state is consistent with the observation of a rapid kinetic phase in the CD experiment. Here the relative amplitude of the fast phase was approximately 3 times larger than that of the fast phase in the fluorescence experiments. One should caution that any signal too fast to be resolved by the stopped-flow method, may be due to the pH-dependent shift of the baselines. However, there is evidence that these signals are parts of the chain-folding reaction (Elöve et al., 1992; Kuwajima et al., 1991a,b; Neri et al., 1992; Perry et al., 1989). Wavelength dependence of fast unresolved signals revealed spectroscopic properties similar to the CD spectrum of an α-helix (Kuwajima et al., 1991a; Perry et al., 1989).

The main difference between kinetics by the CD detection and those by the fluorescence detection is that in the former, there is a slow reaction (much slower than 300 s) that is not seen in the latter. This slow reaction has been labeled the cis-trans isomerization of Pro117 in other studies (Evans et al., 1987, 1989; Fox et al., 1986; Kuwajima et al., 1991b).

Even for the three time-resolved kinetic phases, there are still some noticeable differences between the CD and the fluorescence results. The midrange reaction seems to be several times slower for the CD (τ_2') than for the fluorescence (τ_2) in both folding and unfolding directions. Obviously, these relaxation times have detected different reactions. Notwithstanding, the τ_1 reaction, in which the peptide gains its conformational energy (ΔG and ΔH) in folding, has similar time constants and amplitudes by the two methods. These results suggest that the main folding/unfolding reaction is highly cooperative; differences were observed only for reactions that are energetically peripheral to the main structural change. However, as was emphasized in our previous reports, despite the small energy differences among the three substates of the unfolded protein, the folding followed a unique pathway. It was shown that this was so because of the differences in the activation processes. In other words, the pathway of folding was kinetically controlled at least in the early stage.

Comparisons of the results in Table 1 allow us to derive some useful information. First, the acid-induced folding/unfolding of SNase is much more complex than Scheme 1 can explain. Beside the introduction of an I state to account for the fast reaction (<25 ms), another slow reaction (>300 s) would have to be added (Alexandrescu et al., 1989; Evans et al., 1987, 1989; Fox et al., 1986; Kuwajima et al., 1991b). It is interesting, however, that this slow rearrangement or adjustment of the protein's overall structure, which presumably resulted from proline isomerization, does not alter the hydrophobic environment of the Trp-140 significantly. Second, the amplitudes and the time constants of the three time-resolved kinetic phases are significantly different and these differences cannot be attributed to experimental uncertainty. The greatest difference is seen for the D_2 to D_1 transition of Scheme 1 (with the fluorescence detection), which was interpreted as the chain condensation reaction because of the dependence of its rate on the solvent viscosity (Chen, Markin and Tsong, to be published). This reaction was not detected with the CD signal. Instead the CD signal measured a reaction fivefold slower, suggesting that formation of a hydrophobic environment proceeds with different rate than the accumulation of helices. Third, for the fast unresolved reaction(s), CD has detected formation of 15% of the 22% helix content of SNase within 25 ms. This might be interpreted as due to formation of helix framework in folding. However, the changes in CD amount to an equivalent of only five amino acid residues in a 149-residue protein. Note, however, that the acid-denatured protein retains considerable sheet-like chain configuration, which, although has no ΔH_{cal} of stabilization (Chen et al., 1991, 1992a,b), may function as the framework. Fourth, for the step that the protein acquires most of the free energy and enthalpy of stabilization, i.e., the D_1 to N_0 transition, both detections gave identical results. This reaction involves approximately 50% of signal and with a

time constant of approximately 160 ms in both cases. One should also mention that the stopped-flow CD has been employed to study folding of other proteins, with similar results (Elöve et al., 1992; Kuwajima et al., 1991a).

There could be many mechanisms that would be consistent with the above observations. But, a unique mechanism would be difficult to determine without more experimental information and rigorous analysis. We favor a more cautious approach, to consider only signals that are time-resolved between 2 ms and 300 s. In this limited time range, only the τ_2 and the τ_2' reactions shown in Table 1 are significantly different for the two different detections. Apparently the two relaxation times detected two different steps in the chain-folding reactions, one was invisible by the tryptophan fluorescence and the other had little change in helicity. These considerations would suggest that the minimal model consistent with the time-resolved signals is,

$$D_4 \rightleftarrows D_3 \rightleftarrows D_2 \rightleftarrows D_1 \rightleftarrows N_0$$

SCHEME 2

In Scheme 2, fluorescence signal did not see the D_3 to D_2 transition, and CD signal did not see the D_2 to D_1 transition. The slow reaction seen by the CD (slower than 300 s) has been assigned to the *cis/trans* isomerization of Pro117 (Evans et al., 1987, 1989; Fox et al., 1986; Kuwajima, 1991b). Apparently the isomerization can take place in any of the five states because the amplitude of this phase was 25% in unfolding and 19% in folding. To account for results on the slow reaction, one would have to include a parallel sequence to Scheme 2, with all five species having their Pro117 in trans-form, assuming that all five specis in Scheme 2 have their Pro117 in cis-form. Each species in the two parallel sequences is allowed to interchange.

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REFERENCES

Alexandrescu, A. T., E. L. Ulrich, and J. L. Markley. 1989. Hydrogen-1 NMR evidence for three interconverting forms of staphylococcal nuclease: effects of mutations and solution conditions on their distribution. *Biochemistry.* 28:204–211.

Anfinsen, C. B., A. N. Schechter, and H. Taniuchi. 1971. Some aspects of the structure of staphylococcal nuclease. Part II. Studies in solution. *Cold Spring Harbor Symp. Quant. Biol.* 36:249–255.

Chen, H. M., J. L. You, V. S. Markin, and T. Y. Tsong. 1991. Kinetic analysis of the acid and the alkaline unfolded states of staphylococcal nuclease. *J. Mol. Biol.* 220:771–778.

Chen, H. M., V. S. Markin, and T. Y. Tsong. 1992a. pH-induced folding/unfolding of staphylococcal nuclease: determination of kinetic parameters by the sequential-jump method. *Biochemistry.* 31:1483–1491.

Chen, H. M., V. S. Markin, and T. Y. Tsong. 1992b. Kinetic evidence of microscopic states in protein folding. *Biochemistry.* 31:12369–12375.

Cuatrecasas, P., H. Taniuchi, and C. B. Anfinsen. 1968. The structural basis of the catalytic function of staphylococcal nuclease. *Brookhaven Symp. Biol.* 21:172–180.

Dill, K. A. 1990. Dominant forces in protein folding. *Biochemistry.* 29: 7133–7154.

Dolgikh, D. A., A. P. Kolomiets, J. A. Bolotina, and O. B. Ptitsyn. 1984. "Molten-globule" state accumulates in carbonic anhydrase folding. *FEBS Lett.* 165:88–92.

Elöve, G. A., A. F. Chaffotte, H. Roder, and M. E. Goldberg. 1992. Early steps in cytochrome c folding probed by time-resolved circular dichroism and fluorescence spectroscopy. *Biochemistry.* 31:6876–6883.

Evans, P. A., Dobson, C. M., Kautz, R. A., Hatfull, G., and Fox, R. O. 1987. Proline isomerization in staphylococcal nuclease characterized by NMR and site-directed mutagenesis. *Nature (Lond.).* 329:266–268.

Evans, P. A., R. A. Kautz, R. O. Fox, and C. M. Dobson. 1989. A magnetization-transfer nuclear magnetic resonance study of the folding of staphylococcal nuclease. *Biochemistry.* 28:362–370.

Fox, R. O., P. A. Evans, and C. M. Dobson. 1986. Multiple conformations of a protein demonstrated by magnetization transfer NMR spectroscopy. *Nature (Lond.).* 320:192–194.

Kanehisa, M. I., and T. Y. Tsong. 1978. Mechanism of multiphasic kinetics in the unfolding and refolding of proteins. *J. Mol. Biol.* 124:177–194.

Kanehisa, M. I., and T. Y. Tsong. 1980. Local hydrophobicity stabilizes secondary structures in proteins. *Biopolymers.* 19:1617–1628.

Kim, P. S., and R. L. Baldwin. 1982. Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding. *Annu. Rev. Biochem.* 51:459–489.

Kim, P. S., and R. L. Baldwin. 1990. Intermediates in the folding reactions of small proteins. *Annu. Rev. Biochem.* 59:631–660.

Kuwajima, K. 1989. The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure. *Proteins Struct. Funct. Genet.* 6:87–103.

Kuwajima, K., E. P. Garvey, B. E. Finn, C. R. Matthews, and S. Sugai. 1991. Transient intermediates in the folding of dihydrofolate reductase as detected by far-ultraviolet circular dichroism spectroscopy. *Biochemistry.* 30:7693–7703.

Kuwajima, K., N. Okayama, K. Yamamoto, T. Ishihara, and S. Sugai. 1991. The pro117 to glycine mutation of staphylococcal nuclease simplifies the unfolding-folding kinetics. *FEBS Lett.* 290:135–138.

Neri, D., M. Billeter, G. Wider, and K. Wüthrich. 1992. NMR determination of residual structure in urea-denatured protein, the 434-repressor. *Science (Washington DC).* 257:1559–1563.

Perry, K. M., J. J. Onuffer, M. S. Gettelman, L. Barmat, and C. R. Matthews. 1989. Long-range electrostatic interactions can influence the folding, stability, and cooperativity of dihydrofolate reductase. *Biochemistry.* 28: 7961–7968.

Privalov, P. L. 1979. Stability of proteins. *Adv. Protein Chem.* 33:167–241.

Privalov, P. L., and S. J. Gill. 1988. Stability of protein structure and hydrophobic interaction. *Adv. Protein Chem.* 39:191–234.

Roder, H., G. A. Elöve, and S. W. Englander. 1988. Structural characterization of folding intermediates in cytochrome c by H-exchange labeling and proton NMR. *Nature (Lond.).* 335:700–704.

Sharp, K. A., and B. Honig. 1990. Electrostatic interactions in macromolecules: theory and applications. *Annu. Rev. Biophys. Biophys. Chem.* 19: 301–332.

Shortle, D., and A. K. Meeker. 1986. Mutant forms of staphylococcal nuclease with altered patterns of guanidine hydrochloride and urea denaturation. *Proteins Struct. Funct. Genet.* 1:81–89.

Sugawara, T., K. Kuwajima, and S. Sugai. 1991. Folding of staphylococcal nuclease Astudied by equilibrium, and kinetic circular dichoism spectra. *Biochemistry.* 30:2698–2706.

Tsong, T. Y., R. P. Hearn, D. P. Wrathall, and J. M. Sturtevant. 1970. A calorimetric study of the thermally-induced conformational transitions of ribonuclease A and certain of its derivatives. *Biochemistry.* 9:2666–2677.

Tsong, T. Y., R. L. Baldwin, and P. McPhie. 1972. A sequential model of nucleation-dependent protein folding: kinetic studies of ribonuclease A. *J. Mol. Biol.* 63:453–475.

Udgaonkar, J. B., and Baldwin, R. L. 1988. NMR evidence for an early framework intermediate on the folding pathway of ribonuclease A. *Nature (Lond.).* 335:694–699.