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NMR analysis of staphylococcal nuclease thermal quench refolding kinetics

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Abstract

Thermally unfolded staphylococcal nuclease has been rapidly quenched to temperatures near 0 °C and the refolding behavior examined using an NMR kinetic experiment. Unfolded protein, exhibiting random coil chemical shifts, persists following the quench and refolds in two distinct kinetic phases. A protein folding intermediate with a *trans* Lys 116–Pro 117 peptide bond is transiently overpopulated and relaxes to the predominantly *cis* native *cis–trans* equilibrium. The rate of *trans* → *cis* isomerization in the nativelike nuclease intermediate is approximately 100-fold faster than that observed in a Lys–Pro model peptide. The activation enthalpy of 20 kcal/mol observed for the nuclease Lys 116–Pro 117 peptide bond is comparable to that observed for other X-Pro isomerizations.

Keywords: NMR; proline isomerization; protein folding; refolding phases; staphylococcal nuclease

Proline isomerization is responsible for some slow protein refolding phases (Brandts et al., 1975; Kim & Baldwin, 1982; Nall, 1989). The peptide bonds of proteins may exist in either the *cis* or *trans* isomer, but the isomeric equilibria for all non-prolyl residues strongly favor the *trans* configuration. In X–Pro peptide bonds, steric constraints introduced by the imino ring of proline decrease the free energy difference between the *cis* and *trans* conformers (Schultz & Schirmer, 1979; Nall, 1989). The exact point of the isomeric equilibrium depends on local amino acid sequence in peptides and additionally on local structure in folded proteins. Equilibrium values of 5–50% of the *cis* configuration have been observed in peptides (Grathwohl & Wüthrich, 1981; Dyson et al., 1988; Harrison & Stein, 1990; Hsu et al., 1990). Generally *cis* and *trans* isomers have not been observed to coexist at a single site in proteins with the exception of nuclease (Fox et al., 1986) and calbindin D9k (Chazin et al., 1989; Svensson et al., 1992). Strain induced by cyclizing peptides to mimic conformational restrictions found in proteins can both shift the *cis–trans* equilibrium and increase the isomerization rate (Grathwohl & Wüthrich, 1981; Sukumaran et al., 1990). The activation enthalpy for isomerization is relatively high (18–25 kcal/mol), and thus the rates of isomerization are slow (0.02–0.05 s^{-1} at 25 °C) and are highly temperature dependent (Grathwohl & Wüthrich, 1981; Nall, 1989). Isomerization rates change by a factor of 2 with a 7 °C change from 25 °C, following Arrhenius behavior.

Staphylococcal nuclease has two folded conformations which can be distinguished by ^{1}H NMR spectroscopy (Fox et al., 1986). Distinct pairs of sharp resonances were observed for three of the four His $H^{\varepsilon 1}$ protons (H8, H121, and H124). The major form with a 85–95% population was designated as the N state, and a minor form with a 5–15% population designated as the N* state. Magnetization transfer NMR studies have demonstrated that the two species can interconvert directly, or indirectly via the unfolded state. The rates of interconversion suggested this heterogeneity could be due to *cis–trans* isomerism at a single peptide bond between a proline and its preceding residue. Proline 117 was observed to be in the *cis* configuration in the crystal structure (Arnone et al., 1971; Cotton et al., 1979; Loll & Lattman, 1989; Hynes & Fox, 1991) and was suggested as the site of *cis–trans* conformational heterogeneity monitored by NMR (Fox et al., 1986; Evans et al., 1987). Nuclease variants where Pro 117 was replaced with Gly (P117G) or Thr (P117T) resulted in a single conformation, lending support to this hypothesis (Evans et al., 1987; Kautz & Fox, 1991). The P117G variant adopts a type I′ β-turn conformation while

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the P117T variant adopts a type I β-turn geometry (Hynes & Fox, in prep.). Substitution of the preceding residue to a glycine (K116G) relaxed the equilibrium to a predominantly *trans* conformation as observed by NMR spectroscopy and confirmed in the crystal structure (Hodel et al., 1993). The substitution of Ala, Met, and Glu for Lys 116 did not perturb the equilibrium observed by NMR spectroscopy, however the Asp substitution (K116D) does shift the *cis–trans* equilibrium. A crystal structure of the K116D variant indicates that the Asp 116 side chain makes a specific hydrogen bond favoring the *trans* configuration (Hodel, Kautz, & Fox, in prep.).

Extensive magnetization transfer experiments on staphylococcal nuclease revealed heterogeneity in the unfolded state, thereby facilitating the measurement of the folding and unfolding rates of both the *cis* and *trans* Pro 117 species, and of the rates of isomerization in both folded and unfolded protein within the thermal unfolding transition (Alexandrescu et al., 1989; Evans et al., 1989). The two unfolded states, U and U*, are in direct equilibrium with N and N* native states, respectively (Evans et al., 1989). The U* state represents 95%, and U 5%, of the unfolded material. States U* and N* were proposed to possess a *trans*, and U and N a *cis*, Lys 116–Pro 117 peptide bond. The rate-limiting step of folding was assumed to be independent of the *cis* → *trans* isomerization of the Lys 116–Pro 117 peptide bond. The rate of U → N was found to be fivefold faster than that for U* → N* at T_m in the equilibrium kinetic experiment (Evans et al., 1989). Whereas the isomerization rates in unfolded nuclease are comparable to rates observed in peptide studies, the *trans* → *cis* isomerization rate of Pro 117 in folded nuclease is over an order of magnitude faster. Thermodynamic considerations indicated that the major folded conformation, harboring a *cis* Pro 117 (N), is favored enthalpically, whereas the minor *trans* Pro 117 conformation (N*) is favored entropically. Comparing the folding and unfolding rates of the N (*cis*) and N* (*trans*) forms further indicated that the interactions stabilizing the *cis* Pro 117 conformation occurred late in the folding process, relative to a rate-determining step. The experiments described above provided strong support for a proline isomerism model, where Pro 117 is 95% *trans* in thermally unfolded nuclease, while it is 10% *trans* in folded nuclease (Evans et al., 1989). Further, the major folding pathway was shown to be from the unfolded, *trans* state U*, through the minor folded *trans* form N*, and then to the major folded *cis* form N.

The prolyl isomerism model for nuclease structural heterogeneity predicts that if a sample of thermally unfolded nuclease were cooled rapidly, a predominantly *trans* Pro 117 species of folded nuclease (N*) could be transiently populated. The high activation enthalpy of X-Pro peptide bond isomerization should result in a greater reduction in isomerization rate than the rate reduction for the faster refolding phases not involving prolyl isomerization. The kinetics of isomerization could thus be monitored in a series of NMR spectra collected at low temperature. This type of experiment was successful in characterizing a similar transient refolding intermediate of ribonuclease A (Blum et al., 1976). The determination of isomerization rates as a function of temperature allow an estimation of the activation enthalpy, which is characteristically high for prolyl isomerization (Grathwohl & Wüthrich, 1981).

Results

Spectra of the histidine $H^{\varepsilon 1}$ proton resonances of amide exchanged staphylococcal nuclease A at 49 °C (slightly above its T_m of 48 °C), 25 °C, and 10 °C are shown in Figure 1. Resonances are labeled as previously assigned (Alexandrescu et al., 1988; Torchia et al., 1989; Kautz et al., 1990). The small resonances labeled with an asterisk downfield of His 121 and His 124 and upfield of His 8 arise from a conformationally distinct state of nuclease, proposed to result from protein with a *trans* Lys 116–Pro 117 peptide bond (Fox et al., 1986; Evans et al., 1987). The major resonances (His 8, His 121, His 124), which identify the predominant folded form (N), arise from native nuclease in which the Lys 116–Pro 117 peptide bond is in the *cis* conformation, whereas the minor resonances (His 8*, His 121*, His 124*) identify the minor folded form (N*) with a *trans* Lys 116–Pro 117 peptide bond. The minor peak of His 46 labeled ○ is attributed to *cis–trans* X-Pro isomerism with the adjacent Pro 47. Resonance F76 is a downfield-shifted aromatic, assigned to Phe 76 H^{δ} (Torchia et al., 1989). Above T_m the spectrum is dominated by the resonances of the four histidines in unfolded protein (U) clustered about 8.50 ppm. As the temperature is lowered, the unfolded resonances diminish while the folded resonances gain in intensity. Some residual intensity at U (U_{res}) persists even at low temperature and is attributed to chemically modified, fragmented, or irreversibly unfolded protein. At lower temperatures the unfolded resonances move slightly downfield, together with those of His 8 and His 46. Histidines 121 and 124 move significantly more, reducing their chemical shift separation from the unfolded resonances by 20%. The relative intensity of the minor form diminishes from 12% at 40 °C to less than 5% at 10 °C. The chemical shift differences between the major (N) and minor (N*) forms diminish significantly at lower temperatures. All resonances broaden significantly at lower temperatures.

Thermally unfolded nuclease was rapidly quenched to 0 or 10 °C and quickly examined in sequential NMR spectra. A series of spectra from a sample equilibrated at 60 °C for 30 min and quenched to 10 °C is shown in Figure 2. The spectra are Fourier transforms of single FID transients resulting in a modest signal-to-noise ratio. Two resonances of His 121 of nearly equal intensity can clearly be seen at 8 s. At progressive times the downfield peak (H-121*) diminishes while the upfield peak (H-121) gains in

magnitude. A similar behavior is also seen for His 8 and His 124, but with less spectral resolution. Close inspection of the chemical shifts of the His $H^{\varepsilon 1}$ resonances in Figure 2 reveal slight shifts with time. The drift in the chemical shift of His-121 is consistent with a 2–3 °C change in temperature of the sample, when calibrated against spectra of nuclease equilibrated at 0 and 10 °C. Kinetic series of spectra like those shown in Figure 2 were obtained from several trials, quenching from 60 °C to 10 °C and from 55 °C to 0 °C.

A significant quantity of protein remains with unfolded histidine chemical shifts (U) immediately following the quench. The height of the unfolded resonances diminish with time, indicating the presence of slow refolding phases. The equilibrium refolding kinetic experiments indicated that 95% of the unfolded protein has a *trans* Lys 116–Pro 117 peptide bond, thus the observed refolding is at-

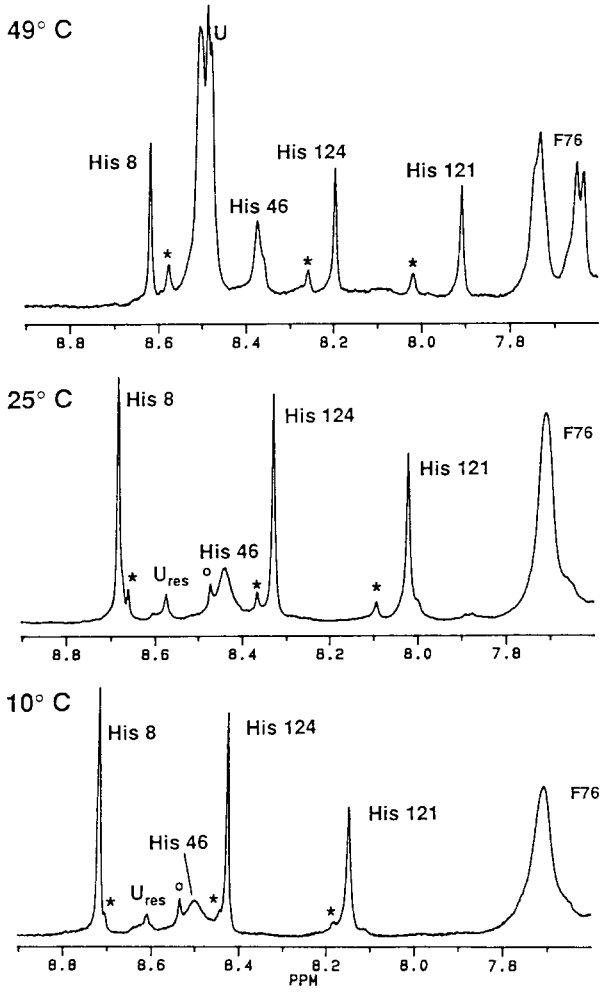

Fig. 1. Proton NMR spectra of nuclease A at 49 °C, 25 °C, and 10 °C. This spectral region of D_2O-exchanged protein shows the resonances of the four histidine $H^{\varepsilon 1}$ protons. Resonances from folded protein are marked with residue number; U is the cluster of resonances from unfolded protein. Small resonances marked with asterisks are minor resonances of the same proton as their adjacent major resonances, arising from an alternative folded conformation of the protein. Spectra at 49 °C and 25 °C have been apodized with 0.5 Hz of line broadening. The spectrum at 10 °C has been resolution-enhanced with −1 Hz to resolve the small minor resonances on His 8 and His 124. All spectra are of 3 mM nuclease in 200 mM deutero-acetate buffered D_2O, pH* 5.3, acquired on a Bruker AM-500 spectrometer.

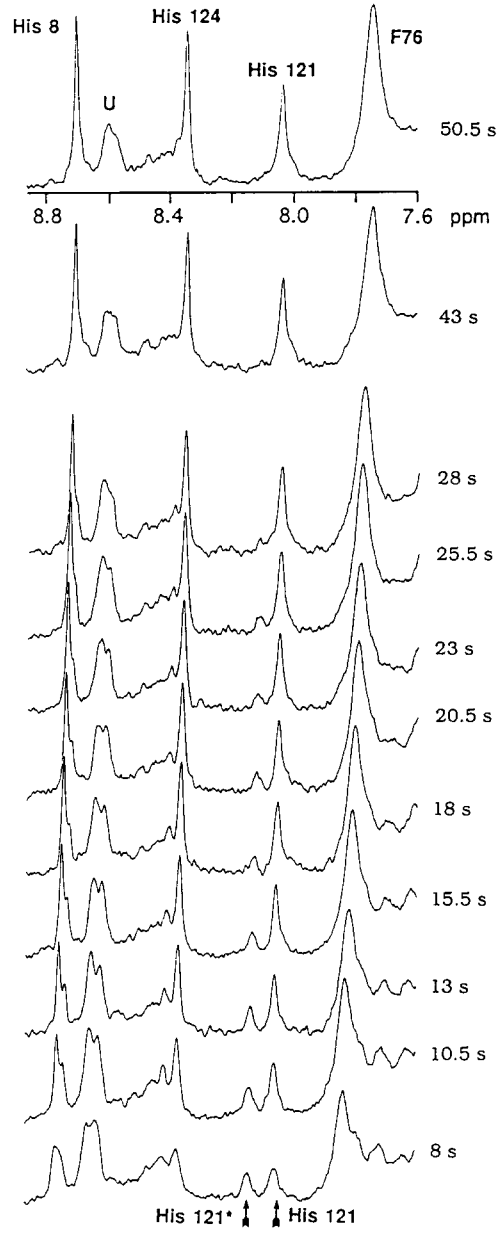

Fig. 2. Kinetic series of NMR spectra of wild type nuclease A after a temperature quench from 60 °C to 10 °C. This region details resonances of the four histidine residues ($H^{\varepsilon 1}$) as in Figure 1. Arrows indicate the major and minor resonances of His 121. The minor resonances diminish in time, whereas the major resonances increase in intensity. The unfolded resonances (U) also decrease over time, indicating slow folding phases.

tributed to the *trans* Lys 116–Pro 117 state (U_s^*) as defined below. The intensity of the four histidines in the U region of the spectrum was fitted to the sum of two exponential refolding phases and a constant term:

$$[U_s^*(t)] = [U_a^*]\exp(-tk_a^*) + [U_b^*]\exp(-tk_b^*) + [U_{res}] \quad (1)$$

where $[U_s^*(t)]$ is the fraction of unfolded material with a *trans* Lys 116–Pro 117 peptide bond at time t; $[U_a^*]$ and $[U_b^*]$ are the amplitudes of two slow-folding populations with first-order rate constants k_a^* and k_b^* (s^{-1}); and $[U_{res}]$ is the residual unfolded material remaining at times long relative to $(k_b^*)^{-1}$. The derived values for $[U_b^*]$ and k_b^* were strongly dependent on $[U_{res}]$; uncertainties in the reported values of $[U_a^*]$, k_a^*, $[U_b^*]$, and k_b^* were determined by independent least-squares optimizations with $[U_{res}]$ fixed at the extremes of its confidence limits. Least-squares fits of $[U_a^*]$ and k_a^* were largely independent of $[U_{res}]$. The magnitude of the two observed slow refolding phases, when back-extrapolated to $t = 0$, account for only 27% of the refolding protein (excluding U_{res}). Thus, 73% of the sample must fold in the dead-time of the experiment (U_f and U_f^*), either in faster kinetic phases or via the slow folding pathways but with accelerated rates during the cooling process. The kinetic data and least-squares curves are shown in Figure 3. The derived kinetic constants are reported in Table 1.

Quantitation of the native $N^* \rightarrow N$ transition rate using the H-121* and H-121 resonances was complicated by the presence of slow refolding phases, requiring a kinetic model involving additional transitions. The relationships between the conformational states proposed in this kinetic scheme are presented in Figure 4. An asterisk signifies that a state has a *trans* Lys 116–Pro 117 peptide bond. The U_f and U_f^* states differ in the isomeric form of the Lys 116–Pro 117 peptide bond but are both proposed to fold rapidly in the dead-time of the experiment. The U_a^* and U_b^* states represent the two slow refolding phases observed in the kinetic experiment. They represent the bulk of the slow refolding material given the 95% population of *trans* Lys 116–Pro 117 observed in thermally unfolded nuclease (Evans et al., 1989). The U_a and U_b states (with a *cis* Lys 116–Pro 117 peptide bond) will make a negligible contribution to the refolding kinetics given their com-

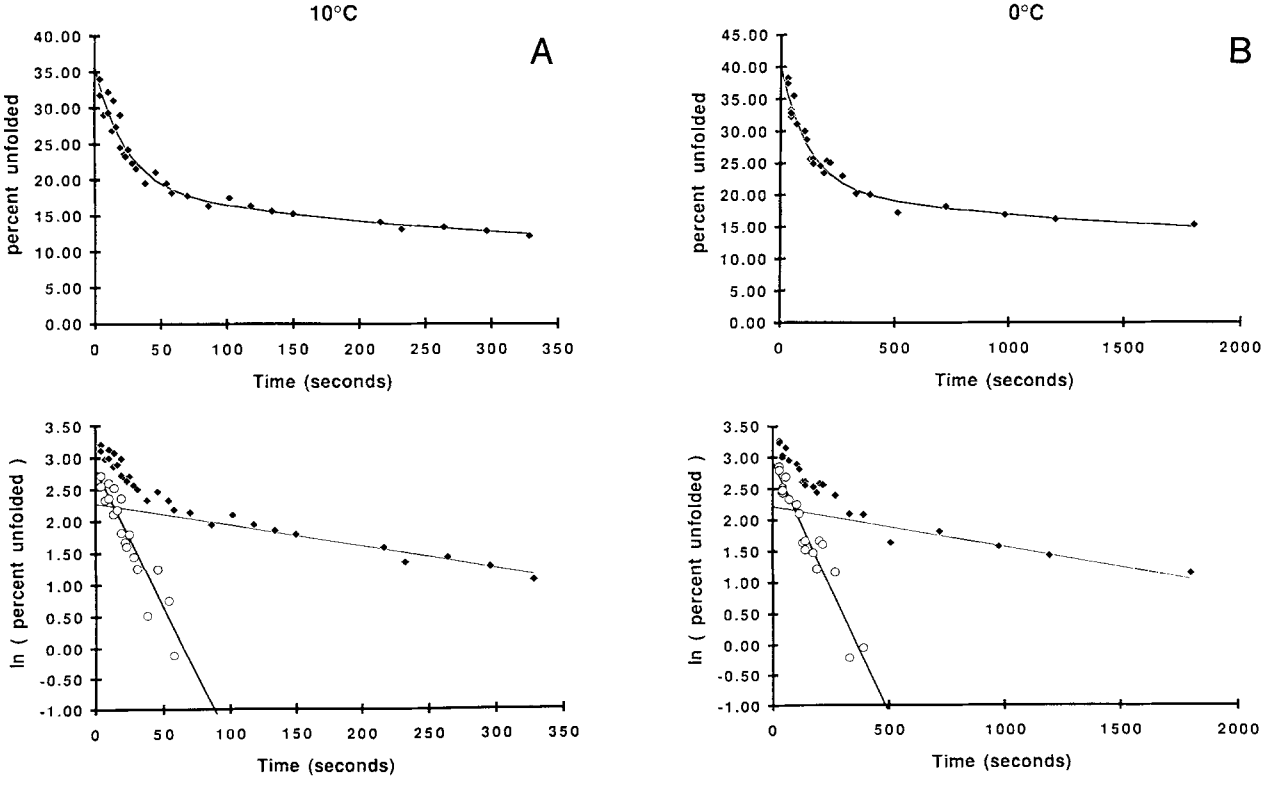

Fig. 3. Refolding kinetics of nuclease after temperature quench. Resonance intensities were derived from spectra such as those shown in Figure 2 and fitted to Equation 1 by least squares. The percentage of unfolded protein is plotted as a function of time after a temperature quench from 60 ° to 10 °C (**A**) and 55 ° to 0 °C (**B**) with both linear (upper panel) and semilog (lower panel) displays. Solid lines in the upper panels are least-squares fits to two exponential phases and a constant term. The open circles in the lower semilog panels represent the subtraction of the fitted slow phase and constant term from the kinetic data. Rate constants are given in Table 1.

Table 1. *Summary of temperature jump kinetic constants*

	Quench from 60 °C to 10 °C		Quench from 55 °C to 0 °C	
Transition (rate)	Amplitude[a]	Rate (s^{-1})	Amplitude	Rate (s^{-1})
$U_a^* \rightarrow N^*$ (k_a^*)	17%	0.045 (0.005)	19%	0.0081 (0.0002)
$U_b^* \rightarrow N^*$ (k_b^*)	10%	0.0038 (0.001)	10%	0.0006 (0.0001)
$U_f + U_f^* \rightarrow N + N^*$ (k_f)	73%	$\gg$0.1	71%	$\gg$0.1
$N^* \rightarrow N$ ($k_{trans \rightarrow cis}$)	28%	0.06 (0.003)	31%	0.015 (.005)

[a] The amplitude refers to the fraction of protein that refolds at long times and excludes the U_{res} component.

bined population of only 5%. The interconversion rates of $N^* \rightarrow N$ and $N \rightarrow N^*$ are indicated by $k_{trans \rightarrow cis}$ and $k_{cis \rightarrow trans}$, respectively. Under the conditions of the experiment N^* is transiently overpopulated, allowing a determination of $k_{trans \rightarrow cis}$. The equilibrium between N and N^* indicates that $k_{trans \rightarrow cis} \gg k_{cis \rightarrow trans}$.

Based on the kinetic scheme presented in Figure 4, the time dependence of $[N^*]$ is given by:

$$\frac{d[N^*]}{dt} = -[N^*]k_{trans \rightarrow cis} + [N]k_{cis \rightarrow trans} + [U_a^*]k_a^* + [U_b^*]k_b^* + [U_f^*]k_f^* \quad (2)$$

and that for [N] by:

$$\frac{d[N]}{dt} = -[N]k_{cis \rightarrow trans} + [N^*]k_{trans \rightarrow cis} + [U_a]k_a + [U_b]k_b + [U_f]k_f \quad (3)$$

The time dependencies of N and N^* were generated numerically and the values for $k_{trans \rightarrow cis}$, $k_{cis \rightarrow trans}$, N(0), and $N^*(0)$ were fit by least squares. The contribution from $[U_f]$ and $[U_f^*]$ occur in the dead time of the experiment and the values of k_f and k_f^* were not determined. $[U_a^*]$ and $[U_b^*]$ were assumed to be 95% of the slow-refolding material as had been reported from the equilibrium kinetic experiment. The relative amplitudes and kinetic constants were taken from the refolding kinetic experiments fitted to Equation 1 as given in Table 1. $[U_a]$ and $[U_b]$ together represent only 5% of the refolding material, and thus make little contribution to the analysis. Similar results for the $N \rightarrow N^*$ rate were obtained if it was assumed that $k_a = k_a^*$ and $k_b = k_b^*$, or if the relative rates were derived from the equilibrium folding experiments ($k_a = 5k_a^*$ and $k_b = 5k_b^*$). The integrated intensities for the His 121 (N) and His 121* (N^*) resonances are plotted in Figure 5 along with the curves fitted to these data using Equations 2 and 3. The first-order rate constants and amplitudes are presented in Table 1.

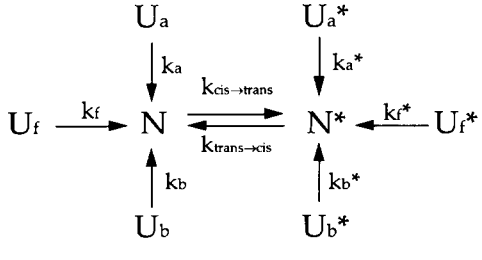

Fig. 4. Kinetic model of states contributing to the observed folding and isomerization phases as described in the text.

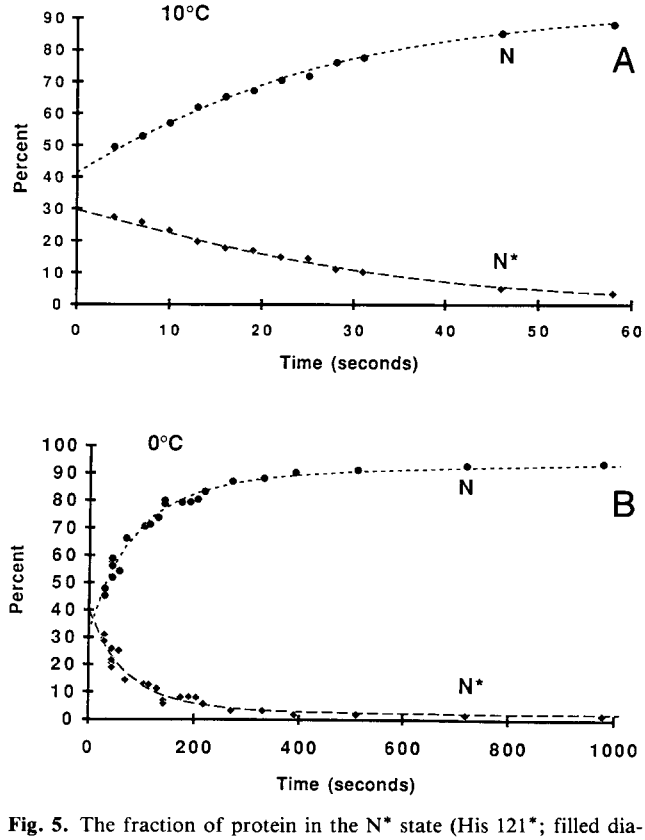

Fig. 5. The fraction of protein in the N^* state (His 121*; filled diamonds), and N state (His 121; filled circles) plotted as a function of time following a quench to 10 °C (**A**) and 0 °C (**B**). The dotted lines are least-squares fits to Equations 2 and 3. Rates are presented in Table 1.

The *trans* → *cis* isomerization rates were derived from least-squares fits to kinetic series collected at 10 and 0 °C as reported in Table 1. These measurements allowed a crude estimation of the activation enthalpy of $\Delta H^{\ddagger} = 18$–25 kcal/mol, assuming Arrhenius behavior. The uncertainties of the equilibration temperature, resonance intensities, and fitted kinetic constants contributed to a relatively large estimate of the uncertainty of the activation enthalpy; however, the large magnitude observed for $\Delta H^{\ddagger}$ is consistent with other estimates for X-Pro peptide bond isomerization (Grathwohl & Wüthrich, 1981).

Discussion

The nuclease thermal quench refolding kinetic experiments reported herein reveal two slow refolding phases and the transient overpopulation of a native-like intermediate (N*). Earlier equilibrium magnetization transfer NMR experiments at T_m indicated that two separate unfolded states were in direct equilibrium with the two native states (U* → N* and U → N) (Evans et al., 1989). While [N] ≫ [N*], the population of the unfolded states was inverted with [U*] ≫ [U]. The equilibrium concentrations and interconversion rates were consistent with the folded and unfolded populations differing in an X-Pro *cis-trans* configuration. The observed populations and the strong temperature dependence of X-Pro peptide bond isomerization led to the hypotheses that the N* intermediate should be overpopulated in a thermal quench experiment, as borne out in this communication. The hypothesis is further strengthened by the high activation enthalpy observed for the N* → N transition that is characteristic of X-Pro isomerization events.

The observed rate of *trans* → *cis* isomerization derived from this experiment (6.0×10^{-2} s^{-1}) is significantly faster than that observed for Lys-Pro in model peptide studies (5.4×10^{-4} s^{-1}) at 10 °C (Harrison & Stein, 1990). This rate enhancement suggests that the isomerization event takes place late in the folding pathway, when the very native-like N* state is able to impose a stress on the Lys 116-Pro 117 peptide bond. Similar rate enhancements have been observed in constrained cyclic peptide systems (Grathwohl & Wüthrich, 1981), for a ribonuclease A folding intermediate (Cook et al., 1979) and in energy calculations of proline isomerization in transition states of bovine pancreatic trypsin inhibitor (Levitt, 1981).

At short times following the quench a significant fraction of the sample remains in the unfolded states (U_a, U_a^*, U_b, and U_b^*). This material refolds slowly to N largely via N*. The His $H^{\varepsilon 1}$ chemical shifts are near the random coil value consistent with either a highly unstructured state akin to the type III state proposed by Levitt (1981), or the rapid formation of a molten globule-like slow folding intermediate (Flanagan et al., 1992). The activation energies for these processes derived from the kinetic data (Table 1) are large $\Delta H^{\ddagger}_{U_a^* \rightarrow N^*} = 26 \pm 1$ kcal/mol and $\Delta H^{\ddagger}_{U_b^* \rightarrow N^*} = 28 \pm 1$ kcal/mol, consistent with an X-Pro peptide bond isomerization process at sites distinct from Pro 117. The slow dissociation of unfolded protein dimers or larger oligomers might also explain the observed slow folding material, given the high protein concentrations necessary for this study, although the high activation enthalpy observed may not be expected for a dimerization process (Taniuchi et al., 1967; Taniuchi & Anfinsen, 1971). The significant breadth of the resonances due to the size of the protein, the high protein concentration, and the low temperature preclude the identification of other native-like intermediates.

Nuclease refolding kinetics from the acid denatured state have been monitored by tryptophan fluorescence (Epstein et al., 1971; Davis et al., 1979; Nakano & Fink, 1990). Refolding of the urea denatured state of nuclease has been examined using stopped-flow circular dichroism (Kuwajima et al., 1991; Sugawara et al., 1991). These studies report multiphasic kinetics with slow folding rates from 0.001 to 2.7 s^{-1}. The observed rates and amplitudes reflect variation with temperature, the denaturation and refolding buffer conditions, and the spectroscopic probe used. Our slowest refolding phase displays an amplitude and rate constant comparable to that reported by Sugawara et al. (1991).

In conclusion, results from NMR thermal quench refolding experiments are consistent with the existence of an equilibrium native state (N*) that is also a major intermediate in the refolding of staphylococcal nuclease under strong native conditions. The observed kinetic rates and activation enthalpy of the N* → N transition provide further support to the conclusion that the *trans* → *cis* conversion of the Lys 116-Pro 117 peptide bond is responsible for the NMR observations, and that the isomerization takes place late in the folding process. Kinetic heterogeneity in the unfolded state has also been identified by this experiment. The slow refolding intermediates (U_a^* and U_b^*) may be investigated by applying this experiment to nuclease proline variants.

Materials and methods

Staphylococcal nuclease A was prepared from a pAS1 expression construct in *Escherichia coli* and purified by ion-exchange chromatography as previously described (Kautz et al., 1990). Fifty milligrams of lyophilized protein was suspended in 1.0 mL D_2O and adjusted to pH* 5.3 with addition of sodium deuteroxide and deuterium chloride. (pH* refers to glass electrode meter reading uncorrected for deuterium isotope effects [Bundi & Wüthrich, 1979].) The sample was then thermally unfolded at 65 °C for 5 min to facilitate exchange of labile protons. Any precipitate formed during heating was removed by centrifugation. The deuterated protein was lyophilized again, and resuspended in 0.5 mL of 200 mM deuterated acetic acid, pH* 5.3, with 1 mM TSP (trimethylsilylpropionate; Aldrich)

as a chemical shift standard to yield a sample 4.3 mM (70 mg/mL) in nuclease.

The sample was transferred to a 5-mm NMR tube and secured in the spectrometer turbine with tape. The spectrometer (General Electric GN-500) probe was tuned, the lock optimized, and the magnet shimmed on the sample at the quench temperature. The sample and turbine were then removed from the spectrometer and immersed into a hot water bath at 60 or 65 °C as noted, through a hole in a styrofoam sheet, and allowed to equilibrate for at least 30 min. At time $t = 0$ the sample was stirred in a dry ice/ethanol bath for 4 or 6 s as required to reach the final temperature (see below), quickly wiped clean, and transferred into the spectrometer magnet. Initial spectra were obtained at $t = 8$–12 s after initiation of the quench. Subsequent FIDs were acquired at 2.5-s intervals, limited by the time required to write the data to disk and allowing time for the magnetization to recover by spin-lattice relaxation. A 70° pulse was used, giving 95% of the available magnetization as signal yet leaving over 30% of M_z to hasten recovery. Spectra of early time points were Fourier transformed from single FIDs to avoid time-averaging. For later time points (>1 min) four or more FIDs were averaged to improve the signal-to-noise ratio.

To determine the immersion time in the dry ice/ethanol bath, the thermistor of a digital electronic thermometer was secured in an NMR tube with an identical amount of buffer as the actual sample. Trials were made with a series of immersion times in dry ice/ethanol before transferring the sample to an ice-water bath (for final temperature = 0 °C) or a precooled styrofoam block (for final temperature = 10 °C), where it was allowed to equilibrate. It was determined to require 6 s for a quench from 55 °C to 0 °C and 4 s from 60 °C to 10 °C. The sample temperature was consistently within 3 °C of target in the seconds immediately after the quench. Inserting a warm sample into a cold spectrometer probe showed a half-time of sample equilibration of about 20 s. The set temperature of the spectrometer probe was confirmed to be accurate within 0.2 °C using the digital electronic thermometer.

Intensities of resonances in spectra from the kinetic experiment were measured using the program GEMCAP, supplied with the GN-500 spectrometer, which permits the user to interactively match displayed experimental and calculated spectra, and minimize a difference spectrum. Intensities thus measured were further processed using spreadsheets (Microsoft EXCEL implemented on a Macintosh IIci). The fraction of folded protein was determined from the relative intensities of the unfolded resonances and the sum of all His $H^{\varepsilon 1}$ resonances. Some additional data including longer times were added, in which the fraction of folded protein was obtained from the intensity of the unfolded resonance relative to the sum of the His 121 resonances, and assuming the intensity of His 121 represented one-quarter of the net histidine intensity from folded protein. Both methods gave consistent estimates of the fraction of folded protein. The fraction N*/N was calculated from the relative intensities of His 121 and His 121* only, because this pair is most well resolved. Kinetic constants for the equations defined below were optimized by least-squares using Newton's method as implemented in the SOLVER module of Microsoft Excel.

Spin-lattice relaxation times (T_1) for the His $H^{\varepsilon 1}$ hydrogens were measured by inversion recovery. T_1 values for His 46, 124, and 121 are 1.0 ± 0.2 s, and that of His 8 is 2.1 ± 0.2 s at 10 °C. The 2.5-s interval between FIDs in the kinetic experiment is not sufficiently long compared to all T_1 values to ensure complete longitudinal relaxation. Insufficient time for the magnetization to recover by spin-lattice relaxation could cause a diminution in the apparent intensities of the resonances. This effect was studied on a sample equilibrated at 10 °C. FIDs were acquired after zero to seven dummy scans (70 °C pulses without acquisition separated by 2.5 s). It was found that there was a 15% loss of intensity from His 8 and a 7% loss from all other resonances, between the first and second spectra. There was an additional 2% loss from His 8 between the second and third spectra, and no measurable change in any resonances in all subsequent spectra. This is exactly as predicted by the T_1 relaxation times measured at 10 °C. Intensity data were corrected for this effect prior to curve fitting. It was not possible to obtain the relaxation times for resonances of transiently unfolded nuclease at low temperatures. The relaxation times of the folded nuclease histidines have been observed to be relatively temperature independent (Evans et al., 1989), changing less than 20% between 40 °C and 10 °C. The T_1 relaxation time of thermally unfolded resonances measured at 50 °C, 0.76 s, was used in the correction. It should be noted that the rate measurements depend on changes in the relative intensities of resonances and these corrections are uniform for all data after the first two points. The intensity corrections only significantly change the first time point. The intensity corrections made for incomplete longitudinal relaxation did not result in significant changes in derived kinetic constants.

Acknowledgments

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