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Folding of Staphylococcal Nuclease A Studied by Equilibrium and Kinetic Circular Dichroism Spectra†

Tatsuro Sugawara,‡ Kunihiro Kuwajima,* and Shintaro Sugai§
Department of Polymer Science, Faculty of Science, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060, Japan
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ABSTRACT: The urea-induced unfolding of staphylococcal nuclease A has been studied by circular dichroism both at equilibrium and by the kinetics of unfolding and refolding (pH 7.0 and 4.5 °C), as a function of Ca^{2+} and thymidine 3′,5′-diphosphate (pdTp) concentration. The results are as follows. (1) The unfolding transition is shifted to higher concentrations of urea by Ca^{2+} and pdTp, and the presence of both ligands further stabilizes the protein. (2) In the first stage of kinetic refolding, the peptide ellipticity changes rapidly within the dead time of stopped-flow measurement (15 ms), indicating accumulation of a transient intermediate. This intermediate is remarkably less stable than those of other globular proteins previously studied. (3) Dependence of the folding and unfolding rate constants on urea concentration indicates that the critical activated state of folding ("transition state") has considerable structural organization. The transition state does not, however, have the capacity to bind Ca^{2+} and pdTp, as indicated by the effects of these ligands on the unfolding rate constant. (4) There are at least four different phases in the refolding kinetics in native conditions below 1 M urea. In the absence of pdTp, there are two phases in unfolding, while in the presence of pdTp the unfolding kinetics show a single phase. Some characteristics of the transient intermediate and of the transition state for folding are discussed.

Elucidation of the mechanism of protein folding remains one of the major challenges in biochemistry. Two important approaches to this problem are (1) characterization of transient intermediates formed early in refolding and (2) examination of the kinetics of folding and unfolding to investigate the nature of the transition state for folding (Kim & Baldwin, 1982, 1990). Kinetic circular dichroism (CD)[1] measurements in the

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* Author to whom correspondence should be addressed.
‡ Present address: Institute of Biological Science, Mitsui Pharmaceuticals Inc., Mobara, Chiba 2974, Japan.
§ Present address: Institute of Life Science, Soka University, Tangicho, Hachioji, Tokyo 192, Japan.

[1] Abbreviations: CD, circular dichroism; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; NMR, nuclear magnetic resonance; pdTp, thymidine 3′,5′-diphosphate; SNase, staphylococcal nuclease; UV, ultraviolet.

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far-UV (peptide region) have been shown to be useful in both of these approaches (Labhardt, 1986; Kuwajima et al., 1987). Early transient folding intermediates have been detected for several globular proteins including lysozyme, α-lactalbumin, and parvalbumin; these intermediates have an appreciable amount of backbone secondary structure (Kuwajima et al., 1985, 1987, 1988). Also, these intermediates are related to the so-called "molten globule" state, whose characteristics have been investigated at equilibrium by various techniques (Kuwajima, 1989; Ptitsyn et al., 1990). Attention has recently been focused on the nature of the transition (A^*) state that may exist between the transient intermediate and the native state. Effects of specific ligands and of mutations, as well as environmental variables such as temperature and pH, have been studied to characterize the structure of the transition state (Segawa & Sugihara, 1984a,b; Matthews, 1987; Kuwajima et al., 1989; Chen et al., 1989; Matouschek et al., 1989, 1990).

Staphylococcal nuclease (SNase) A is a well-characterized globular protein consisting of 149 amino acid residues in a single peptide chain without disulfide bonds or cysteinyl residues (Tucker et al., 1978). This protein binds specifically Ca^{2+} and its inhibitor thymidine 3′,5′-diphosphate (pdTp). The X-ray structure of the liganded enzyme–Ca^{2+}–pdTp complex is known at 1.5-Å resolution (Tucker et al., 1979a,b; Cotton et al., 1979) and has recently been refined by Loll and Lattman (1989). Studies of the folding of SNase A were started some 20 years ago by Anfinsen's group (Anfinsen et al., 1972; Tucker et al., 1979c), and Taniuchi and his co-workers later used complementation by fragments of SNase to study the kinetics of unfolding by a fragment exchange technique (Taniuchi, 1973; Taniuchi & Bohnert, 1975; Taniuchi et al., 1986). Recent developments in the cloning and expression of SNase A and its mutants in *Escherichia coli* have made this protein particularly attractive for studies of protein folding (Shortle, 1983; Shortle & Meeker, 1986, 1989; Shortle et al., 1988). So far as we are aware, however, little is known about the structure and stability of the transient folding intermediate or of the A^* state in SNase A.

In this study, the equilibrium and kinetics of urea-induced unfolding and refolding of SNase A have been investigated in order to characterize the transient intermediate and the A^* state. The results are analyzed quantitatively, while taking account of effects of the specific ligands, Ca^{2+} and pdTp, and the stopped-flow CD technique is used to follow the kinetics of folding. As a first step, results are reported for the wild-type enzyme from the Foggi strain of *Staphylococcus aureus*. The transient intermediate found in the folding of SNase A is shown to be remarkably less stable than the intermediates previously characterized in other proteins. The A^* state of folding is shown to be well organized but unable to bind either Ca^{2+} or pdTp.

Materials and Methods

Chemicals. Urea was the specially prepared reagent grade for biochemical use from Nacalai Tesque, Inc. (Kyoto). The stock solution of urea was deionized on a mixed-bed column of Amberlite IR-120B and IRA-402 and used within a day. The concentration of urea was determined from the refractive index at 589 nm (Pace, 1986). The nucleotide pdTp was purchased from Calbiochem and also from Pharmacia. Its concentration was determined spectrophotometrically by using a molar extinction coefficient of 9700 $M^{-1}\cdot cm^{-1}$ at 267 nm (provided by Calbiochem) (Parker et al., 1981). All other chemicals were reagent grade.

Purification of SNase A. Cultures of *Staphylococcus aureus* Foggi strain (ATCC 27735) close to the stationary phase were prepared on a 100-L scale by continuous fermentation. The culture medium was essentially that of Taniuchi and Bohnert (1975) except for addition of 0.27 g of $CaCl_2$/L of the medium and NZ-amine A (Sheffield) was used instead of NZ-amine B. Crude SNase (~30% pure) was prepared by adsorption of the protein to phosphocellulose (Taniuchi & Bohnert, 1975) and was further purified by ion-exchange chromatography on a column of CM-Sephadex C-25 with a linear gradient of NaCl from 0 to 0.5 M in the presence of 50 mM Tris-HCl (pH 8.5) and by gel chromatography on a Sephadex G-100 column equilibrated with 0.1 M ammonium acetate (pH 8.0). The purity and the molecular weight of the protein were checked by SDS–polyacrylamide gel electrophoresis, and samples more than 95% pure were used. The final yield of purified SNase A was 400 mg from a 100-L culture. The protein concentration was determined spectrophotometrically by using an extinction coefficient $E^{1\%}_{1cm} = 9.3$ at 280 nm (Tucker et al., 1978).

Equilibrium Measurements. Equilibrium CD spectra were taken on a Jasco J-20 or J-500A spectropolarimeter as described previously (Kuwajima et al., 1985, 1988). The path length of the optical cuvette was 1.0 or 0.1 mm for measurements in the peptide region and 10.0 mm for measurements in the aromatic region. The CD data were reported as mean residue ellipticity by taking 113 as the mean residue weight (Tucker et al., 1978). All CD measurements were carried out at 4.5 °C. The protein concentration was typically 3.5×10^{-5} M. The protein solutions at neutral pH contained 50 mM sodium cacodylate and 50 mM NaCl to maintain pH and ionic strength. The solutions also contained urea as a denaturant and the indicated amounts of $CaCl_2$ and pdTp. To solutions without $CaCl_2$ was added 1 mM EGTA to assure complete removal of the protein-bound Ca^{2+}. The CD spectra in the acid state of SNase A were measured at pH 2.1 in 10 mM HCl plus 100 mM NaCl. The pH values are pH-meter readings at room temperature.

Kinetic Measurements. Refolding and unfolding were induced by concentration jumps of urea at pH 7.0 and 4.5 °C, by means of a stopped-flow apparatus (whose dead time of mixing is 15 ms) or by manual mixing (whose dead time is 0.5 or 30 s, depending on the path length of the cuvette; see below), and the kinetics were followed by the ellipticity in the peptide region using a Jasco J-500A spectropolarimeter (Kuwajima et al., 1985, 1987, 1988; Ikeguchi et al., 1986a). Usually, the reactions up to 1000 s were measured by the stopped-flow method, and longer reactions were measured by manual mixing. For refolding experiments, the protein was kept unfolded until equilibrium was reached. The unfolding conditions and the time required for equilibrium were 4 M urea and 2 h in the absence of ligand at 4.5 °C or in the presence either of Ca^{2+} or of pdTp alone, and 6 M urea and 3 h in the presence of both ligands (4.5 °C) (see Results). The path length of the optical cuvette in the stopped-flow measurements was 1.0 mm, and the protein concentration in the final solution was the same as in the equilibrium measurements. In manual mixing, the path length used was 10.0 mm when pdTp was absent, and the protein concentration was 10-fold more dilute than in the stopped-flow experiments. No dependence of the kinetics on protein concentration was found in the concentration range used in this study. Two solutions, a protein solution and a diluent, were mixed within the cuvette by a magnetic stirring mixer, and the dead time of mixing was ~0.5 s (Ikeguchi et al., 1986a). When pdTp was present, the path length of the cuvette must be 1.0 mm because of the absorbance of pdTp. The manual mixing was made in a test tube,

and then the solution was transferred quickly into the cuvette set in the spectropolarimeter (the dead time is ≤30 s).

Refolding kinetics starting from the acid state of SNase A were also measured in the same manner by the stopped-flow method. The reaction was induced by a pH jump after the acid protein solution (pH 2.1 to 2.5) was mixed with an alkaline solution to give the final pH of 7.0 in 0.1 M NaCl. Refolding from the acid state was measured in the absence of urea and at 0.4 M urea, and the final solutions contained 1 mM EGTA.

The kinetic data were fitted by nonlinear least-squares with the equation:

$$\theta(t) = \theta(\infty) + \sum_i \Delta\theta_i e^{-k_i t} \quad (1)$$

where $\theta(t)$ and $\theta(\infty)$ are the observed ellipticities at time t and infinite time, respectively, k_i is the apparent first-order rate constant of the ith kinetic phase, and $\Delta\theta_i$ is the ellipticity change in the ith phase.

Results

CD Spectra. The CD spectra of SNase A are shown in Figure 1. The spectra of native SNase A agree with those reported in previous studies (Omenn et al., 1969; DiBello & Griffin, 1975; Griko et al., 1988; Shortle & Meeker, 1989), and they do not depend on the presence or absence of 10 mM $CaCl_2$. Addition of 1 mM pdTp increases the intensity at 220 nm but only by 10%, so that most of the ellipticity in the peptide region must arise from the protein. Addition of 8 M urea leads to the loss of all the native CD bands, indicating that the protein is fully unfolded in concentrated urea. In the acid state (pH 2.1), the aromatic CD spectrum is identical with that in the unfolded state in 8 M urea, but the peptide spectrum is remarkably different from both the native and the unfolded-state spectra. The acid state is thus partially unfolded.

Equilibrium Unfolding. The unfolding transition was followed by measuring the ellipticity at 225 nm (θ_{225}) as a function of urea concentration (pH 7.0 and 4.5 °C). The ligands Ca^{2+} and pdTp are known to bind native SNase A (Tucker et al., 1979a; Calderon et al., 1985), and so the unfolding equilibrium was investigated under four different conditions, in which the solutions contained (1) no ligand but 1 mM EGTA as a chelator, (2) 10 mM $CaCl_2$, (3) 1 mM pdTp and 1 mM EGTA, and (4) 1 mM pdTp plus 10 mM $CaCl_2$ (Figure 2). Reversibility of the transitions was checked by kinetic experiments using concentration jumps of urea (see below). The transition curves at 1 mM EGTA and at 10 mM $CaCl_2$ were also measured by the ellipticity at 276 nm, and they were found to coincide with those in Figure 2a. Addition of 10 mM $CaCl_2$ or 1 mM pdTp shifts the transition to a higher concentration of urea by 0.3–0.5 M, and addition of 1 mM pdTp together with 10 mM $CaCl_2$ further stabilizes SNase A.

The unfolding equilibria are analyzed by a two-state approximation in which only the native (N) and the unfolded (U) states are populated in the transition zone, as

$$N \rightleftharpoons U \quad (2)$$

Here, K_{NU} is the apparent equilibrium constant for unfolding and is related to the free energy change of unfolding, ΔG_{NU}, by

$$K_{NU} = e^{-\Delta G_{NU}/RT} \quad (3)$$

where R denotes the gas constant and T is the absolute temperature. For many globular proteins, ΔG_{NU} is known to vary linearly with denaturant concentration, c, in the transition zone (Pace, 1986; Pace et al., 1989), so that

$$\Delta G_{NU} = \Delta G_{NU}^{H_2O} - mc \quad (4)$$

where $\Delta G_{NU}^{H_2O}$ is found by linear extrapolation to 0 M urea and m is a measure of the dependence of ΔG_{NU} on c and represents a cooperativity index for the unfolding transition. In order to estimate $\Delta G_{NU}^{H_2O}$ and m, the values of the apparent fractional extent of unfolding, f_{app}, at various c values were calculated from the data of Figure 2 by the equation:

$$f_{app} = \frac{\theta_{obs} - \theta_N}{\theta_U - \theta_N} \quad (5)$$

where θ_{obs} represents the observed θ_{225} at equilibrium and θ_N and θ_U are the values in the N and U states, respectively. The θ_N values outside the native region were obtained experimentally from kinetic unfolding curves, using concentration

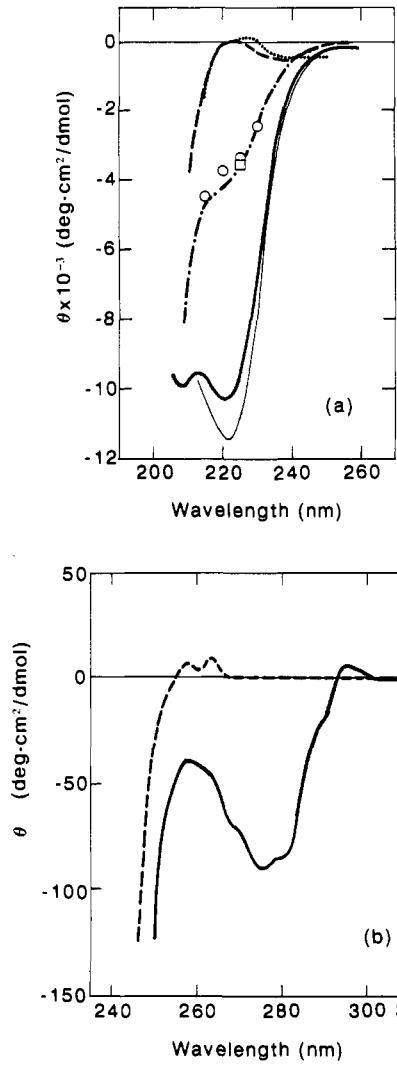

FIGURE 1: CD spectra of SNase A in the peptide (a) and aromatic (b) regions (4.5 °C). (a) Native state at 1 mM EGTA and pH 7.0 (thick curve); native state at 1 mM pdTp plus 10 mM $CaCl_2$ at pH 7.0 (—); unfolded state in 8 M urea at 1 mM EGTA and pH 7.0 (– –); unfolded state in 8 M urea at 1 mM pdTp plus 10 mM $CaCl_2$ at pH 7.0 (···); acid state at 100 mM NaCl and pH 2.1 in the absence of urea (–·–); $\theta(0)$ values in refolding induced by concentration jump of urea from 4 to 0.38 M at 1 mM EGTA and pH 7.0 (○) and by pH jump from pH 2.1 to 7.0 at 0 M urea in 0.1 M NaCl (□). (b) Lines refer to the same state as in (a), but the dashed line represents the spectra of both the urea-unfolded state at 1 mM EGTA and the acid state.

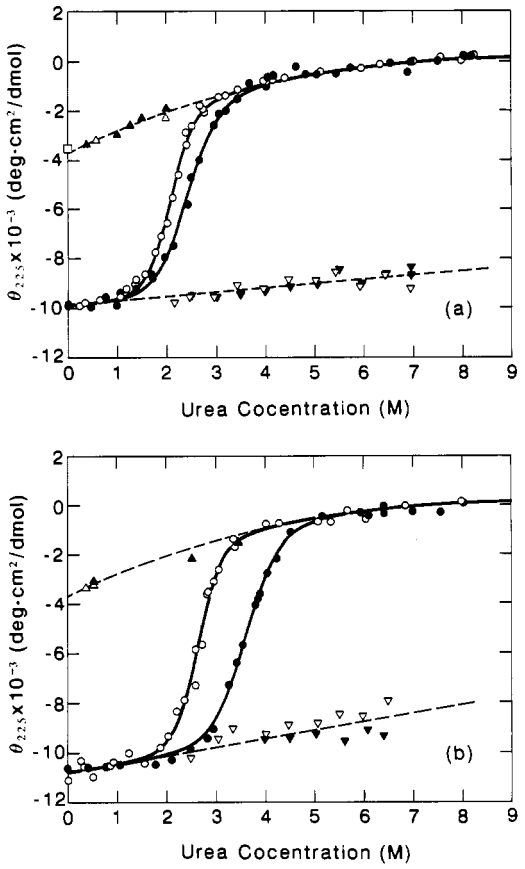

FIGURE 2: Unfolding transitions of SNase A measured by θ_{225} as a function of urea concentration at pH 7.0 and 4.5 °C. (a) Open symbols refer to the values in the absence of ligand at 1 mM EGTA and closed symbols to those at 10 mM $CaCl_2$. (b) Open symbols refer to the values at 1 mM pdTp with 1 mM EGTA and closed symbols to those at 1 mM pdTp plus 10 mM $CaCl_2$. In both (a) and (b), solid lines show the equilibrium unfolding curves (○, ●), and dashed lines show the dependence of the $\theta(0)$ values on urea concentration obtained from the kinetic refolding (△, ▲) and unfolding (▽, ▼) curves. An open square in (a) shows the $\theta(0)$ of refolding from the acid state induced by pH jump at 0 M urea.

jumps of urea from the N state (see below) (Figure 2). Similarly, the θ_U values were obtained from kinetic refolding curves. On the basis of the two-state model for unfolding, f_{app} is related to K_{NU} and hence to $\Delta G_{NU}^{H_2O}$ and m by the equation:

$$f_{app} = \frac{K_{NU}}{1 + K_{NU}} = \frac{\exp[(-\Delta G_{NU}^{H_2O} + mc)/RT]}{1 + \exp[(-\Delta G_{NU}^{H_2O} + mc)/RT]} \quad (6)$$

Values of $\Delta G_{NU}^{H_2O}$ and m were calculated from eq 6 by the method of nonlinear least squares, and they are summarized in Table I. The value of $\Delta G_{NU}^{H_2O}$ in the absence of ligand is a little larger than the value (3.7 kcal/mol at 4.5 °C) calculated from the thermodynamic parameters of the thermal transition reported by Griko et al. (1988), but it is smaller than the value (6.1 kcal/mol at 20 °C) previously reported for the urea-induced unfolding of SNase A by Shortle and Meeker (1986). The former difference in $\Delta G_{NU}^{H_2O}$ may be caused by a possible deviation from the linear relationship of eq 4 at low c values [see Shortle et al. (1989)], and the latter may arise from the difference in temperature, as the enthalpy change of unfolding is negative below 15 °C (Griko et al., 1988).

The difference in stability of SNase A, $\Delta(\Delta G_{NU})$, between the two conditions, in the presence and in the absence of a ligand, has been estimated by the equation:

$$\Delta(\Delta G_{NU}) = \Delta G_{NU,1}(\bar{c}) - \Delta G_{NU,0}(\bar{c}) \doteq \Delta c_{1/2}(m_0 + m_1)/2 \quad (7)$$

Table I: Parameters for Urea-Induced Unfolding of SNase A (pH 7.0 and 4.5 °C)

	$\Delta G_{NU}^{H_2O}$ (kcal·mol^{-1})	m (kcal·mol^{-1}·M^{-1})	$\Delta(\Delta G_{NU})$ (kcal·mol^{-1})
1 mM EGTA	4.64 ± 0.19	2.24 ± 0.09	
10 mM Ca^{2+}	4.17 ± 0.19	1.74 ± 0.08	$\Delta(\Delta G_{NU})_{Ca} = 0.65$ ($\bar{c}$ = 2.24 M)
1 mM pdTp, 1 mM EGTA	6.08 ± 0.47	2.31 ± 0.17	$\Delta(\Delta G_{NU})_{pdTp} = 1.28$ ($\bar{c}$ = 2.35 M)
1 mM pdTp, 10 mM Ca^{2+}	6.01 ± 0.24	1.67 ± 0.06	$\Delta(\Delta G_{NU})_{pdTp-Ca} = 2.99$ ($\bar{c}$ = 2.84 M)

where subscripts 0 and 1 refer to the absence and presence of ligand, respectively, $\Delta c_{1/2}$ is the difference in $c_{1/2}$ (the c at the midpoint of unfolding transition) between the two conditions, and $\Delta G_{NU,0}(\bar{c})$ and $\Delta G_{NU,1}(\bar{c})$ are the ΔG_{NU} values at a mean urea concentration given by $\bar{c} = (c_{1/2,0} + c_{1/2,1})/2$. This approach to evaluating $\Delta(\Delta G_{NU})$ was introduced by Pace et al. (1989) and may be useful for quantitating a real increase in protein stability caused by ligand binding [see Pace and Grimsley (1988)]. Because m depends on the presence or absence of Ca^{2+} (Table I), and also because of a possible deviation from the linear relationship of eq 4 at low c values in SNase A (Shortle et al., 1989), the $\Delta G_{NU}^{H_2O}$ calculated may not represent the real stability of the protein in the absence of urea, so that $\Delta(\Delta G_{NU})$ should be more reliable as a measure of the change in stability. The $\Delta(\Delta G_{NU})$ values were calculated for the following three sets of conditions: (1) 10 mM $CaCl_2$ vs no ligand; (2) 1 mM pdTp vs no ligand; and (3) 1 mM pdTp plus 10 mM $CaCl_2$ vs no ligand, and these are denoted by $\Delta(\Delta G_{NU})_{Ca}$, $\Delta(\Delta G_{NU})_{pdTp}$, and $\Delta(\Delta G_{NU})_{pdTp-Ca}$, respectively (Table I). $\Delta(\Delta G_{NU})_{Ca}$ and $\Delta(\Delta G_{NU})_{pdTp}$ are measures of stabilization of the protein by Ca^{2+} alone and by pdTp alone, respectively, and $\Delta(\Delta G_{NU})_{pdTp-Ca}$ provides a measure of synergistic stabilization by 1 mM pdTp plus 10 mM $CaCl_2$. The binding constants of the ligands to native SNase A were calculated from the respective $\Delta(\Delta G_{NU})$ values on the assumption that the unfolded molecule does not bind the ligands (data not shown), and they were found to be consistent with the known binding constants to the native protein reported by other researchers [summarized in Tucker et al. (1979a) and also see Serpersu et al. (1986)]. The stabilization of SNase A in the presence of Ca^{2+} and/or pdTp observed in this study is thus caused by simple displacement of the unfolding equilibrium by ligand binding to the N state.

Kinetic Folding and Unfolding Curves. Figure 3a,b shows a kinetic refolding curve of SNase A at 1 mM EGTA measured by θ_{225} in a stopped-flow experiment with a concentration jump of urea from 4.0 to 0.38 M. Essentially the same refolding curves were observed under other conditions in the presence of ligand. Curve fitting with eq 1 reveals that the kinetics are composed of at least four phases, with the fastest phase occurring within 1 s and the slowest phase having a time constant of 500 s. Because of the small amplitude of the third phase, the calculation with three exponentials also gave a reasonable fit, but nevertheless the presence of the fourth phase was apparent, because it was clearly discernible at a higher c value where the amplitude of the phase was increased (see Figure 5). A five-exponential fit to the observed curve did not show convergence in the nonlinear least-squares calculation. Multiphasic refolding of SNase has also been reported for the kinetics followed by tryptophan fluorescence (Davis et al., 1979; Nakano & Fink, 1990). The $\theta(\infty)$ obtained by the four-exponential fit agrees well with the value of native SNase A, indicating that the unfolding transition is fully reversible. The ellipticity value extrapolated to zero time [$\theta(0) = -3300$ deg·cm²/dmol) is, however, smaller than the value of the

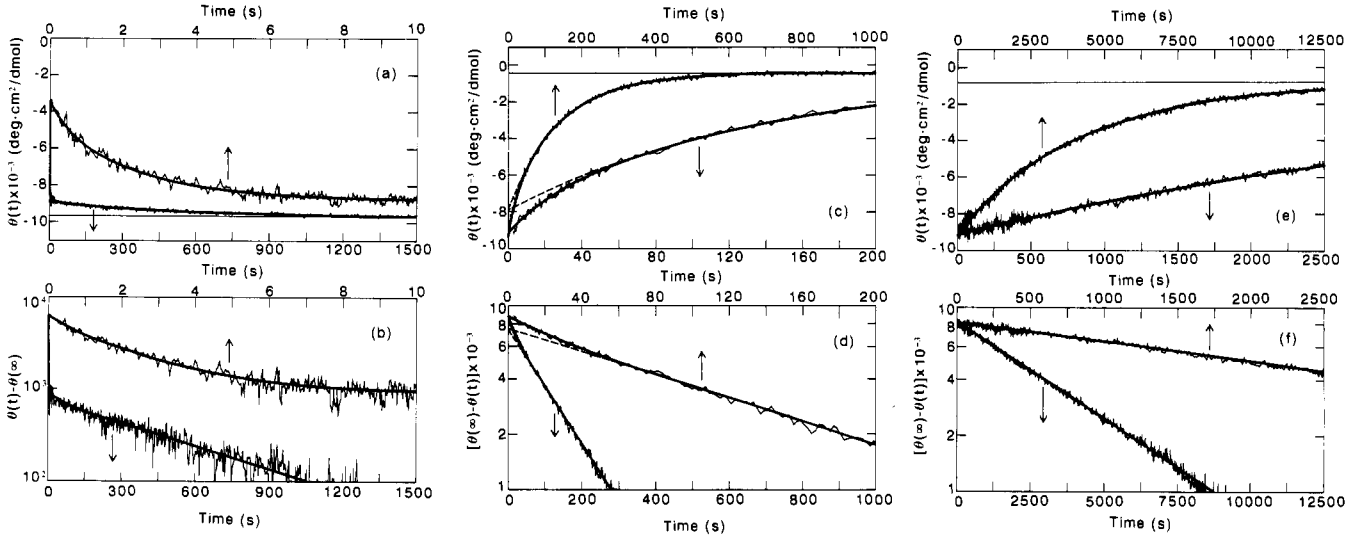

FIGURE 3: Kinetic progress curves of refolding [(a) and its logarithimic form in (b)] and unfolding [(c) and (e), and their logarithmic forms in (d) and (f), respectively] of SNase A measured by θ_{225} (pH 7.0 and 4.5 °C). Final conditions: (a) 0.38 M urea and 1 mM EGTA; (c) 5.9 M urea and 1 mM EGTA; and (e) 6.1 M urea and 1 mM pdTp plus 10 mM $CaCl_2$. Each reaction curve is shown in two different time scales. A thick solid line in each figure represents the theoretical curve according to eq 1. The best-fit parameter values are the following: (a) $\Delta\theta_1 = 1.0 \times 10^3$, $\Delta\theta_2 = 4.4 \times 10^3$, $\Delta\theta_3 = 2.1 \times 10^2$, $\Delta\theta_4 = 7.5 \times 10^2$, $k_1 = 2.7\ s^{-1}$, $k_2 = 0.45\ s^{-1}$, $k_3 = 0.022\ s^{-1}$, $k_4 = 0.0019\ s^{-1}$, and $\theta(\infty) = -9.7 \times 10^3$; (c) $\Delta\theta_1 = -1.2 \times 10^3$, $\Delta\theta_2 = -7.5 \times 10^3$, $k_1 = 0.049\ s^{-1}$, $k_2 = 0.0073\ s^{-1}$, and $\theta(\infty) = -4 \times 10^2$; (e) $\Delta\theta = -8.3 \times 10^3$, $k_1 = 2.4 \times 10^{-4}\ s^{-1}$, and $\theta(\infty) = -8 \times 10^2$. Dashed lines in (c) and (d) show the slow phase of the unfolding.

unfolded molecule at 4 M urea ($\theta_U = -800$ deg·cm²/dmol). The $\theta(0)$ values obtained at different wavelengths are compared with the equilibrium CD spectra in Figure 1a. The values coincide well with the spectrum of the acid state. The refolding kinetics of SNase A, starting from the acid state, were investigated by the pH-jump method. The kinetics observed were essentially identical with those in Figure 3a, so that the full difference in θ_{225} between the N and the acid states was observed kinetically; the $\theta(0)$ value and the values of k_i and $\Delta\theta_i$ of the four phases are shown in Figure 1a and Figures 4 and 5, respectively (see below). The results thus strongly suggest that the acid state in SNase A is identical with the transient state formed within the dead time of the stopped-flow method (15 ms), when refolding is measured starting from the urea-unfolded state. Such a transient state may be regarded as a folding intermediate which is still unfolded but nevertheless differs strongly in peptide structure from the unfolded state in concentrated urea (see Discussion). In Figure 2, the $\theta(0)$ values of refolding at different c values are plotted against c, and they were used as θ_U in the region where the protein was not unfolded at equilibrium to calculate the f_{app} based on the two-state approximation (see above). The validity and limitations of such a two-state analysis will be discussed later.

Kinetic unfolding reactions, brought about by concentration jumps of urea from 0 M to high concentrations, were also measured by θ_{225}. The rate of unfolding was found to be strongly retarded by ligand (see below), and also the kinetics depend on the ligand species present. Figure 3c,d shows kinetic unfolding curves in the absence of ligand at 5.9 M urea. Although the major kinetic phase occurs with a time constant of 140 s, the observed kinetics deviate from a single exponential and are well fitted with two exponentials. Similar behavior was also observed in unfolding at 10 mM $CaCl_2$. The presence of 1 mM pdTp, however, makes the kinetics obey a single-phase reaction. Figure 3e,f presents examples of the unfolding reaction observed at 1 mM pdTp plus 10 mM $CaCl_2$. A single-phase reaction was also observed at 1 mM pdTp without $CaCl_2$. The biphasic unfolding in the absence of pdTp may arise from the presence of two interconverting native species of SNase A, which has been suggested in the previous NMR

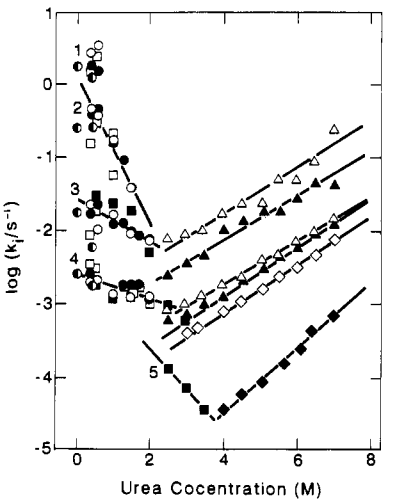

FIGURE 4: Dependence of the logarithmic rate constants of refolding and unfolding of SNase A on urea concentration (pH 7.0 and 4.5 °C). Conditions in refolding from the urea-unfolded state: 1 mM EGTA (○), 10 mM $CaCl_2$ (●), 1 mM pdTp with 1 mM EGTA (□), and 1 mM pdTp plus 10 mM $CaCl_2$ (■). Half-filled circles refer to the refolding reactions from the acid state induced by pH jump. Conditions in unfolding: 1 mM EGTA (△), 10 mM $CaCl_2$ (▲), 1 mM pdTp with 1 mM EGTA (◇), and 1 mM pdTp plus 10 mM $CaCl_2$ (◆).

studies and is caused by cis–trans isomerization around the Lys116–Pro117 peptide bound (Markley & Jardetzky, 1970; Fox et al., 1986; Evans et al., 1989; Alexandrescu et al., 1990). As this peptide bond is close to the binding site of pdTp, it may be cis only when the inhibitor is bound by the protein, as observed in the X-ray structure of liganded SNase A (Cotton et al., 1979). The curve-fitting analysis was thus made with a biphasic or single-phase function, depending on the conditions, and the $\theta(0)$ values obtained are plotted against c in Figure 2. Both in the absence and in the presence of pdTp, $\theta(0)$ varies linearly with c and is extrapolated to values coincident with θ_N at low c values. The value of $\theta(0)$ was thus regarded as θ_N in the region where the protein unfolds.

Effect of Urea on the Folding and Unfolding Kinetics. The observed rate constants for refolding and unfolding the SNase

Table II: Kinetic Parameters for Unfolding of SNase A (pH 7.0 and 4.5 °C)[a]

	log ($k_u^{H_2O}$/s^{-1})	m_{k_u} (M^{-1})	m_u^* (kcal·mol^{-1}·M^{-1})	m_u^*/m (%)	$\Delta(\Delta G_u^*)$ (kcal·mol^{-1})
1 mM EGTA	−3.93 ± 0.04	0.300 ± 0.007	0.38	17	
10 mM Ca^{2+}	−4.15 ± 0.05	0.324 ± 0.009	0.41	24	$\Delta(\Delta G_u^*)_{Ca} = 0.21$
1 mM pdTp, 1 mM EGTA	−4.45 ± 0.04	0.326 ± 0.006	0.41	18	$\Delta(\Delta G_u^*)_{pdTp} = 0.59$
1 mM pdTp, 10 mM Ca^{2+}	−6.46 ± 0.17	0.469 ± 0.027	0.60	36	$\Delta(\Delta G_u^*)_{pdTp-Ca} = 2.61$

[a] The values in the absence of pdTp refer to those of the slow phase that accounts for more than 85% of the total kinetics.

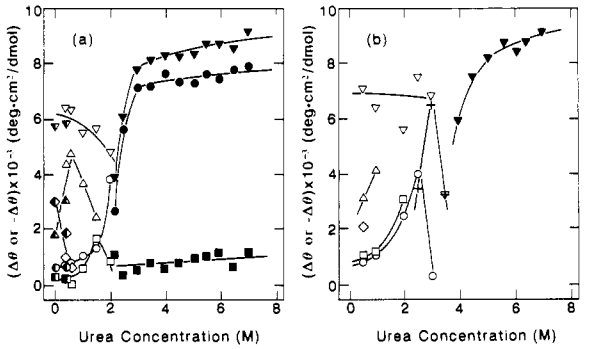

FIGURE 5: Dependence of the total amplitude and the amplitudes of different phases in refolding and unfolding kinetics of SNase A on urea concentration, at 1 mM EGTA (a) and at 1 mM pdTp plus 10 mM $CaCl_2$ (b) (pH 7.0 and 4.5 °C). Symbols for refolding: total (▽, ▼) and the first (◇, ◆), second (△, ▲), third (□, ■), fourth (○, ●), and fifth (+) phases. Half-filled symbols in (a) refer to the refolding from the acid state. Symbols for unfolding: total (▼) and the fast (■) and slow (●) phases. The fifth phase of refolding was observed only in the condition of (b), and the biphasic unfolding only in the condition of (a).

A are shown as a function of c in Figure 4. The amplitudes of the individual phases are plotted against c for two conditions, in the absence of ligand and at 1 mM pdTp plus 10 mM $CaCl_2$ (Figure 5a,b). Although the data are not shown, a similar dependence of the amplitudes on c was observed in the other conditions. At low c values (<1 M), where four phases are detected in refolding, the second phase from the fastest phase predominates in the kinetics (50–70% of the total kinetics), but in the kinetics of refolding from the acid state at 0 M urea, the first phase is bigger than the second phase. With increasing c, the slower phases become progressively predominant, and the faster phases diminish in amplitude. Above 2.5 M urea, a fifth phase that is even slower than the slowest phase observed at low c values appears in refolding at 1 mM pdTp plus 10 mM $CaCl_2$, and it predominates in the kinetics above 3 M urea under these conditions. In unfolding at 1 mM EGTA or at 10 mM $CaCl_2$, where the kinetics are biphasic, the slow phase accounts for more than 85% of the total kinetics at any urea concentration above 2.2 M.

The rate constant of any phase in refolding decreases with an increase in c except the first phase, for which the dependence on c is not discussed because the phase is detectable only in a narrow range of c (Figure 4). The unfolding rate increases with an increase in c, and its dependence on c is more or less similar for both the fast and the slow phases when the two phases are observed, and also for the single phase when pdTp is present. In summary, the changes with c of the individual rate constants and the amplitudes are characterized by a V-shaped dependence of the folding–unfolding rate with a minimum near the midpoint of the equilibrium unfolding transition. This phenomenon has been observed in a number of globular proteins and may be general in the kinetics of protein folding and unfolding (Kuwajima, 1989).

By analogy with the theory of absolute reaction rates, the highest free energy state (the critical activated state or transition state, A*) exists between the U and N states, and the rate constants of folding and unfolding (k_f and k_u) are related to the activation free energies (ΔG_f^* and ΔG_u^*) required for getting across A* in folding and unfolding, respectively:

$$\Delta G_f^* = (\text{constant}) - RT \ln k_f \quad (8)$$

$$\Delta G_u^* = (\text{constant}) - RT \ln k_u \quad (9)$$

The V-shaped dependence of the apparent rate constant on c arises from the linear relationships between ΔG_f^* and ΔG_u^* and c; such a linear dependence of the activation free energy on c is useful for characterizing the structure in the A* state (Kuwajima et al., 1989; Matouschek et al., 1989). As discussed above, however, the refolding kinetics of SNase A are complicated by the presence of several phases, so that it is difficult to evaluate the true k_f. Therefore, in the following, we only investigate ΔG_u^*. When unfolding is biphasic, we consider only the slow phase, and the contribution of the fast phase is ignored. Because the slow phase accounts for more than 85% of the kinetics, and also because both the fast and slow phases show similar dependences of the rate constant on c, any error introduced by this treatment must be trivial. The apparent rate constant for unfolding, in the region where the protein is fully unfolded, i.e., above 3.4 M urea in the absence of ligand, above 4 M urea in the presence of Ca^{2+} or pdTp, and above 5 M urea in the presence of both, is taken as k_u. From Figure 4, it is apparent that log k_u depends linearly on c, and so

$$\log k_u = \log k_u^{H_2O} + m_{k_u} c \quad (10)$$

Thus, ΔG_u^* is expressed by

$$\Delta G_u^* = \Delta G_u^{*H_2O} - m_u^* c \quad (11)$$

where $m_u^* = 2.303RTm_{k_u}$. The values of log $k_u^{H_2O}$, m_{k_u}, and m_u^* are summarized in Table II. Equation 11 is analogous to eq 4, and m and m_u^* in these equations are expected to be proportional to increases in solvent-accessible surface area of the protein molecule during unfolding from N to U and in the activation process from N to A*, respectively (Schellman, 1978, 1987; Kuwajima et al., 1989). Therefore, the fractional increase in the surface area of the protein in the A* state for unfolding relative to that on complete unfolding is given by m_u^*/m, and the values calculated range from 17 to 36% (Table II). If we assume that the A* state is the same for folding and unfolding, it follows that the degree of structural organization in terms of a decrease in accessible surface area in the A* state relative to N is 64–83% for folding from the U state. The A* state need not be the same for folding as for unfolding, because the conditions for folding and unfolding are not the same. A similar approach for characterizing the A* state has been used for α-lactalbumin and also for barnase [Kuwajima et al., 1989; Matouschek et al., 1989; see also Fersht et al. (1990)].

Effect of Ligand Binding on the Folding and Unfolding Kinetics. From Figure 4, only the unfolding rate constant is affected by ligand. The difference in ΔG_u^* in the presence

Table III: Restoration of the Peptide Structure at the First Stage of Refolding Measured by the CD Ellipticity in Various Globular Proteins

	α (%)	wavelength (nm)
β-lactoglobulin (pH 3.2, 4.5 °C)[a]	170	219
chymotrypsinogen A (pH 3.0, 4.5 °C)[b]	100	220
lysozyme (pH 1.5, 4.5 °C)[a]	90	222
α-lactalbumin (pH 7.0, 4.5 °C)[a]	80	222
ferricytochrome *c* (pH 7.0, 25 °C)[a]	80	222.5
parvalbumin (pH 7.0, 4.5 °C)[a]	60	222.5
tryptophan synthase β_2 (pH 7.8, 12 °C)[c]	60	225
staphylococcal nuclease A (pH 7.0, 4.5 °C)	30	225

[a] Kuwajima et al. (1985, 1987, 1988). [b] Kuwajima et al. (unpublished data). [c] Goldberg et al. (1990).

and in the absence of ligand has been estimated by the equation:

$$\Delta(\Delta G_u^*) = -RT \ln (k_{u,1}/k_{u,0}) \quad (12)$$

where subscripts 0 and 1 refer to the absence and presence of ligand, respectively, and the respective k_u values ($k_{u,0}$ and $k_{u,1}$) are given by eq 10. The $\Delta(\Delta G_u^*)$ values for the three sets of conditions [$\Delta(\Delta G_u^*)_{Ca}$, $\Delta(\Delta G_u^*)_{pdTp}$, and $\Delta(\Delta G_u^*)_{pdTp-Ca}$] are listed in Table II. Considering the number of approximations used to estimate these parameters, $\Delta(\Delta G_u^*)$ values are consistent with the corresponding values of $\Delta(\Delta G_{NU})$. Quantitatively, each $\Delta(\Delta G_u^*)$ is smaller than the $\Delta(\Delta G_{NU})$ by 0.4–0.7 kcal/mol, but the change in refolding kinetics caused by ligand binding has not been seen in refolding in native conditions (see Figure 4). This discrepancy may be explained by the structure of the A* state being slightly different in unfolding conditions and in native conditions [see Kuwajima et al. (1989)]. Consequently, the protein molecule does not bind any ligand to a detectable extent in the A* state of folding, and therefore the specific tertiary structure around the ligand binding sites is not yet organized in the A* state during folding.

Discussion

Two important questions for elucidating the folding mechanism of a protein are as follows: (1) What kind of structure is assumed early in refolding, and how stable is this structure; (2) what is the nature of the A* state between the early intermediate and the N state? The purpose of this study is to compare results for SNase A with those of other proteins studied previously, to obtain a general picture of the mechanism of protein folding.

Transient Folding Intermediate. For a number of globular proteins, a transient intermediate with an appreciable amount of backbone secondary structure is known to accumulate rapidly, within the dead time of measurement in most cases, in refolding from the fully unfolded state [Goldberg et al., 1990; Ptitsyn et al., 1980; Kiefhaber et al., 1990; Tandon & Horowitz, 1990; other references cited in Kuwajima (1989)]. The present results indicate that a transient folding intermediate also accumulates rapidly in refolding of SNase A. The peptide structure observed at this first stage of refolding is, however, remarkably less ordered than the structure observed for other globular proteins. An empirical measure (α) of the restoration of the structure within the dead time of measurement of refolding is given by

$$\alpha = \frac{\theta(0) - \theta_D}{\theta_N - \theta_D} \times 100 \quad (13)$$

where θ_D is the ellipticity in the fully unfolded state in a concentrated denaturant solution (6–8 M urea or 5–6 M guanidine hydrochloride). Table III summarizes the α values of various proteins that have been investigated by peptide CD spectra. It appears that the extent of forming the secondary structure varies strongly with protein species. The observation of stable, nativelike secondary structure at the first stage of refolding has been reported for several proteins (Kuwajima et al., 1985, 1987; Kuwajima, 1989), but this is clearly not a general rule in globular protein folding.

The values of $\theta(0)$ for refolding and also the equilibrium values of θ_U show gradual increases in θ_{225} with increasing *c*, similar to a saturation curve (Figure 2). A similar dependence of $\theta(0)$ on guanidine hydrochloride concentration was observed previously for parvalbumin (Kuwajima et al., 1988), while a sigmoidal dependence of $\theta(0)$ on *c* has been reported for α-lactalbumin and lysozyme (Ikeguchi et al., 1986a). There are two alternative interpretations for the gradual dependence on *c* of $\theta(0)$. (1) It reflects a gradual change in peptide structure in the unfolded state without cooperativity. (2) There is a cooperative transition between the fully unfolded (D) state and the transient folding intermediate (I), but nevertheless, a gradual transition curve is observed because the curve is truncated at low *c* owing to limited stability of the I state. Regarding these two possibilities, one should refer to the elegant work of Shortle and Meeker (1989). They have studied effects of various amino acid substitutions on the residual structure formed by large fragment of SNase A. Because the C-terminal 21 residues have been deleted, SNase(1–128) does not fold into the native structure, and the residual structure in this fragment may be a good model of the transient structure formed at the first stage of refolding of SNase A. The dependence of θ_{222} on the concentration of guanidine hydrochloride for wild-type SNase(1–128) reported by Shortle and Meeker is similar to the dependence of $\theta(0)$ on *c* observed in this study. A similar dependence of θ_{222} on *c* has been found in many mutants of SNase(1–128), but there are a few mutations that give rise to distinct sigmoidal transition curves with increased stability of the structure formed by the fragment. Cooperative structure formation in the wild-type and mutant fragments is indicated by coincidence of the transitions measured by θ_{222} and by the molecular-size parameter in gel filtration (Shortle & Meeker, 1989). The gradual increase in θ_{222} values of the fragments, or in the $\theta(0)$ values of intact SNase A, thus results from truncation of a cooperative transition curve between D and I. Protein folding may in general be represented by a two-stage process with a rapidly formed I state as an intermediate (eq 14), but nevertheless the stability

$$D \rightarrow I \rightarrow N \quad (14)$$

of the I state strongly depends on the protein species. This conclusion implies that the two-state theory, as used in this study, does not adequately describe the equilibrium unfolding behavior of globular proteins and that more rigorous analysis must be made by considering the presence of the I state. It is, however, impossible to make such a three-state analysis without knowledge of the molecular parameters for the pure I state. Thus, the equilibrium unfolding of SNase A in this study has been analyzed on the basis of the two-state theory by regarding both the I and D states as essentially unfolded (U) states. Because the population of the I state is sufficiently small in the transition zone, the thermodynamic parameters derived from this analysis do not introduce serious errors for wild-type SNase A, but this may not be the case for some mutants (Shortle & Meeker, 1986, 1989; Shortle et al., 1988). For proteins with a sufficiently stable I state, the three-state theory of equilibrium unfolding by guanidine hydrochloride has been well established (Robson & Pain, 1976; Kuwajima

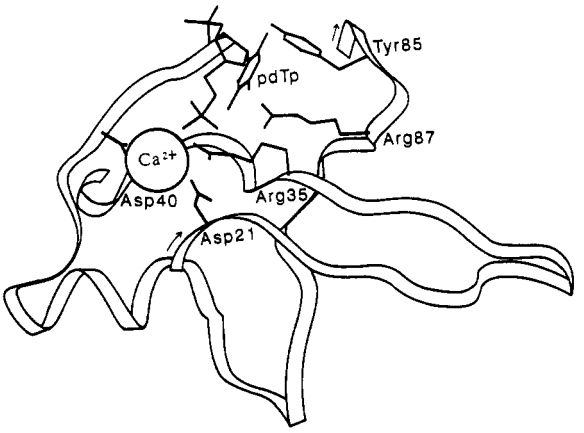

FIGURE 6: Structures around the ligand-binding site in SNase A (Cotton et al., 1979). The two peptide segments of (20–43) and (83–115), the Ca^{2+} ion, pdTp, and five side chains are shown. The file name in the Brookhaven Protein Data Bank used to draw the figure is 2SNS, and it has been obtained from Osaka University.

et al., 1976; Ikeguchi et al., 1986b; Kuwajima, 1989).

The A State.* In previous studies, the A* state of folding has been characterized by investigating the effects of denaturant and specific Ca^{2+} binding on the rate constants of folding and unfolding, in α-lactalbumin and parvalbumin (Kuwajima et al., 1988, 1989). Both of these are Ca^{2+}-binding proteins with binding constants of the order of 10^6–10^9 M^{-1}; α-lactalbumin has a single Ca^{2+}-binding site while parvalbumin has two binding sites (Hiraoka et al., 1980; Mitani et al., 1986; Hamano et al., 1986; Kretsinger, 1980). For α-lactalbumin, the effect of selective cleavage of a disulfide bond (Cys6–Cys120) on the rate constants has also been investigated (Kuwajima, 1989; Ikeguchi et al., unpublished results). The acceleration of the folding rate by Ca^{2+} has been taken as an indication that the specific structure responsible for Ca^{2+} binding is already restored in the A* state in these proteins. The denaturant guanidine hydrochloride affects both rate constants in both proteins, leading to a V-shaped dependence of the apparent rate constant on the denaturant concentration and indicating partial organization of the structure in the A* state, in terms of the accessibility to the denaturant molecule. It has been demonstrated that the structure in the A* state is characterized by local, specific, tertiary structure. The secondary structure segments preformed in the I state may be organized as in the native structure but only in a localized region; such local "native" structure in the A* state has been termed *critical substructure* (Kuwajima et al., 1989; Kuwajima, 1989).

The essential difference between SNase A and the two proteins mentioned above is that the specific structure required for ligand binding is not yet organized in the A* state in SNase A unlike the other proteins. In spite of a remarkable organization in terms of the accessible surface area (64–83% estimated from m and m^*), the A* state of SNase A does not have the capacity to bind the ligands Ca^{2+} and pdTp, as indicated by the selective effects of these ligands on the unfolding rate constant. Therefore, inspection of the structures around the ligand-binding sites in the three proteins may give us further insight into the structure of the A* state. The Ca^{2+}-binding sites of α-lactalbumin and parvalbumin are both helix–loop–helix substructures that are constructed from continuous peptide segments (Acharya et al., 1989; Moews & Kretsinger, 1975; Kretsinger, 1980). In contrast, the substructure for ligand binding in SNase A is constructed from discontinuous peptide segments (Cotton et al., 1979; Tucker et al., 1979b; Serpersu et al., 1987; Pourmotabbed et al., 1990). Figure 6 shows the X-ray structure of SNase A in its liganded form. Calcium ion coordinates with the carboxyl groups of two aspartyl residues (Asp21 and Asp40) and the carbonyl oxygen of Thr41. One of the two phosphate groups of pdTp is in close proximity to the side chains of Arg35 and Arg87, the other phosphate is close to Tyr85, and a hydrophobic pocket on the surface of SNase A may also be required for the interaction. Therefore, the difference in the effect of a ligand on folding between SNase A and the other two proteins is likely to arise from differences in construction of the ligand-binding substructures. The substructure constructed by a continuous peptide segment is possibly organized at an early stage of folding and can take part in organization of the critical substructure, but the substructure constructed by discontinuous peptide segments may be organized only at a late stage of folding after the A* state.

Conclusions. The early transient intermediate formed within 15 ms during refolding of SNase A has been characterized and compared with the intermediates of other globular proteins previously studied. The intermediate has a substantial amount of secondary structure, but its stability has been shown to depend on the protein species. The effects of ligand binding on folding and unfolding kinetics provide information about the specific substructure formed in the A* state. Comparison of the present result with those for two Ca^{2+}-binding proteins previously studied has given a picture of protein folding consistent with the sequential model of folding and with the hierarchical nature of protein structure.

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Registry No. pdTp, 2863-04-9; CA, 7440-70-2; staphylococcal nuclease, 9013-53-0.

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