Effect of Proline Mutations on the Stability and Kinetics of Folding of Staphylococcal Nuclease†

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ABSTRACT: The role of proline in the stability and kinetics of folding of wild-type staphylococcal nuclease and its P117G, P117T, and P31A mutants was examined as a function of guanidinium thiocyanate (Gdn·SCN) concentration. Replacement of Pro-117 with Gly or Thr caused small increases in stability, whereas substitution of Pro-31 by Ala led to a small decrease in stability. The slopes of the plots of ΔG against denaturant concentration (m) for the mutant proteins are significantly smaller than for the wild-type, suggesting a decrease in the solvent-accessible surface area of the denatured state relative to that of the wild-type. The rates of unfolding and refolding were monitored using tryptophan fluorescence. The kinetic traces for refolding in the presence of Gdn·SCN were triphasic for the wild-type protein and P31A but biphasic for P117G and P117T mutants. The slower phases were typically 10% of the total amplitude except in the transition region. The rates of the fastest and medium phases of the wild-type were essentially unaffected by the mutations. Double-jump experiments in which the protein was unfolded in a high concentration of denaturant for a short time period and then refolded to final Gdn·SCN concentrations near the C_m revealed a fast increase in fluorescence emission corresponding to formation of the native state, followed by a slower decrease with an amplitude that varied with the guanidine concentration and time of unfolding. The slower phase is ascribed to proline isomerization and was observed for wild-type and mutants at both proline positions, indicating the presence of a second, nonnative proline conformation in the unfolded protein. We conclude that the isomerization of Pro-117, which is in the less stable cis conformation in the native state, is responsible for the guanidine-independent slow-folding kinetic phase seen with the wild-type. An additional proline isomerization (or isomerizations), in which a significant amount of the nonnative (cis) conformation is formed in the unfolded state, is responsible for the intermediate kinetic phase seen with the wild-type and P31A mutant and the slow phase for the P117 mutants as well as the proline isomerization seen in the double-jump assays with the P117 mutants.

A number of hypotheses have been put forward to account for observations of multiphasic kinetics in the refolding of monomeric, single-domain proteins. One common explanation is the involvement of proline cis/trans isomerization. That the folding of small monomeric proteins may be complicated by cis/trans isomerization of proline has been known for some time (Brandts et al., 1975). It has usually been assumed in such cases that at least one proline is in the thermodynamically less stable cis conformation in the native state and that unfolding allows isomerization to the more stable trans conformation. As a consequence the refolding kinetics may appear multiphasic. Some characteristics of proline isomerization in refolding kinetics include its high energy of activation (approximately 20 kcal/mol), susceptibility to acid catalysis, and lack of sensitivity to the presence of denaturants such as urea or guanidine hydrochloride (Garel et al., 1976; Schmid & Baldwin, 1978).

Previous reports are consistent with proline isomerization being important in the refolding of staphylococcal nuclease (SNase).[1] The kinetics of refolding are triphasic (Davis et al., 1979; Nakano & Fink, 1990), and NMR spectra of the histidine aromatic region suggest at least two interconverting populations of the native state (Fox et al., 1986; Evans et al., 1987, 1989). Recently, this NMR heterogeneity has been shown to disappear on forming the ternary complex with calcium and the inhibitor pdTp and by replacement of Pro-117 with Gly (Evans et al., 1987).

SNase is a particularly well-suited protein for investigations of folding. Its advantages include high solubility, a single domain, the absence of disulfide bridges, reversibility of folding, the availability of high-resolution crystal structures (Hynes & Fox, 1991) and extensive NMR assignments and analysis, high-yield expression systems for the cloned gene (Shortle & Lin, 1985; Fox et al., 1986), and a representative structure consisting of both helices and β-sheets. The present investigation was undertaken to probe the role of proline isomerization in the folding process.

MATERIALS AND METHODS

Proline Mutants of Staphylococcal Nuclease. Staphylococcal nuclease A was produced using a recombinant expression system in *Escherichia coli*. The production and purification of the wild-type enzyme and mutants were as described in Evans et al. (1989).

Preparation of Guanidinium Thiocyanate Solutions. A 10 mM cacodylate buffer was used for all experiments.

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[1] Abbreviations: Gdn·SCN, guanidine thiocyanate; SNase, staphylococcal nuclease; pdTp, thymidine 3′,5′-bisphosphate; C_m, denaturant concentration at which half of the protein appears unfolded.

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Sodium cacodylate (Sigma) was dissolved in distilled, deionized water, and the pH was adjusted to 7.0 using HCl or NaOH. The guanidinium monothiocyanate (Gdn·SCN, Boehringer Mannheim Biochemicals) solutions for the unfolding and refolding experiments were made by diluting a 2 M stock solution of Gdn·SCN in 10 mM sodium cacodylate buffer, pH 7.0, to give solutions ranging from 0 to 0.8 M Gdn·SCN. All buffer solutions were filtered using an MF-Millipore filter, 5-μm pore size.

Preparation of Stock Protein Solutions. The stock protein solution was prepared by dissolving 5 or 10 mg of protein in 1 mL of 10 mM cacodylate buffer, pH 7.0. The exact protein concentration was determined by measuring the absorbance at 280 nm using an extinction coefficient of 15 624 M^{-1} cm^{-1} ($A^{1\%}$ = 9.3; Fuchs et al., 1967). The 5 mg/mL stock protein solution was used for the unfolding experiments, and the 10 mg/mL stock was diluted 1:1 with 2 M Gdn·SCN and 10 mM cacodylate, pH 7.0, for the refolding experiments. The unfolded protein was prepared fresh for each set of experiments to ensure there was no aggregated material.

Experimental Instrumentation. The folding of SNase was monitored using a Perkin-Elmer Model MPF-4 fluorescence spectrophotometer, equipped with a computer interface, using an excitation wavelength of 295 nm and an emission wavelength of 330 nm. The brass cell block was cooled to 0 °C using a Neslab Endocal refrigerated circulating bath. The cell compartment was purged with dry nitrogen to prevent condensation of water on the faces of the cell.

C_m Determinations. A typical C_m determination was carried out as follows: 800 μL of buffer (10 mM cacodylate, pH 7.0) and 30 μL of protein (4–5 mg/mL in buffer) were pipetted into a quartz fluorometer cell. The cell was placed into the thermostated cell compartment of the instrument and was cooled to 0 °C. Aliquots (5–10 μL) of 2 M Gdn·SCN were added, and the fluorescence at equilibrium was recorded as a function of Gdn·SCN concentration. Corrections of the fluorescence were made to account for protein and Gdn·SCN dilution.

The C_m for the N → D transition was determined from the data, readjusting the relative fluorescence values to give the fraction of native protein, $Fr_N = (F - F_D)/(F_N - F_D)$, where Fr_N is the fraction native at some Gdn·SCN concentration, F is the fluorescence at that concentration, F_D is the fluorescence intensity of the completely denatured protein, and F_N is the fluorescence intensity of the native protein. In some cases where the initial or final points gave a sloping baseline, the baseline was extrapolated across the graph to five corrected values of F_N or F_D. The C_m is defined as the Gdn·SCN concentration corresponding to a 1:1 mixture of native and denatured molecules (i.e., Fr_N = 0.5).

Refolding and Unfolding Kinetics Monitored by Fluorescence. One milliliter of the Gdn·SCN solution (0–0.8 M) was pipetted into a quartz fluorometer cell and cooled to 0 °C. An aliquot (20–40 μL) of stock protein solution was injected into the cell using a Hamilton gas tight syringe, and the contents were stirred for about 10 s with a small battery-powered stirrer. The refolding or unfolding was monitored until the fluorescence intensity leveled off.

The measurement of the fastest phase of refolding for the wild-type SNase was carried out at 6.3 °C using a Rapid Kinetic Accessory SFA-11 (Hi-Tech Scientific, Inc.).

Double-Jump Experiments. If a proline residue is cis in the native state and isomerizes to the more stable trans conformation in the unfolded state, this should be demonstrable by appropriate double-jump experiments. In particular, the relatively slow rate of proline isomerization at 0 °C (reflecting the high energy of activation of proline isomerization) compared to the rates of conformational changes in folding suggested the following experiment. The protein would be unfolded in a high concentration of denaturant. The conformational process should be complete in seconds or less, but the proline isomerization would take place on a time scale of minutes. Thus the proline residues would retain their native conformations for a short period of time. In terms of eq 1, N would be converted to U_f.

$$N \leftrightarrow U_f \leftrightarrow U_s \quad (1)$$

If the sample was then jumped to conditions near the C_m, the protein would initially fold into the native state but then more slowly, at a rate determined by the rate of proline isomerization, would establish an equilibrium with some of the native state unfolding to provide molecules in state U_s at a rate corresponding to that of $U_f \rightarrow N$ (see eq 1).

The experiments were carried out as follows. Aliquots of 50–80 μL of the 5 mg/mL protein solution were pipetted into a small Eppendorf vial on ice. An equal amount of 2 M Gdn·SCN in 10 mM cacodylate, pH 7.0, at 0 °C was added to the vial, and the contents were stirred. About 20–30 s after the addition of the Gdn·SCN, 80 μL of the mixture was pipetted into a preequilibrated fluorometer cell containing 1 mL of a Gdn·SCN solution (0.2–0.5 M), and the contents were stirred. The change in fluorescence was measured over time.

Analysis of Kinetic Data. The raw data from the kinetic experiments was first plotted in semilog form to determine the number of phases present. The amplitude and rate constants for the slowest phase were determined using the kinetic analysis program REDUCE (Koerber & Fink, 1987), and this phase was then stripped from the data. The kinetic parameters for the remaining phases were determined by curve fitting using REDUCE for either a mono- or biphasic reaction.

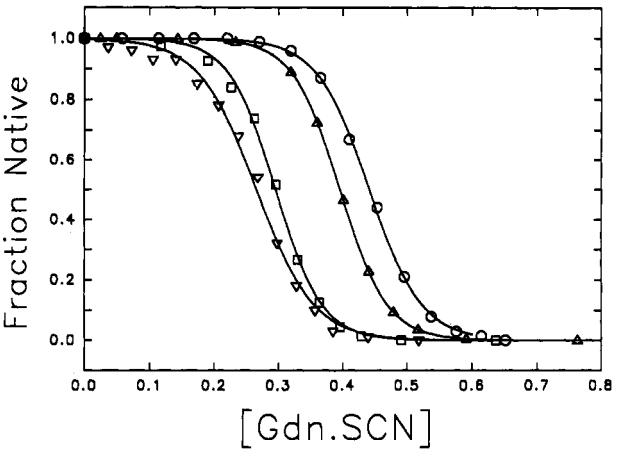


FIGURE 1: Stability of SNase and its mutants as a function of Gdn·SCN concentration. Conditions were pH 7.0 and 10 mM sodium cacodylate buffer, at 0 °C. The wild-type is represented by squares, P117G by circles, P117T by triangles, and P31A by inverted triangles.

RESULTS

Stability of Wild-Type SNase and Mutants. The stability of SNase toward Gdn·SCN was monitored by fluorescence emission from the single Trp residue (Trp-140). The C_m's were determined to be 0.30, 0.45, 0.40, and 0.27 M for the wild-type SNase, P117G, P117T, and P31A, respectively (Figure 1). A two-state approximation was used to analyze the equilibrium constants in the transition region, and ΔG, $\Delta G(H_2O)$, and the dependence of ΔG on denaturant con-

Table I: Thermodynamic Parameters[a] Associated with Equilibrium of SNase and Its Proline Mutants

	wild-type	P117G	P117T	P31A
C_m (M)	0.30	0.45	0.40	0.27
m	−14.0 ± 0.6	−13.6 ± 0.3	−14.6 ± 0.6	−12.0 ± 0.7
m/m_{wt}	1.0	0.97	1.04	0.86
$\Delta G(H_2O)$ (kcal/mol)	4.6	6.2	5.7	4.0
$\Delta\Delta G(H_2O)$	0	+1.6	+1.1	−0.6
m'(r)	−5.76 ± 0.99	−8.77 ± 0.99	−4.73 ± 1.0	−6.16 ± 0.6
m'(u)	7.70 ± 0.74	6.10 ± 0.73	6.40 ± 1.2	6.54 ± 0.83
m^*(r)	−3.12 ± 0.54	−4.76 ± 0.54	−2.57 ± 0.56	−3.34 ± 0.32
m^*(u)	4.18 ± 0.40	3.31 ± 0.40	3.47 ± 0.65	3.55 ± 0.45
m_r^*/m	0.22 ± 0.05	0.35 ± 0.05	0.18 ± 0.05	0.28 ± 0.04
m_u^*/m	−0.30 ± 0.04	−0.24 ± 0.03	−0.24 ± 0.05	−0.30 ± 0.05

[a] The values of m were determined from the slope of a plot of ΔG (unfolding) against denaturant concentration. $\Delta G(H_2O)$ is the value of ΔG extrapolated to zero denaturant concentration, and $\Delta\Delta G(H_2O)$ is the difference in ΔG for the mutant relative to that of the wild-type at zero denaturant. The values of m'(r) and m'(u) correspond to the slope of a plot of ln k_{obs} vs [Gdn·SCN], where k_{obs} is the first-order rate constant for the major phase observed in refolding (r) or unfolding (u), and $m^* = -RTm'$ (see the text) (note that in the figures the kinetic data are shown as ln τ).

centration, m ($m = d(\Delta G)/d[\text{Gdn·SCN}]$), are shown in Table I. A linear relationship between ΔG and denaturant concentration was assumed, i.e., $\Delta G = \Delta G(H_2O) + mc$, where $\Delta G(H_2O)$ is the free energy of unfolding in the absence of denaturant and c = [Gdn·SCN].

Kinetics of Unfolding and Refolding of Wild-Type SNase with Gdn·SCN Monitored by Fluorescence. The dependence of the apparent rate constants and their relative amplitudes on Gdn·SCN concentration is shown in Figure 2. The relative amplitude is the ratio of the observed amplitude to the total expected at that particular denaturant concentration (i.e., the difference between the fully unfolded or folded protein and the equilibrium mixture at that denaturant concentration). Three phases were observed in refolding, accounting for all the expected amplitude in the fluorescence emission: two Gdn·SCN concentration-dependent faster phases and one Gdn·SCN concentration-independent slow phase. The amplitude of the fastest phase in refolding was largest (80% of the total amplitude) at low guanidine concentrations (0–0.2 M) and diminished as the Gdn·SCN concentration increased. This phase practically disappeared at a Gdn·SCN concentration above 0.5 M. The amplitude of the intermediate phase showed the opposite tendency; the relative amplitude was ≤10% at low Gdn·SCN concentration and increased substantially in the transition region. The slowest phase had an apparent rate of 1.5×10^{-3} s^{-1}, which was independent of the Gdn·SCN concentration. The amplitude of this phase was quite low (~10%) except in the transition region where it became significant. In unfolding experiments a single kinetic phase was observed, except in the transition region, where biphasic kinetics were seen. The small amplitudes of the slower phases in both refolding and unfolding led to significant scatter in the results of the analysis of both the rate constants and the amplitudes of these phases. All of the expected amplitude change was observed by fluorescence, i.e., no faster hidden phases were seen.

Kinetics of Unfolding and Refolding of P117G and P117T with Gdn·SCN Monitored by Fluorescence. The dependence of the apparent rate constants on Gdn·SCN concentration for the mutants of Pro-117 is shown in Figures 3A and 4A. Biphasic kinetics were observed for both mutants, and the corresponding rate constants were very similar. For the P117G and P117T mutants, both refolding kinetic phases were dependent on the Gdn·SCN concentration. The relative amplitudes as a function of Gdn·SCN concentration are shown in Figures 3B and 4B. Outside the transition region the amplitudes of the fast phase in unfolding and refolding were about 90% of the total expected amplitude. Near the transition region, the amplitude of the slow phase increased to about

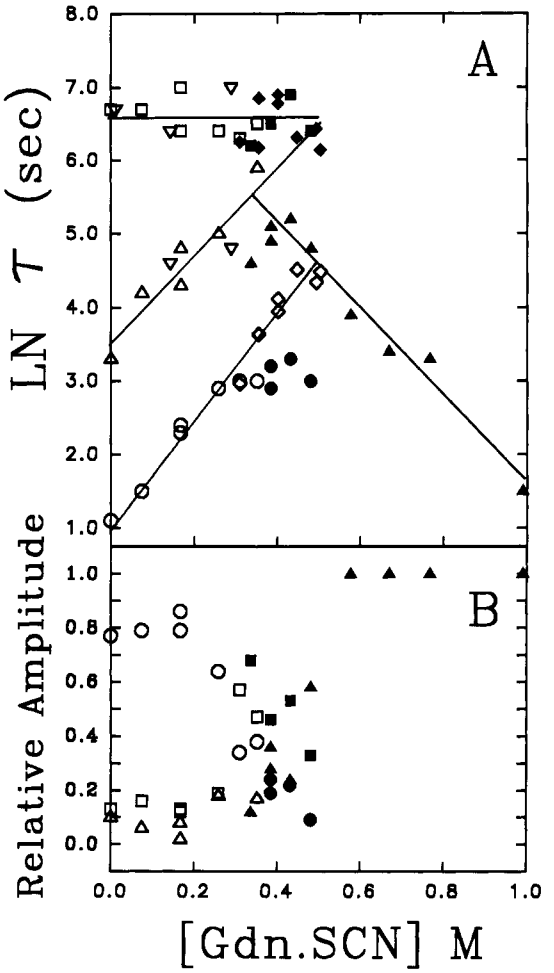


FIGURE 2: Kinetics of folding of wild-type SNase. Panel A represents the kinetics of unfolding (filled symbols) and refolding (open symbols), and panel B represents the relative amplitude data for each kinetic transient. The fastest phase is represented by circles, the slowest by squares, and the intermediate by triangles. The diamonds represent data from the double-jump folding assays (see text); open diamonds represent the initial increase in fluorescence corresponding to folding, and filled diamonds correspond to the subsequent decrease in fluorescence. The lines are drawn as a guide. All studies were carried out at 0 °C, in 10 mM cacodylate buffer, pH 7.

20% of the total expected amplitude for the P117G mutant and 15% for the P117T mutant.

Kinetics of Unfolding and Refolding of P31A SNase Monitored by Fluorescence. The effect of Gdn·SCN on the kinetics of unfolding and refolding of the P31A mutant is shown in Figure 5A along with the corresponding relative

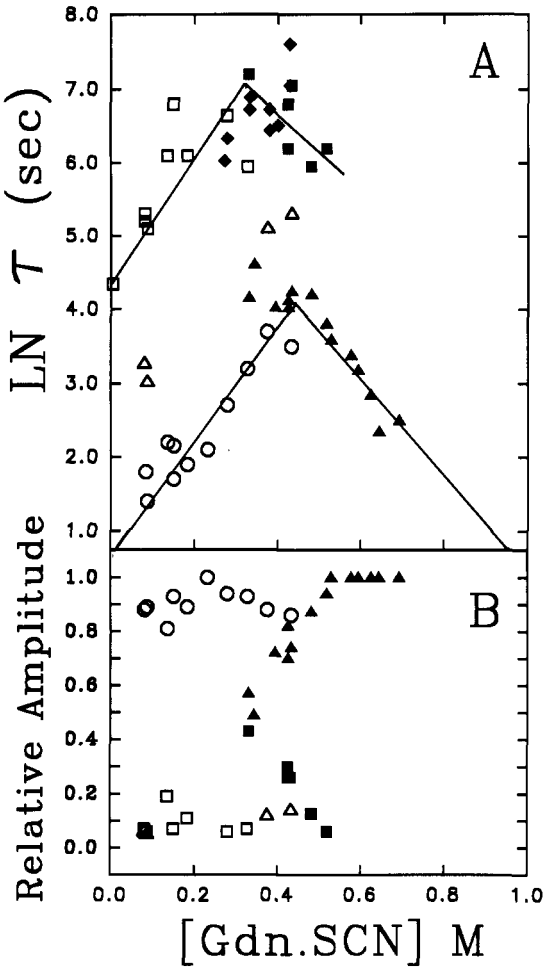


FIGURE 3: Kinetics of folding of the P117G mutant of SNase. Panel A represents the kinetics of unfolding (filled symbols) and refolding (open symbols), and panel B represents the relative amplitude data for each kinetic transient. See the legend to Figure 1 for details.

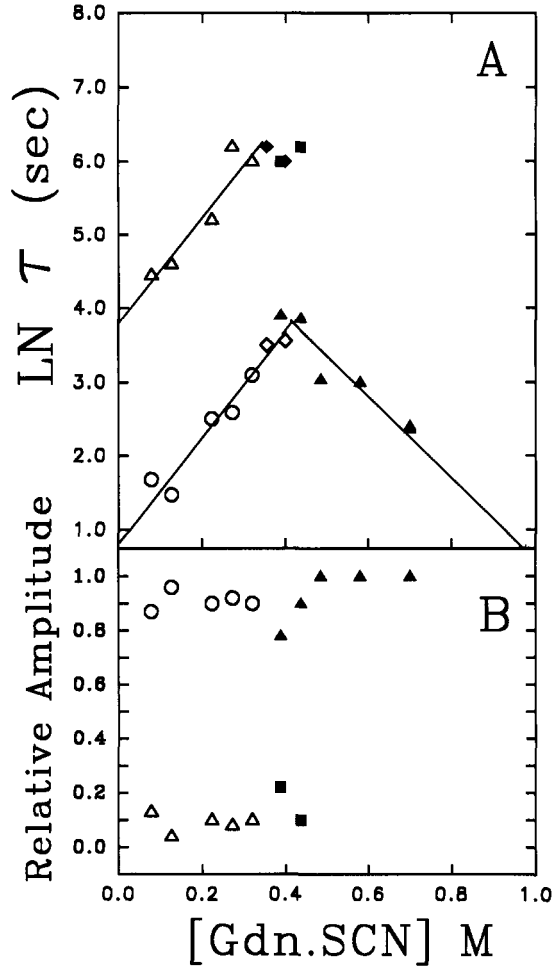


FIGURE 4: Kinetics of folding of P117T. Panel A represents the kinetics of unfolding (filled symbols) and refolding (open symbols), and panel B represents the relative amplitude data for each kinetic transient. See the legend to Figure 1 for details.

amplitudes in Figure 5B. The kinetic profile of this mutant was similar to that of the wild-type.

Double-Jump Refolding Experiments with Wild-Type SNase. Double-jump experiments were carried out using Trp fluorescence emission to monitor the reaction. A typical trace showing the fluorescence intensity against time is shown in Figure 6. This plot shows an initial rapid increase in fluorescence intensity corresponding to the formation of the native state, followed by a much slower decrease in intensity as the denatured states equilibrate due to proline isomerization. The kinetics of the rate of increase in fluorescence were in good agreement with those for the faster phase obtained in the previously described refolding experiments for the same concentrations of denaturant (open diamonds in Figure 2A). The kinetics of the decrease in fluorescence were independent of the guanidine concentration and had the same rates as those observed for the slowest phase in the refolding experiments, namely, 1.1×10^{-3} s^{-1} (solid diamonds in Figure 2A). The amplitude of the fluorescence decrease was strongly dependent upon the Gdn·SCN concentration (Figure 7A) and the length of time the sample was maintained under strongly denaturing conditions (Figure 7B). In the double-jump experiments, the relative amplitude corresponds to the ratio of observed amplitude to the total fluorescence difference between denatured and native protein. The amplitude of the fluorescence decrease as a function of denaturant concentration present under the refolding conditions (Figure 7B) indicates that at 0.40 M Gdn·SCN, conditions under which at equilibrium only a very few percent of the native protein is present, the ratio of N to U_f prior to the equilibration of the unfolded states is 30:70.

Double-Jump Refolding Experiments with Proline Mutants. When samples of P117G, P117T, or P31A were subjected to double-jump refolding experiments, similar results were obtained in that a fast increase in fluorescence was followed by a slower phase involving a decrease in fluorescence intensity when refolding was carried out at denaturant concentrations in the vicinity of the C_m. A summary of double-jump data for the wild-type and mutants is shown in Table II. As with the wild-type protein, the rates of the fluorescence increase for the mutants agreed with those for the fast phase of refolding at the same concentration of denaturant (Figures 3A, 4A, and 5A), and the rates for the decrease in fluorescence were independent (within experimental error) of denaturant concentration. The slow fluorescence decrease had a rate of $(1.2–1.3) \times 10^{-3}$ s^{-1}, in good agreement with the slow phase observed in refolding with the wild-type, which has been attributed to proline isomerization. The presence of a decreasing fluorescence phase in the double-jump refolding experiments of the Pro-117 mutants indicates that there is another proline, in addition to Pro-117, whose isomerization affects folding. Analysis of the relative amplitudes in the double-jump assays indicates that the slow phase involves 5% of the protein for P117G and P117T, whereas the corresponding figure for the wild-type and P31A is 20% (Table II).

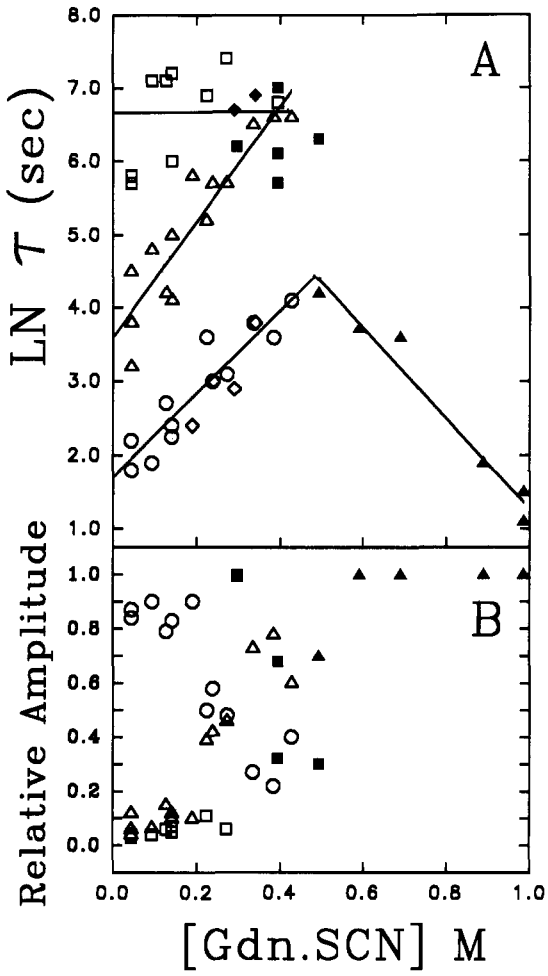


FIGURE 5: Kinetics of folding of P31A. Panel A represents the kinetics of unfolding (filled symbols) and refolding (open symbols), and panel B represents the relative amplitude data for each kinetic transient. See the legend to Figure 1 for details.

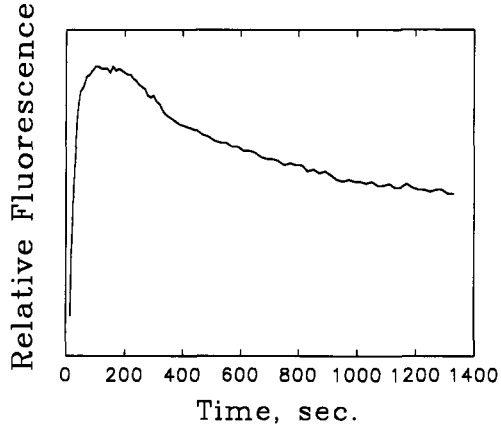


FIGURE 6: Double-jump folding assay, kinetic trace for wild-type, in 0.40 M Gdn·SCN. The protein was first unfolded for 20–40 s in 1 M Gdn·SCN, pH 7.0. An aliquot was then allowed to equilibrate in a solution of Gdn·SCN in the vicinity of the C_m (see the text for further details).

DISCUSSION

It is interesting to note that the Pro-117 mutants were considerably more stable toward denaturation by Gdn·SCN than the wild-type protein. This suggests that the presence of the proline at that position is maintaining the protein in a higher energy conformation than that accessible to the other more flexible amino acid residues or, alternately, destabilizing

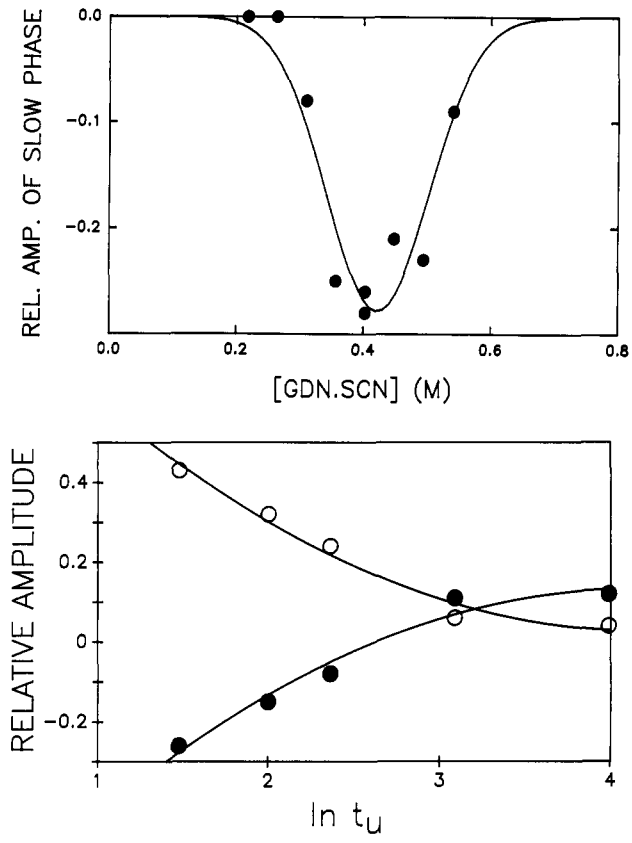


FIGURE 7: Relative amplitudes of the decrease in fluorescence observed in the double-jump assays for wild-type SNase as a function of time of unfolding (panel A, top) and Gdn·SCN concentration, pH 7.0, during the subsequent equilibration (panel B, bottom) (see the text).

the unfolded state. This is surprising in view of the fact that the cis conformation is apparently favored at Pro-117. Recent crystal structure analysis indicates that the Pro-117 substitutions result in a change in β-turn type, type I′ for P117G, and type I for P117T, both with trans peptide bonds (T. R. Hynes and R. O. Fox, unpublished observations).

The value of m, the dependence of ΔG on denaturant concentration, for all three mutants is significantly smaller than that for the wild-type protein. Shortle and co-workers (Shortle & Meeker, 1986, 1989; Shortle et al., 1989) have suggested that a decrease in m is associated with a decrease in the solvent-accessible surface of the denatured state, suggesting a change in the amount of residual structure in the denatured state of the mutants relative to the wild-type. The smaller values of m for the mutants relative to the wild-type indicate that the unfolding transition is less cooperative for the mutants.

Kinetics of Folding of SNase. The NMR experiments which demonstrate the presence of two interconverting forms of the native state in the wild-type protein cannot be carried out under the exact conditions used in these kinetics experiments due to the titration of the histidines in the vicinity of pH 7. However, at pH 5.3 the His resonances attributed to the minor form of the native state (Pro-117 in the trans conformation) disappear as the temperature is lowered (Evans et al., 1989), suggesting that under our experimental conditions all the Pro-117 residues in the native state may be cis. Pro-117 has been observed to be the only proline in SNase which is in the cis conformation in crystallographic studies (Cotton et al., 1979; Loll & Lattman, 1989; Hynes & Fox, 1991). The NMR results are quite consistent with this proline residue being responsible for the alternate forms of native and unfolded

Table II: Double-Jump Rate Constants and Amplitude Data for Wild-Type SNase and Mutants[a]

	[Gdn·SCN] (M)	A_f[b]	k_f[c] (s^{-1})	A_s[d]	k_s[e] (s^{-1})	$\Delta F_s/\Delta F_t$[f]
wild-type	0.402	958	1.9×10^{-2}	222	1.2×10^{-3}	0.188
P117G	0.402	2244	3.3×10^{-2}	108	1.2×10^{-3}	0.046
P117T	0.402	1204	2.9×10^{-2}	70	2.5×10^{-3}	0.055
P31A	0.335	1215	2.2×10^{-2}	307	1.3×10^{-3}	0.202

[a] See the text regarding experimental details. [b] Relative amplitude (arbitrary units) for the fluorescence increase of the fast phase. [c] Rate constant for the fast phase (increase in fluorescence intensity). [d] Relative amplitude for the fluorescence decrease of the slow phase. [e] Rate constant for the slow phase. [f] Fractional amplitude of the slow decreasing phase ($\Delta F_t = |A_f| + |A_s|$).

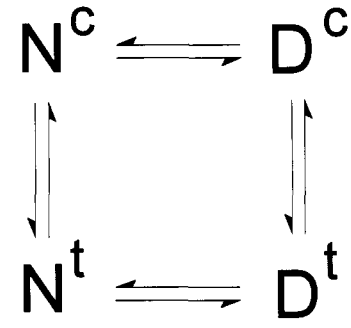
SNase and show that Pro-117 is in the trans conformation in the major unfolded species at pH 5.3, 49 °C (Evans et al., 1987, 1989; Torchia et al., 1989). For P117G and P117T only a single native species is seen by NMR (Evans et al., 1987).

Comparison of the kinetics of folding of P117G and P117T with those of the wild-type protein in Gdn·SCN (e.g., Figures 2A and 3A) shows that the major difference in the mutants is the loss of the guanidine-independent, slowest phase. The properties of this phase are indicative of rate-limiting proline isomerization in the wild-type SNase. The absence of this guanidine-independent phase in the Pro-117 mutants is strong evidence that this phase is due to cis/trans isomerization about the Lys-116–Pro-117 bond. Since the patterns for the folding kinetics are simpler for the Pro-117 mutants, we will consider them prior to those of the wild-type protein. What is of particular interest, as discussed below, is the evidence for a nonnative proline isomer in the unfolded states of the P117 mutants, implying that in wild-type SNase at least one proline in addition to Pro-117 is significantly populated in a nonnative isomer in the unfolded state.

Folding Kinetics for Pro-117 Mutants. In interpreting the folding kinetics for the Pro-117 mutants, we must account for the two guanidine-dependent kinetic phases observed in refolding kinetics experiments and the results of the double-jump assays. The latter are most simply interpreted as indicating the existence of a proline isomerization in the unfolded state. Consistent with this interpretation are the facts that the rates of the decrease in fluorescence in the double-jump experiments are similar to those observed for the slow phase in refolding of the wild-type protein, which we believe corresponds to proline isomerization, and the amplitude of the decrease is smaller for the P117 mutants than for the wild-type (Table II). The crystallographic structure of wild-type SNase shows that, except for Pro-117, all of the other proline residues are in the trans conformation in the native state. Thus if we assume that this situation also exists in solution under our experimental conditions, the double-jump assay results imply that one (or more) nonnative, *cis*-proline exists in the unfolded state. An alternative possibility is that, in solution under our experimental conditions, a proline in addition to Pro-117 is in the cis conformation. On the basis of NMR observations, Wang et al. (1990) have shown that in the SNase mutant H124L, Pro-47 is 15% in the cis conformation. However, on the basis of model peptide studies (Grathwohl & Wüthrich, 1981), one would expect a comparable amount of the cis isomer in the unfolded state at equilibrium, so it is likely that it would be some other proline. A further intriguing possibility is that there is a small population of non-proline peptide bonds which are in the cis conformation in the unfolded state and that these contribute to the slow folding.

The biphasic kinetics observed in the folding of P117G and P117T exhibit patterns similar to the two guanidine-dependent kinetic transients in the folding of the wild-type protein.

Scheme I



Biphasic refolding kinetics in a single-domain protein such as SNase are most simply interpreted as the result of either a populated intermediate state or a proline isomerization. The results of the double-jump experiments indicate the involvement of proline isomerization; thus a simple three-step scheme with an intermediate state is unlikely. In the present case the denaturant dependence of the slower kinetic phases with the P117 mutants indicates that proline isomerization itself is not the rate-limiting step. Either the proline isomerization is silent in the folding kinetics experiments (under strongly native conditions, at least), i.e., it does not affect the folding kinetics and presumably occurs after completion of the formation of the native-like conformation without affecting the fluorescence, or the slower observed phase is due to an indirect effect of the proline isomerization. The simple model

$$N \leftrightarrow U_f \leftrightarrow U_s$$

can be eliminated by the guanidine dependence of the slower phase as well as by the fact that one would not expect to observe fluorescence changes for just the proline isomerization from U_s to U_f.

The best interpretation of the folding kinetics for the P117 mutants (Scheme I) involves the assumption that there is one or more proline residue in a nonnative conformation in the denatured protein. In addition, we assume that it is in the trans conformation in the native state and that its presence in the cis conformation in the unfolded state slows the folding of the protein. In model peptide studies, proline residues are often present in the less stable cis conformation to the extent of 10–15% (Grathwohl & Wüthrich, 1981). In such model systems the amount of cis proline is also quite context-dependent.

In Scheme I we assume that D_S^c differs from D_F^t by having one proline in the nonnative cis conformation. The superscript refers to the state of the critical proline residue which isomerized from the trans conformation in the native state to the cis conformation in the denatured state. N^c corresponds to a native-like intermediate with the nonnative proline conformation (or possibly a corresponding alternative native form). We choose to use the symbol D for the denatured state, rather than U for unfolded, to indicate the possibility that there may be residual structure present in the denatured

state (Shortle et al., 1988). The subscript F is used to denote fast-folding species due to their having the native proline conformation; conversely, the subscript S implies a nonnative proline conformation and potentially slower refolding kinetics. The conformational process of folding must still be faster than the isomerization itself in order to account for the guanidine dependence of the slower phase and its absolute rate.[2] Conceptually, one can picture the later stages of refolding as involving local metastable regions of structure coalescing to form the tertiary structure; different proline conformations could result in significant differences in the relative spatial disposition of some of these metastable regions of structure and hence lead to effects on the rate of formation of the native state.

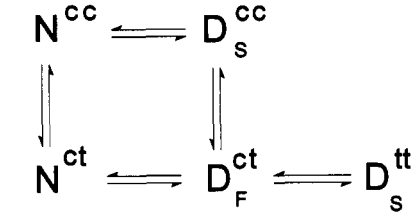
Scheme I accounts for the observed kinetic and amplitude effects as follows. We assume that the equilibrium between the two folded species is strongly in favor of N^t. Similarly, in the denatured state, the equilibrium favored D_F^t by approximately 90:10, based on the observed amplitudes of the refolding kinetics and double-jump experiments (Figure 3B). Unfolding is monophasic at higher denaturant concentrations, corresponding to $N^t \rightarrow D_F^t$. The subsequent slow formation of D_S^c is not observed due to the similarity in spectral properties of the denatured states. Refolding is biphasic with the faster phase reflecting the $D_F^t \rightarrow N^t$ transition, confirmed by the identity in the rate of formation of the native state in the double-jump assays and that of the fastest phase in refolding. The slower phase in refolding corresponds to the $D_S^c \rightarrow N^c$ transition. The subsequent conversion of N^c to N^t, which will be relatively slow, is not observed by fluorescence due to the similarities in their fluorescence properties. The data do not allow distinction between the refolding pathway for the D_S^c species involving direct conversion to the corresponding N^t species. Kuwajima et al. (1991) have recently reported that the slowest phase observed by CD in refolding (0.4 M urea) of the urea-unfolded wild-type SNase is absent in the P117G mutant, which is in accordance with our results.

Model for Wild-Type Folding Kinetics. The major difference between the folding kinetics for the wild-type SNase and the Pro-117 mutants in Gdn·SCN was the presence of a guanidine-independent kinetic phase with a rate constant consistent with that expected for proline isomerization. Such denaturant independence is a hallmark of a folding process in which the rate-limiting step is proline isomerization (Nall, 1985). As noted above, the absence of this slowest phase in the Pro-117 mutants indicates that this phase can be attributed to isomerization of Pro-117. Confirmation of this comes from the amplitude data from the slow phase in the double-jump assays which indicate that 75% of the amplitude observed with the wild-type protein is absent in the P117 mutants. The two faster phases observed in refolding of the wild-type can therefore be assumed to correspond to the same processes observed with the Pro-117 mutants.

The kinetic and amplitude data for the wild-type protein are best interpreted by a kinetic model analogous to that of Scheme I, but taking the isomerization of two proline residues into consideration. The simplest model is shown in Scheme II.

In the model of Scheme II we make the same assumptions as for Scheme I. We assume that the transition between N^{ct} (the native state) and the other native-like species is "silent" in that all native-like species have similar fluorescence

Scheme II



properties. The first superscript refers to the isomerization state of Pro-117 and the second superscript to another (or possibly more than one) proline which is trans in the native structure but becomes cis in the denatured state. The equilibria favor the vast majority of the protein in the N^{ct} state in strongly native conditions. This species is responsible for the single, fast phase seen in unfolding at high guanidine concentrations (although the possibility that small amounts of the other N species are present cannot be ruled out at this time).

In refolding experiments the observed fast phase with the major amplitude corresponds to the transformation of $D_F^{ct} \rightarrow N^{ct}$. This is supported by the observation that the rate of formation of the native state in the double-jump assays is the same as the fastest phase observed in the refolding experiments (Figure 2A). The intermediate kinetic phase corresponds to the transition $D_S^{cc} \rightarrow N^{cc}$ as observed in the Pro-117 mutants and is followed by the "silent" transformation to N^{ct}. The species responsible for the intermediate kinetic phase, D_S^{cc}, has one nonnative proline isomer in the non-P117 position. The slow phase corresponds to the isomerization of Pro-117 in the D_S^{tt} to D_F^{tt} transition. The refolding of the D_S^{tt} species is rate-limited by proline isomerization regardless of the concentration of denaturant.

Recently, Sugawara et al. (1991) have reported the results of an investigation of the folding of wild-type SNase monitored by CD in the presence of urea. In general, their findings are consistent with those reported here, with the exception of a faster phase detectable by stopped-flow CD. This phase is not observed by fluorescence, indicating that it involves formation of secondary but not tertiary structure.

Model for the Folding Kinetics of P31A. The folding kinetics data for P31A resemble those for the wild-type. Thus the model of Scheme II is also applicable to P31A SNase. These results indicate that P31 is not one of the proline residues involved in the isomerizations detected in the unfolded state.

Transition State for Folding. The kinetic data can be analyzed to provide information about the transition state for the folding process (Kuwajima et al., 1989; Matouschek et al., 1989). The slopes of plots of ln k_{obs} vs denaturant concentration, m', are given in Table I. Only the data for the kinetic phases corresponding to the major amplitude phase (>80%) were used in this analysis. For refolding, the value of m' was greater than that for the wild-type for P117G, similar to that for the wild-type for P117T, and less than that of the wild-type for P31A. It is believed that m' or m^* ($= -RTm'$) reflects changes in the solvent-accessible surface area between the transition state and the native or denatured states (Kuwajima et al., 1989; Matouschek et al., 1989). Information about differences in the transition state for folding between the wild-type and mutants can thus be found by comparing the values of m^*. The data in Table I indicate that there are significant differences in the degree of solvent exposure, i.e., folding, in the transition states for the different mutants and the wild-type. The ratios of m^* to m indicate that the transition state is substantially folded in all cases. The value for the

[2] It is possible that, in the center of the transition, proline isomerization becomes rate-limiting (compare Figure 2A with Figure 3A).

wild-type protein for unfolding agrees with that reported by Sugawara et al. (1991) for unfolding in the absence of calcium.

Structural Effects of the Mutations. Pro-117 occurs in an exposed type I′ turn which attaches the C-terminal helix to the rest of the molecule. Pro-31 occurs in a type VI turn, in this case linking two strands of antiparallel sheet.

Since the substitution of Pro-117 by either Gly or Thr leads to a more stable protein, it is clear that there is considerable latitude in the nature of the residue at this position, especially since the replacement of proline in such a turn is likely to lead to a significantly different local conformation. Although the substitutions at Pro-117 lead to both equilibrium and kinetic effects in folding, these are quite small. The kinetic effects indicate that the amino acid substitution leads to an effect on the free energy of the key activated complex in the folding process (this is the conformational state equivalent to a transition state in a chemical reaction involving bond breaking/reforming). Thus either the residue at position 117 plays an important role in the folding process or, more likely, the rate-limiting step in refolding involves a structure close to that of the native state, and any residue substitution which affects the stability of the native state also affects the rate of folding. This is supported by the fact that the other mutations also cause changes in both rates of folding and stability.

For the substitution of Pro-31, the rate of the major phase becomes slower than that of the wild-type in refolding, but faster in unfolding. As a consequence the mutant is somewhat less stable than the wild-type. Structural studies (T. Hynes and R. O. Fox, unpublished results) reveal that changes in the vicinity of position 31 occur in the P31A mutant relative to the wild-type. The turn containing residue 31 is able to accommodate the substitution of Ala for Pro with little affect on the overall stability, even though there are significant changes in the structure. Thus it is clear that compensating changes in the structure lead to an overall similar free energy to that of the wild-type.

In summary, replacements of Pro-117 and Pro-31 have small but measurable effects on the stability of SNase. The slow denaturant-independent refolding phase observed in the wild-type and P31A mutant is attributed to the rate-limiting trans to cis isomerization of Pro-117 and is absent in the refolding of the Pro-117 mutants. The intermediate kinetic phase observed in the refolding of the wild-type and P31A, accounting for approximately 10% of the total amplitude and corresponding to the slowest observed phase with the Pro-117 mutants, is believed to be the result of the trans to cis isomerization of at least one proline residue other than Pro-117 when the protein is unfolded. The resulting conformers refold to a native-like state more slowly than the denatured species with the correct proline conformation.

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