Early formation of a beta hairpin during folding of staphylococcal nuclease H124L as detected by pulsed hydrogen exchange

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Abstract

Pulsed hydrogen exchange methods were used to follow the formation of structure during the refolding of acid-denatured staphylococcal nuclease containing a stabilizing Leu substitution at position 124 (H124L SNase). The protection of more than 60 backbone amide protons in uniformly ^{15}N-labeled H124L SNase was monitored as a function of refolding time by heteronuclear two-dimensional NMR spectroscopy. As found in previous studies of staphylococcal nuclease, partial protection was observed for a subset of amide protons even at the earliest folding time point (10 msec). Protection indicative of marginally stable hydrogen-bonded structure in an early folding intermediate was observed at over 30 amide positions located primarily in the β-barrel and to a lesser degree in the α-helical domain of H124L SNase. To further characterize the folding intermediate, protection factors for individual amide sites were measured by varying the pH of the labeling pulse at a fixed refolding time of 16 msec. Protection factors >5.0 were observed only for amide positions in a β-hairpin formed by strands 2 and 3 of the β-barrel domain and a single site near the C-terminus. The results indicate that formation of stable hydrogen-bonded structure in a core region of the β-sheet is among the earliest structural events in the folding of SNase and may serve as a nucleation site for further structure formation.

Keywords: Staphylococcal nuclease; pulse labeling; hydrogen exchange; NMR; protein folding

Staphylococcal nuclease (SNase) has long been recognized as an excellent model for studies of protein folding and stability (Anfinsen et al. 1972; Shortle 1995). Much insight has been gained, for example, about the role of proline isomerism in protein folding from studies on SNase (Evans et al. 1987, 1989; Kuwajima et al. 1991; Hinck et al. 1996; Maki et al. 1999) and other model proteins (Brandts et al. 1975; Ernst et al. 1985; Kiefhaber et al. 1990; Herning et al. 1991; Schmid 1993). SNase has also been a source of information on the contribution of *cis- trans* isomerism of prolines to protein conformational heterogeneity and on the interplay between local conformation and global stability as studied by the effects of single-site mutations (Alexandrescu et al. 1990). In particular, the presence of a *cis* peptide bond at position 116-117 in SNase (see Fig. 1) has been found to strongly increase the stability of SNase (Hinck et al. 1996). Although the structure of SNase can be divided into two subdomains (Fig. 1), an N-terminal β-barrel domain comprised of five antiparallel β-strands and a C-ter-

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Abbreviations: C_m, midpoint of a GuHCl denaturation transition; DCl, ^{2}HCl; D_2O, ^2H_2O; GuHCl, guanidine hydrochloride; H124L SNase, staphylococcal nuclease with a histidine-to-leucine substitution at position 124 (sequence equivalent to the staphylococcal nuclease isolated from the V8 strain of *Streptococcus aureus*); *m*, slope of the GuHCl denaturation transition in units of kcal mol^{-1} M^{-1}; HSMQC, heteronuclear single-quantum, multiple-quantum correlation; pdTp, deoxythymidine-3′,5′-bisphosphate; pH*, pH of a sample dissolved in D_2O as determined by an uncorrected glass electrode measurement; Pro$^-$, mutant of SNase with P11A, P31A, P42A, P47G, P56A, and P117G substitutions; SNase, wild-type staphylococcal nuclease with a sequence equivalent to that isolated from the Foggi strain of *S. aureus*).

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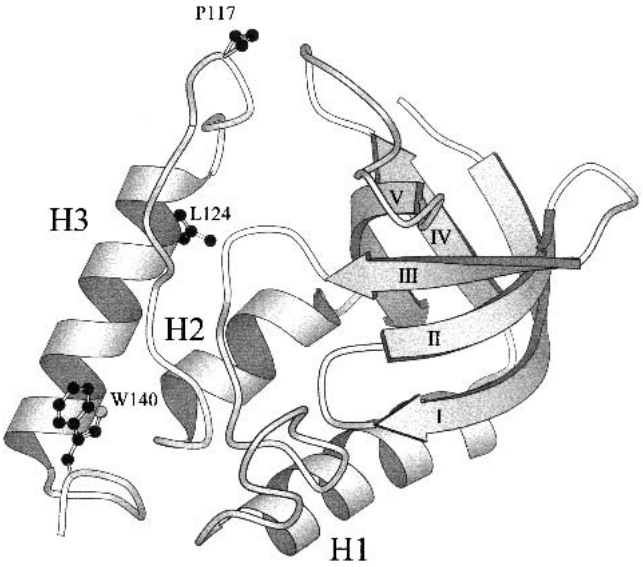


Fig. 1. Ribbon diagram of H124L staphylococcal nuclease (SNase) based on the NMR structure (Wang et al. 1997) and oriented to show the two subdomains and their connecting loops. Trp^{140}, the sole fluorescence probe, is indicated as are the positions of Pro^{117} and Leu^{124}. The β-strands are numbered with Roman numerals I–V. The α-helices are designated by H1–H3. The figure was prepared using the program MOLSCRIPT (Kraulis 1991).

minal cluster of three α-helices, the wild-type protein shows the cooperative unfolding transition characteristic of single-domain proteins (Anfinsen et al. 1972; Shortle and Meeker 1986). Structural and mutational data on SNase and several deletion mutants suggested that residual structure existed, either in the denatured state or in a subdomain of the protein (Shortle et al. 1990; James et al. 1992; Shortle 1992, 1993; Shortle and Abeygunawardana 1993; Alexandrescu et al. 1995).

More recently, several lines of study, including calorimetry (Carra et al. 1994; Carra and Privalov 1995, 1996), NMR (Wang and Shortle 1995; Gillespie and Shortle 1997), pulsed hydrogen exchange (Jacobs and Fox 1994), mutational studies (Kalnin and Kuwajima 1995), and kinetic analysis of a proline-free (Pro^-) SNase variant (Walkenhorst et al. 1997) have converged to indicate that the β-sheet subdomain of the protein is the most stable to denaturation and among the first regions of the protein to fold. By coupling hydrogen exchange labeling techniques with NMR detection of individual amide protons, it is possible to localize folding events to specific regions of the protein (Roder and Wüthrich 1986; Roder et al. 1988; Udgaonkar and Baldwin 1988). In a previous pulse-labeling study on the P117G mutant of SNase, Jacobs and Fox (1994) found a number of amide protons, especially in the β-sheet domain, that were partially protected from solvent exchange at refolding times as early as 5 msec. After 100 msec of refolding, they measured protection factors in the range 10–60 for amide protons throughout the β-sheet domain (except for the first strand) and several residues in each of the three helices.

For further investigation of this system, we chose to work with H124L SNase, the more stable variant originally isolated from the V8 strain of *Streptococcus aureus*, rather than the wild-type enzyme (SNase), which was isolated from the Foggi strain of *S. aureus*. H124L SNase differs from SNase by a single residue substitution in helix 3 (Fig. 1), which stabilizes the protein by 1.3 kcal mol^{-1} (GuHCl denaturation of H124L SNase: $\Delta G = 6.91$ kcal mol^{-1} at 20°C at pH 7; GuHCl denaturation of SNase: $\Delta G = 5.62$ kcal mol^{-1} [Shortle 1986]). We describe below the use of pulsed hydrogen exchange methods to measure the time course and magnitude of protection of amide protons as probes for the development of structure during early stages of folding.

Results

Verification of NH exchange rates under denaturing conditions

Denaturing conditions to initiate refolding in pulse-labeling experiments were established by comparing the real-time exchange of backbone amide protons of H124L SNase following transfer of the protein from H_2O to D_2O as monitored by the time-dependence of peak heights in ^1H-^{15}N HSMQC spectra. Exchange rates were compared for uniformly ^{15}N-labeled H124L SNase samples denatured at pH 3.0 in the presence and absence of 2.5 M GuHCl (data not shown). The exchange rates for the most slowly exchanging amide protons were nearly identical under both sets of denaturing conditions and were within a factor two of predicted rates for unfolded nuclease at pH 3.0 (Bai et al. 1993), indicating that the protein is largely denatured (Filfil and Chalikian 2000) and, most importantly for these studies, shows no significant NH protection at pH 3.0 under conditions of low ionic strength. This is consistent with previous observations by fluorescence that H124L SNase is unfolded at pH 3.0 (Walkenhorst et al. 1997) in the absence of denaturant and by concentrations of GuHCl greater than ~1.5 M ($C_m = 1.08$ M) at pH 7 (Shortle and Meeker 1986). An unfolded control sample (pH 3.0) exposed to the labeling pulse at high pH was fully labeled when compared to a folded control, confirming that H124L SNase has no residual backbone NH protection at acid pH and low ionic strength. The stability of H124L SNase to the conditions of the labeling pulse were verified by equilibrium CD measurements (see Fig. 2) and kinetically by using stopped-flow fluorescence (see Materials and Methods) to monitor the protein following pH jumps from pH 5 to pH 9 and from pH 5 to pH 10.

Initial amplitudes early in refolding

The refolding kinetics for H124L SNase were measured by recording ^1H-^{15}N HSMQC spectra for protein samples that

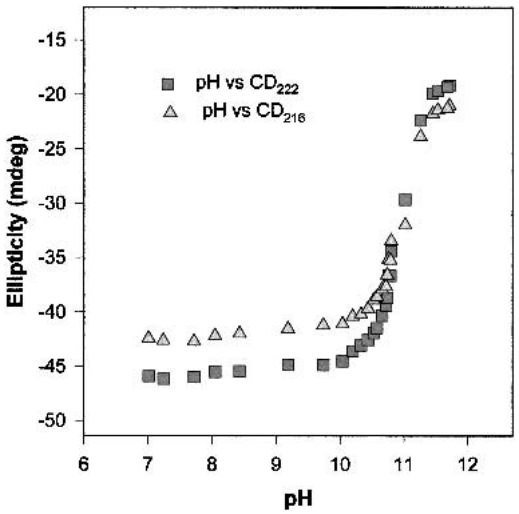


Fig. 2. CD spectra for H124L SNase collected as a function of pH in the alkaline range. The ellipticities at 222 and 216 nm are plotted as a function of pH.

were allowed to refold for varying times (t_f) before application of a high pH pulse to label exposed amide sites. The pulse-labeling data show a gradual increase in protection as folding progresses that is largely complete after 20 sec. The amplitudes of the amide cross peaks in the 2D spectra are diminished when compared with those of the control spectra, even at the earliest folding time (10 msec). Figure 3 shows a plot of the normalized initial amplitudes (A_0) for backbone amide protons in H124L SNase after 10 msec of refolding. The most striking observation is that more than half of the 60 observed amide sites show initial amplitudes significantly lower (A_0 <0.8) than the value expected for a fully labeled amide site in the unfolded state (A_0 = 1). Thus, a species accumulates during the dead time of the quenched flow experiment in which many of the amide sites found to be highly protected in the folded protein are already partially protected against exchange with solvent hydrogens. These rapidly protected sites include positions in all strands of the beta sheet (Fig. 3), in each of the three major α-helices (H1–H3, Fig. 1), and in a minor hydrophobic domain, which includes residues 37–40 and 110–112 (Wang et al. 1990b; Wang and Shortle 1996; Loh et al. 1993). The lowest A_0 values are observed at sites in strands 2 and 3, whereas many positions in the three α-helices show no protection at all (see Figs. 3, 4B). Qualitatively similar results were observed by Jacobs and Fox (1994) for the P117G mutant of SNase for a smaller set of 39 amide protons resolved by homonuclear ^{1}H NMR spectroscopy.

Time course of NH protection during refolding

Representative data for a number of amide sites are plotted in Figure 4 as a function of time. In panel A, the time course of protection for Val99, located in helix 2, is shown; the data are from all 15 samples measured and cover 10 time points between 10 msec and 160 sec. The time course of protection is also shown for additional amide sites located in the helical (Fig. 4B) and β-sheet (Fig. 4C) regions of H124L SNase. The protection observed between 10 msec and 160 sec (in addition to the early events that give rise to A_0) occurs in two kinetic phases of nearly equal amplitude, which are similar for all probes (Fig. 4A–C). The kinetic data for 53 of the 60 observed amide protons were fitted to two exponentials. The rates from the fits are plotted as a function of residue number in Figure 5. The rates of the faster process fall in the range between 2 and 11 sec^{-1}, whereas those of the slower phase range from 0.08 to 0.25 sec^{-1}. The fits indicate a global protection pattern in which all 53 probes show, within error, a nearly identical rate of 5 ± 1 sec^{-1} for the faster phase and a rate of 0.13 ± 0.01 sec^{-1} for the slower process (99% confidence interval from data in Fig. 5).

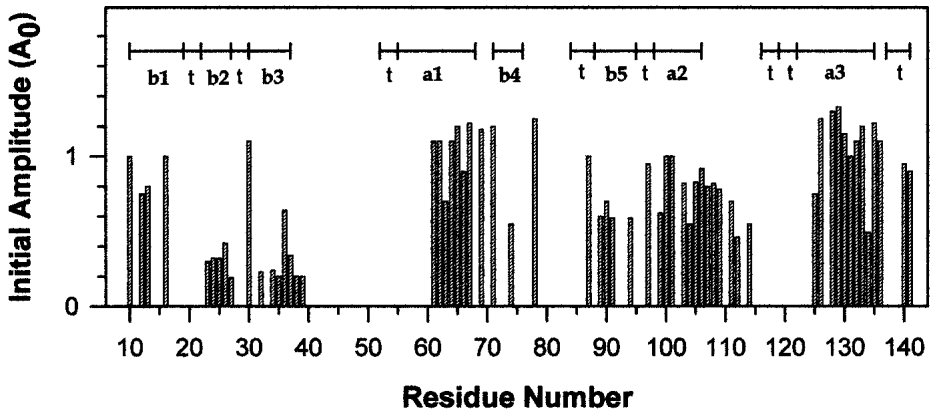
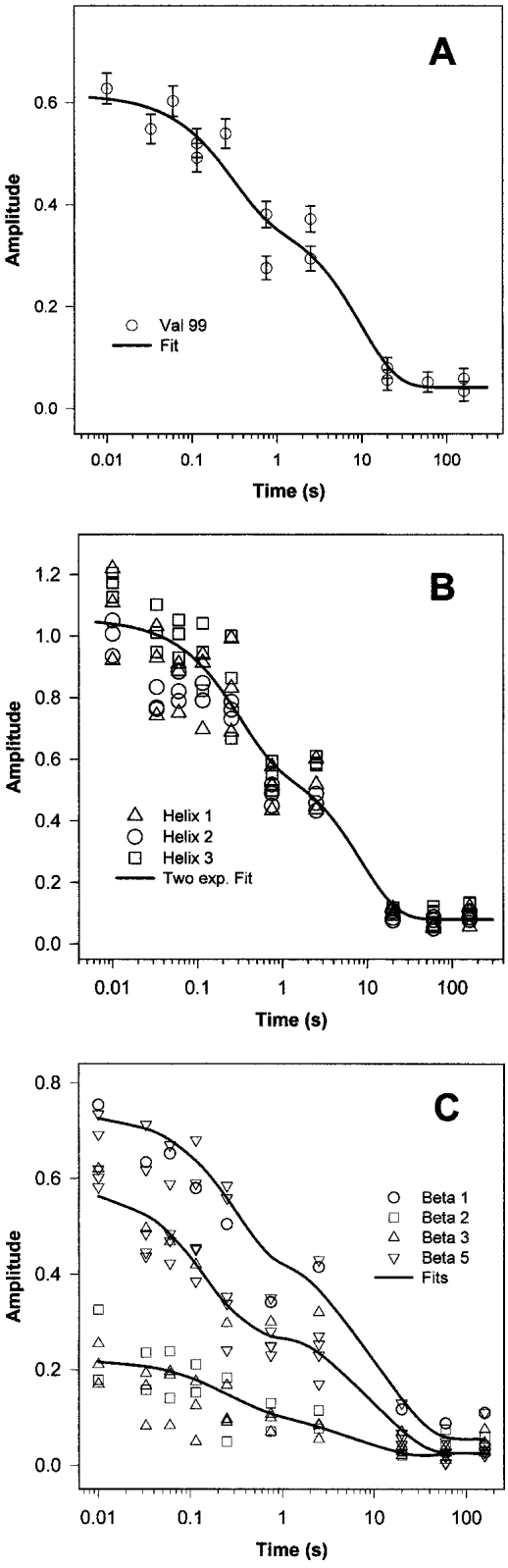


Fig. 3. Initial amplitudes after 10 msec of folding for slowly exchanging amide protons in H124L SNase are indicated in the bar graph. Positions of secondary structural elements are indicated at the top of the panel.

Measurement of protection factors

A decrease in the initial amplitude, A_0 (Fig. 3), can arise from structure formation during refolding, from intrinsically slow exchange for certain amino acid sequences, or through exchange of these sites with solvent deuterons at a later time before NMR data collection. To distinguish among these possibilities, protection experiments were performed at a fixed refolding time as a function of the pH of the labeling pulse. A t_f of 16 msec was chosen as optimal to simultaneously measure a protection factor as early as possible in refolding while allowing for a long-enough labeling pulse (50 msec) to completely exchange most unprotected amide sites at all pH values chosen for the analysis. The proton occupancies compared to fully protonated control spectra were measured after 16 msec of refolding for eight pH values between pH 7.3 and pH 10.7. The data were then fitted as described previously (see Materials and Methods) to extract protection factors for 75 different amide sites in H124L SNase. The fits for sets of amides in β-strand 2 (V23, K24, L25, and M26) and helix 2 (E101 and R105) and the extended hydrophobic region involving helix 2 (L108 and A109) are compared in Figures 6A and 6B, respectively. The amide protons located in β-strand 2 showed measurable protection factors with an average value of P = 6.5 (Fig. 6A), indicating that they take on average about six times as long to exchange with solvent as would be predicted for an unstructured region with the same pro-



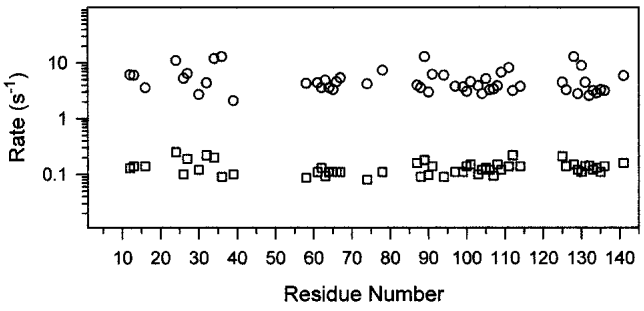


Fig. 5. The rates for the two observable processes following the initial dead-time protection are plotted as a function of residue number. The rates for λ_1 (open circles) fall between 2 and 11 sec^{-1} with an average value of 5 ± 1 sec^{-1}, whereas those for λ_2 (open squares) range from 0.08 to 0.25 sec^{-1} with an average value of 0.13 ± 0.01 sec^{-1} (errors are 99% confidence interval).

Fig. 4. (*A*) Observed time course for the protection of Val99 of H124L SNase, which is located near the beginning of helix 2, plotted for all 15 samples covering 10 time points between 10 msec and 160 sec along with a two-exponential fit for these data. The error bars represent the uncertainty in the peak height for a given NH proton (ΔP_i), $\Delta P_i/P_i = \Delta I_i/I_i(t_f) + \Delta I_i/I_i$ (control), in which P is the peak height and I is the intensity of that peak in the control spectra and at a particular time point in the folding reaction. (*B*) Observed time course for protection of three residues from each of the three major helices in SNase plotted along with a global fit to the data. Error bars are contained within the symbols used and duplicate time points have been averaged. (*C*) Plot of protection data as a function of time for amide sites with varying degrees of missing amplitude from four of the beta-strands in SNase along with global fits to the data. Error bars have been omitted for clarity, and duplicate time points have been averaged.

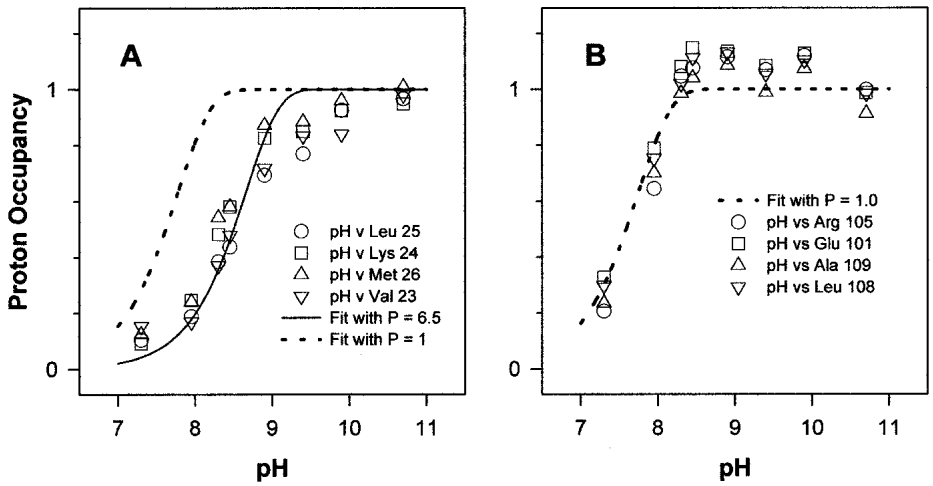


Fig. 6. Protection factors obtained by pH variation of the labeling pulse after a constant refolding time of 16 msec. (*A*) Proton occupancies for residues in β-strand 2 of SNase (Val23 to Met26) are plotted along with a fit (solid line) for a protection factor (P_f) of 6.5 and a fit for $P_f = 1$ (no protection) (dashed line). (*B*) Proton occupancies for residues in the helix H2 region of SNase are plotted along with a fit for $P_f = 1$ (dashed line). Symbols are defined in the figure legends.

tein sequence at this temperature. The amide protons in and near helix 2, on the other hand, show no protection and exchange at the predicted rate for this sequence (Bai et al. 1993). The protection factors for all amide protons measured in this experiment are plotted in Figure 7 along with an indication of the secondary structure in the native protein sequence. Amides with protection factors P_f that fall in the range between 2 and 5 are observed primarily in β-strands 2 and 3, but a few residues in strands 4 and 5 and in each of helices H1–H3 also fall in this range as do several amides near the C-terminus. Amide protons with P_f >5.0 are observed primarily in β-strands 2 and 3. Other residues with slightly elevated protection factors (P_f = 3–7) occur in a minor hydrophobic domain near Trp 140 (Loh et al. 1993), which includes residues 134 in helix 3, and residues 138–141. In Figure 8, amide protons with P_f >5.0 are indicated by black spheres, whereas those with P_f between 2.5 and 5.0 are indicated by gray spheres.

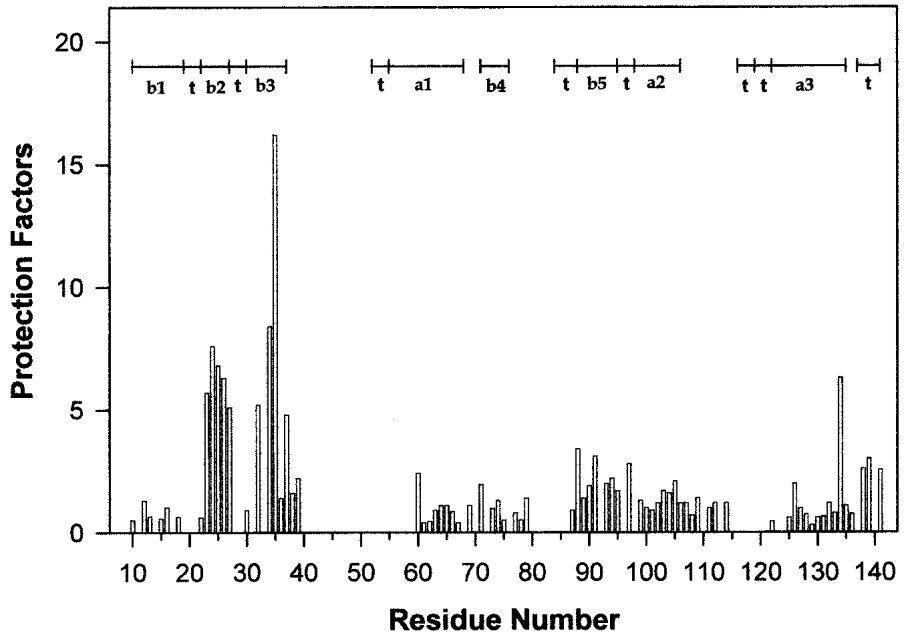


Fig. 7. Plot of protection factors, as a bar graph and as a function of sequence. The secondary structure in SNase is indicated at the *top* of the panel.

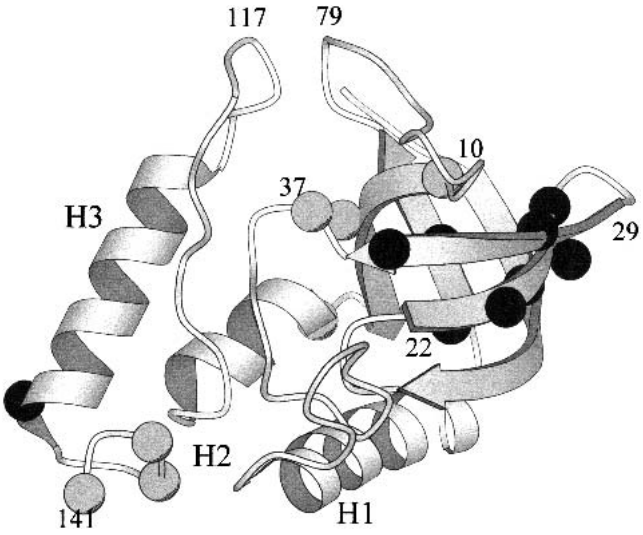


Fig. 8. Ribbon diagram of H124L SNase (Tuckses et al. 1996; PDB Accession code 1SNO) displayed with protection factors (P_f) >5.0 plotted as black spheres and P_f values between 2.5 and 5.0 plotted as gray spheres.

Discussion

Detection of early structural events in protein folding is an important goal of many recent studies (for review, see Eaton et al. 1997; Roder and Colón 1997; Callender et al. 1998; Roder et al. 2000). The use of conventional and ultrarapid mixing methods combined with optical detection has been especially important for characterizing the kinetics of protein folding (Roder and Shastry 1999). Hydrogen exchange labeling techniques offer an important advantage in that they can detect specific structural events at the level of individual residues (Roder and Wüthrich 1986; Roder et al. 1988; Udgaonkar and Baldwin 1988). The present studies provide the most comprehensive set of such data available to date for staphylococcal nuclease.

To detect protection of amide protons during refolding, NH groups must be shielded from solvent, either by being buried away from solvent or as is more often the case, by becoming involved in an intramolecular hydrogen bond during refolding. A second prerequisite is that the amides protected in refolding must also be protected in the native structure. Despite this requirement, the patterns of protected amide protons can provide information even on nonnative structure in folding intermediates. This was shown recently for β-lactoglobulin, in which the formation of nonnative helical structure was detected preceding the formation of native β-sheet (Kuwata et al. 2001). More generally, observation of nonnative structure requires that amide positions be protected in both the intermediate and the native structures and that the patterns or rates of protection contain clues that allow the existence of nonnative structure to be inferred. For example, if a β-hairpin is formed in an intermediate, alternating amide sites in that region would be protected. If this region converted to an α-helix (or a pair of α-helices) in the fully folded protein, the resulting pattern of protection would differ from that of a sequence that contained an α-helix in both the intermediate and the native protein.

To establish that protection of amide positions is attributable to the formation of structure during folding, it is necessary to verify that there is no residual structure under denaturing conditions. Fluorescence measurements following denaturation by acid (Walkenhorst et al. 1997) or by GuHCl (Shortle 1986) suggested that nuclease was completely unfolded by acid or by GuHCl alone. Previous hydrogen exchange studies by Loh et al. (1993) also suggested that there was no residual structure in denatured SNase. On the other hand, a number of observations by NMR and other methods indicates that SNase and some of its deletion mutants contain residual structure under some denaturing conditions (Wang and Shortle 1995; Gillespie and Shortle 1997; Filfil and Chalikian 2000). To resolve this question for these experiments, we looked for residual NH protection by performing real-time exchange measurements under several sets of denaturing conditions. The observed rates were within a factor two of the predicted rates (Bai et al. 1993) and were identical within error for solutions at pH 3 with or without added GuHCl (2.5 M). This is consistent with previous hydrogen exchange studies on SNase deletion mutants, which found that protection factors for a denatured state were no larger than 2.4 (Mori et al. 1997), whereas those in a structured deletion mutant showed protection factors between 1 and 190 (Alexandrescu et al. 1996). This justified our use of pH 3 alone under low ionic strength conditions to denature SNase.

Our results for protection in the dead time of the pulse-labeling experiment (10 msec) are similar to those of Jacobs and Fox (1994) on the P117G variant of SNase. In particular, both studies show evidence for population of a folding intermediate consisting largely of the β-sheet domain of SNase with some missing amplitude for amide sites outside the β-barrel. In our studies, we were able to observe between 60 and 75 amide protons (depending on field strength), which included many more residues in the helices and turn regions than the 39 amides reported by Jacobs and Fox (1994), despite the addition of stabilizing salts in their buffer solutions. We attribute this to two main factors: (1) The use of heteronuclear ^{1}H-^{15}N HSMQC experiments, rather than the proton NMR used by Jacobs and Fox, allowed us to identify a larger number of signals; (2) the addition of the stabilizing ligands Ca^{2+} and pdTp directly in the quench buffer to prevent the loss of information because of exchange during sample workup.

To measure protection factors in the intermediate, we chose to vary the pH of the labeling pulse in a series of experiments with a fixed refolding time of 16 msec. Using

a refolding delay of 100 msec followed by a 50-msec labeling pulse, Jacobs and Fox (1994) observed measurable protection factors throughout the protein with the largest protection observed for residues in the β-barrel. The main result from our studies is that at an earlier stage of folding (16 msec), we observe protection factors >5.0 only in a β-hairpin consisting of strands 2 and 3 of the β-barrel, suggesting that this region of the protein is the first to acquire stable hydrogen bonds and may act as a nucleation site for structure formation. This is consistent with previous evidence that this β-hairpin is partially structured in a deletion mutant of SNase (Wang and Shortle 1995; Gillespie and Shortle 1997). Recent studies of the pentapeptide N-acetyl-YKGQP-NH_2, which contains the central residues of this β-hairpin, indicated that turn conformations were partially populated in solution (Ramakrishna and Sasidhar 1997). Studies on a longer 15-residue peptide containing the entire β-hairpin were inconclusive because of its strong tendency to aggregate (Ramakrishna and Sasidhar 1998).

Interestingly, residues in strands 2 and 3 contain some of the most slowly exchanging amide protons under native conditions (Loh et al. 1993). In particular, the exchange rates for residues 23–26 are consistent with exchange via global unfolding. Thus, the most stable core region of the native SNase structure is also among the earliest to acquire persistent hydrogen bonds during folding, as is observed for a number of other proteins (e.g., Roder et al. 1988; Woodward 1993; Bai et al. 1994; Raschke and Marqusee 1997; Kuwata et al. 2001).

The global rates of 5 sec^{-1} and 0.13 sec^{-1} observed in our time-resolved labeling experiments are consistent with similar rates detected by other methods (Sugawara et al. 1991; Chen and Tsong 1994; Kalnin and Kuwajima 1995; Maki et al. 1999; Walkenhorst et al. 1997). There appears to be no detectable protection of amides associated with a 1-sec^{-1} phase observed by stopped flow, which is consistent with our assignment of this phase to a nonproline *cis-trans* isomerism in our previous stopped-flow fluorescence studies (Walkenhorst et al. 1997). We also see no additional changes in proton occupancy at folding times >20 sec after Pro117 has isomerized to *cis*, suggesting that this process causes no additional protection detectable in our experiment.

Although we have not directly followed the time course of formation of the early intermediate containing the β-hairpin by pulsed hydrogen exchange, this process may be related to the observation of a lag phase over the 2–10 msec time range in our earlier stopped-flow fluorescence experiments on H124L nuclease and a proline-free variant (Walkenhorst et al. 1997). The amide protection patterns observed at a folding time of 16 msec (Fig. 7) differ substantially from those measured previously at a folding time of 100 msec followed by a 50-msec labeling pulse (Jacobs and Fox 1994). At these longer times, well-protected amide sites (protection factors in the range 10–60) were found not only in β-strands 2 and 3, but also strands 4 and 5, as well as isolated residues in helices 2 and 3. Because the time-resolved pulse-labeling experiments show only minor changes over the 10–100 msec time range (Fig. 4), the increase in the level and number of protected sites is probably attributable to the partial accumulation of a more stable native-like species in the subsequent kinetic phase, which has a time constant of ~200 msec.

Initiation sites for protein folding

An important goal of protein-folding studies is to identify regions of the chain that become structured during early stages of refolding and, therefore, are potential nucleation sites for folding. For many small, single-domain proteins, the first appearance of a nucleus of native-like contacts is also the rate-limiting step in folding, resulting in a concerted (two-state) folding process. However, for many other proteins, including SNase, this is not the case. The initial stabilization of a core region of the structure (in the present case a central pair of β-strands that forms on the 10-msec time scale) is succeeded by a series of slower conformational events, giving rise to transient accumulation of partially structured states. For proteins with two-state folding mechanisms, Plaxco et al. (1998) found a striking (inverse) correlation between the rate of folding and the contact order, a measure of the average sequence separation of contacting residues. Thus, the kinetic barrier encountered in small proteins comprising a single folding unit appears to be dominated by the overall topology of the chain rather than specific aspects of their amino acid sequence. However, structurally more complex proteins that do not follow a simple two-state folding mechanism often fold more slowly than predicted based on their contact order (Plaxco et al. 2000). This is also the case for SNase. The folding rate predicted based on its relatively low contact order (~10%) is several orders of magnitude faster than the experimentally observed rate for the major folding step (5 sec^{-1}). Thus, the rate-limiting barrier in the formation of the native SNase structure is clearly dominated by factors other than the overall topology of the chain, such as specific tertiary interactions or docking of subdomains. On the other hand, topological features such as the β-hairpin in SNase described here are more likely to be an important factor in determining the rate at which the early intermediate is formed.

Materials and methods

Protein samples

H124L SNase was prepared and purified as described previously (Wang et al. 1990a; Royer et al. 1993), except that the cells used

to prepare some samples of unlabeled protein were grown on LB media. Uniformly ^{15}N-labeled protein was obtained by growing the cells in M9 minimal media containing 1 g of $(^{15}NH_4)_2SO_4$ (Isotec) per liter of media. The final purity was >95% by SDS-PAGE in all cases.

Pulsed hydrogen-exchange experiments

A Biologic QFM-5 quenched flow instrument was used to label unprotected backbone amide positions at various times during refolding. A 3-mM solution of H124L SNase in 99.9 % D_2O (Isotec) was unfolded by lowering the pH* (uncorrected glass electrode reading) to below 3.0 by the addition of DCl. All exchangeable NH groups were deuterated by first placing the unfolded protein solution at 65°C for 1 h, followed by incubation overnight at 25°C. Refolding was initiated at 15°C by a 1:2 dilution of the unfolded protein with a refolding buffer containing 150-mM KCl and 75-mM sodium acetate (pH 6.8) such that the final refolding mixture had a pH* of 5.3 and contained 100-mM KCl and 50-mM sodium acetate. All subsequent buffers used in the pulse-labeling experiment contained 100-mM KCl. All pH values were verified by manual mixing at 15°C before running the experiments. Refolding was allowed to continue for a variable length of time for 10 samples covering folding times (t_f) of 10, 33, 60, 115, 250, and 750 msec, and 2.5, 20, 60, and 160 seconds. A number of these time points (115 msec, 750 msec, 2.5 sec, 20 sec, and 160 sec) were repeated in independent experiments as controls for procedural variations such as the choice of continuous or interrupted mixing mode (necessary for time points >200 msec) or variation of solvent composition. For example, the refolding buffer contained H_2O for shorter refolding times, but D_2O was used for samples with a t_f longer than a few seconds to prevent exchange with solvent protons from occurring during folding. As a control, the 2.5-sec time point was run independently under each condition: one trial with refolding buffer containing H_2O and one trial with refolding buffer containing D_2O.

After refolding for the designated amount of time, an H_2O labeling pulse (t_p) was introduced at high pH to exchange deuterons for protons at unprotected amide sites. For samples refolded in H_2O, this was accomplished by a 1:1 dilution of the refolding mixture with a solution containing 100-mM glycylglycine adjusted to provide a pH of 8.8 after mixing. For samples refolded in D_2O, a 1:4 dilution was used to achieve a sufficiently high molar fraction of H_2O during the labeling pulse. Because the initial unfolded protein solution always contained 99.9% D_2O, the final conditions during the labeling pulse were 83% H_2O for the samples refolded in H_2O and 80% H_2O for those refolded in D_2O. The labeling was allowed to continue for 57 msec before quenching and completion of folding at lowered pH was initiated in a third and final mixing event. The quench buffer contained 300-mM sodium acetate in D_2O, adjusted to provide a final pH* of 5.3 after mixing, as well as 20-mM $CaCl_2$ and 1 mM of the competitive inhibitor pdTp (Cal Biochem). The latter components were included because the binding of Ca^{++} and pdTp are known to stabilize SNase and to drastically decrease the rates of hydrogen exchange for many backbone protons (Loh et al. 1993). The samples for each time point were kept on ice before workup. Final concentration of the samples and transfer into a suitable NMR buffer (50-mM d4-acetic acid, 0.1-M KCl, 20-mM $CaCl_2$, 1-mM pdTp in 99.9% D_2O) was accomplished by three sequential cycles of dilution and concentration at 4°C using Centri-Prep concentrators (Amicon) with a MW cutoff of 10 kD.

Measurement of protection factors

Protection factors after 16 msec of refolding were measured on a Biologic stopped flow/quenched flow instrument (SFM-4/QFM-4). The extent of protection for backbone amide sites was probed by systematic variation of the labeling pulse pH in separate experiments at a constant refolding time. The pH of the 50-msec labeling pulse was varied for eight samples in the range between 7.0 and 11.0 following a fixed refolding time of 16 msec. The pH values after mixing were 7.3, 8.0, 8.3, 8.5, 8.9, 9.5, 9.9, and 10.7 at 15°C. The labeling buffers all contained 100-mM KCl in H_2O and were 100 mM in either HEPES (pH 7.3, 8.0), glycylglycine (pH 8.3, 8.5), CHES (pH 8.9, 9.5, 9.9), or CAPS (pH 10.7). All other details are as described above for the time-course data.

Folded and unfolded controls

A separate, protonated control sample was run along with each set of folding data to control for differences in NMR instrumentation and sensitivity. The protonated controls were prepared by dissolving 30 mg of H124L SNase in 2.0 mL of 100-mM glycylglycine buffer (pH 9.0) containing H_2O and D_2O in a 80:20 ratio. The sample was equilibrated by raising the temperature to 65°C for 10 min. The sample was then cooled and the pH lowered to 5.0, followed by dilution with four volumes of cold quench buffer. The control samples were concentrated and worked up in parallel with the experimental samples to ensure that all samples had the same history.

A number of other controls were run to test the unfolding and labeling conditions used in the pulse-labeling studies. To test whether the protein was unfolded by acid pH alone, real-time NH exchange was monitored at 15°C by proton-detected ^1H-^{15}N HSMQC experiments (described below) for protein samples prepared at pH 3.0 in the presence and absence of 2.5-M GuHCl. Exchange was initiated at 15°C using a spinning Sephadex column to transfer the protein into a 99.9% D_2O solution buffered to pH* 3.0 (±GuHCl) with 10-mM citrate, and spectra were collected every 30 min for a period of 4 h to monitor exchange.

To ensure that the labeling pulse was neither too strong nor too weak, both hydrogen exchange and stopped-flow fluorescence controls were run. To assess the stability of the protein to the high pH conditions of the labeling pulse, we measured the unfolding kinetics for H124L SNase by monitoring changes in Trp fluorescence following pH jumps from pH 5 to 9 and pH 5 to 10 using a six-fold dilution into 100-mM glycine buffer. An alkaline titration of H124L SNase by CD was performed over the pH range from 7 to 12 to verify that nuclease remained stable to the pH values used in the labeling pulse. The stability of the folded protein to the labeling pulse was also assessed by the use of a very long t_f of 160 sec preceding the high pH pulse. To verify that the calculated labeling strength (calculated D-H exchange rate at a given pH times the length of the labeling pulse) was sufficient to completely label all unprotected amide positions, an unfolded protein sample was subjected to the labeling pulse and compared to a fully protonated (folded) control sample prepared as described above.

NMR experiments

NMR experiments were run on an AM-500 for the time course and real-time exchange data and on a DMX-600 for the protection factor measurements. The time-course and protection data were collected at 295K, whereas the real-time exchange data were collected at 288K. ^1H-^{15}N HSMQC experiments (Zuiderweg 1990)

with proton detection were run with 256 experiments (t1) consisting of 32 scans and 2048 data points (t2), except for the real-time exchange experiments, which were run with eight scans and 128 experiments. To aid in assignment, HSMQC spectra were also collected between 295K and 315K in increments of 5K. All spectra were processed identically, with apodization by squared sine-bell functions in each dimension before zero-filling to a digital resolution of 2 points/Hz. One-dimensional ^{1}H reference spectra were collected for each folding time for use in normalization between samples.

Data analysis

NMR peak assignments previously made at 310 K and 318K (Wang et al. 1990a; Loh et al. 1993) were extended to 295K via analysis of the temperature titration data described above. Peak heights and volumes were measured using macros written in the Felix software package (Molecular Simulations). The uncertainty in the peak height for a given NH proton (ΔP_i) was calculated from $\Delta P_i/P_i = \Delta I_i/I_i\,(t_f) + \Delta I_i/I_i$ (control), in which P is the peak height and I is the intensity of that peak in the control spectra and in a particular folding time point. Differences in sample concentration were taken into account by normalizing each 2D spectra according to the averaged heights of five nonexchangeable proton resonances in the 1D reference spectra (described above) collected separately for each sample. Time-course data were fit to two exponentials using a nonlinear least-squares algorithm. Protection factors (P_f) were determined by fitting the pH-dependent proton occupancies, f(pH), to the expression $f = 1 - (\exp(-k_c * t_p/P_f))$ (t_p = 50 msec), in which $k_{OH} = 8.063*10^7$ and $k_c = k_{OH}*10^{(pH-14.35)}$, based on (Elöve and Roder 1991). Intrinsic hydrogen exchange rates as a function of pH, temperature, and amino acid sequence were estimated on the basis of the model-peptide data of (Bai et al. 1993) using the program HX-Pred (www.fccc.edu/research/labs/roder).

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References

Alexandrescu, A.T., Hinck, A.P., and Markley, J.L. 1990. Coupling between local structure and global stability of a protein: Mutants of staphylococcal nuclease. *Biochemistry* **29:** 4516–4525.

Alexandrescu, A.T., Gittis, A.G., Abeygunawardana, C., and Shortle, D. 1995. NMR structure of a stable "OB-fold" sub-domain isolated from staphylococcal nuclease. *J. Mol. Biol.* **250:** 134–143.

Alexandrescu, A.T., Dames, S.A., and Wiltscheck, R. 1996. A fragment of staphylococcal nuclease with an OB-fold structure shows hydrogen-exchange protection factors in the range reported for "molten globules". *Protein Sci.* **5:** 1942–1946.

Anfinsen, C.B., Schecter, A.N., and Taniuchi, H. 1972. *Cold Spring Harbor Symposia on Quantitative Biology*, pp. 249–255, Cold Spring Harbor Laboratory, New York.

Bai, Y., Milne, J.S., and Englander, S.W. 1993. Primary structure effects on peptide group exchange. *Proteins* **17:** 75–86.

Bai, Y., Milne, J.S., Mayne, L., and Englander, S.W. 1994. Protein stability parameters measured by hydrogen exchange. *Proteins* **20:** 4–14.

Brandts, J.F., Halvorsen, H.R., and Brennan, M. 1975. Consideration of the possibility that the slow step in protein denaturation reactions is due to *cis-trans* isomerism of proline residues. *Biochemistry* **14:** 4953–4963.

Callender, R.H., Dyer, R.B., Gilmanshin, R., and Woodruff, W.H. 1998. Fast events in protein folding: The time evolution of primary processes. *Annu. Rev. Phys. Chem.* **49:** 173–202.

Carra, J.H. and Privalov, P.L. 1995. Energetics of denaturation and m values of staphylococcal nuclease mutants. *Biochemistry* **34:** 2034–2041.

———. 1996. Thermodynamics of denaturation of staphylococcal nuclease mutants: An intermediate state in protein folding. *FASEB J.* **10:** 67–74.

Carra, J.H., Anderson, E.A., and Privalov, P.L. 1994. Three-state thermodynamic analysis of the denaturation of staphylococcal nuclease mutants. *Biochemistry* **33:** 10842–10850.

Chen, H.M. and Tsong, T.Y. 1994. Comparison of kinetics of formation of helices and hydrophobic core during the folding of staphylococcal nuclease from acid. *Biophys. J.* **66:** 40–45.

Eaton, W.A., Muñoz, V., Thompson, P.A., Chan, C.-K., and Hofrichter, J. 1997. Submillisecond kinetics of protein folding. *Curr. Opin. Struct. Biol.* **7:** 10–14.

Elöve, G.A. and Roder, H. 1991. Structure and stability of cytochrome c folding intermediates. *ACS Symposium Series* **470:** 50–63.

Ernst, J.F., Hampsey, D.M., Stewart, J.W., Rackovsky, S., Goldstein, D., and Sherman, F. 1985. Substitutions of proline 76 in yeast iso-1-cytochrome c Analysis of residues compatible and incompatible with folding requirements. *J. Biol. Chem.* **260:** 13225–13236.

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

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

Filfil, R. and Chalikian, T.V. 2000. Volumetric and spectroscopic characterizations of the native and acid-induced denatured states of staphylococcal nuclease. *J. Mol. Biol.* **299:** 827–842.

Gillespie, J.R. and Shortle, D. 1997. Characterization of long-range structure in the denatured state of staphylococcal nuclease. II. Distance restraints from paramagnetic relaxation and calculation of an ensemble of structures. *J. Mol. Biol.* **268:** 170–184.

Herning, T., Yutani, K., Taniyama, Y., and Kikuchi, M. 1991. Effects of Proline mutations on the unfolding and refolding of human lysozyme: The slow refolding kinetic phase does not result from *cis-trans* isomerization. *Biochemistry* **30:** 9882–9891.

Hinck, A.P., Walkenhorst, W.F., Truckses, D.M., and Markley, J.L. 1996. *Biological NMR Spectroscopy* (eds. J.L. Markley and S.J. Opella), pp. 133–160, Oxford University Press, Oxford.

Jacobs, M.D. and Fox, R.O. 1994. Staphylococcal nuclease folding intermediate characterized by hydrogen exchange and NMR spectroscopy. *Proc. Natl. Acad. Sci.* **91:** 449–453.

James, E., Wu, P.G., Stites, W., and Brand, L. 1992. Compact denatured state of a staphylococcal nuclease mutant by guanidinium as determined by resonance energy transfer. *Biochemistry* **31:** 10217–10225.

Kalnin, N.N. and Kuwajima, K. 1995. Kinetic folding and unfolding of staphylococcal nuclease and its six mutants studied by stopped-flow circular dichroism. *Proteins* **23:** 163–176.

Kiefhaber, T., Grunert, H.-P., Hahn, U., and Schmid, F.X. 1990. Replacement of a *cis* proline simplifies the mechanism of ribonuclease T1 folding. *Biochemistry* **29:** 6475–6480.

Kuwajima, K., Okayama, N., Yamamoto, K., Ishihara, T., and Sugai, S. 1991. The Pro117 to glycine mutation of staphylococcal nuclease simplifies the unfolding-folding kinetics. *FEBS Lett.* **290:** 135–138.

Kuwata, K., Shastry, M., Cheng, H., Hoshima, M., Batt, C., Goto, Y., and Roder, H. 2001. Structural and kinetic characterization of early folding events in b-lactoglobulin. *Nat. Struct. Biol.* **8:** 151–155.

Loh, S.N., Prehoda, K.E., Wang, J., and Markley, J.L. 1993. Hydrogen exchange in unligated and ligated staphylococcal nuclease. *Biochemistry* **32:** 11022–11028.

Maki, K., Ikura, T., Hayano, T., Takahashi, N., and Kuwajima, K. 1999. Effects of proline mutations on the folding of staphylococcal nuclease. *Biochemistry* **38:** 2213–2223.

Mori, S., van Zijl, P.C.M., and Shortle, D. 1997. Measurement of water-amide

proton exchange rates in the denatured state of staphylococcal nuclease by a magnetization transfer technique. *Proteins* **28:** 325–332.

Plaxco, K.W., Simons, K.T., and Baker, D. 1998. Contact order, transition state placement and the refolding rates of single domain proteins. *J. Mol. Biol.* **277:** 985–994.

Plaxco, K.W., Simons, K.T., Ruczinski, I., and Baker, D. 2000. Topology, stability, sequence, and length: Defining the determinants of two-state protein folding kinetics. *Biochemistry* **39:** 11177–11183.

Ramakrishna, V. and Sasidhar, Y.U. 1997. A pentapeptide model for an early folding step in the refolding of staphylococcal nuclease: the role of its turn propensity. *Biopolymers* **41:** 181–191.

———. 1998. Conformational features of a peptide model Ac-DTVKLMYKGQPMTFR-NH2, corresponding to an early folding beta hairpin region of staphylococcal nuclease. *Indian J. Biochem. Biophys.* **35:** 333–338.

Raschke, T.M. and Marqusee, S. 1997. The kinetic folding intermediate of ribonuclease H resembles the acid molten globule and partially unfolded molecules detected under native conditions. *Nat. Struct. Biol.* **4:** 298–304.

Roder, H. and Wüthrich, K. 1986. Protein folding kinetics by combined use of rapid mixing techniques and NMR observation of individual amide protons. *Proteins* **1:** 34–42.

Roder, H. and Colón, W. 1997. Kinetic role of early intermediates in protein folding. *Curr. Opin. Struct. Biol.* **7:** 15–28.

Roder, H. and Shastry, M.C.R. 1999. Methods for exploring early events in protein folding. *Curr. Opin. Struct. Biol.* **9:** 620–626.

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

Roder, H., Elöve, G.A., and Shastry, R.M.C. 2000. *Early Stages of Protein Folding*, Oxford University Press, New York.

Royer, C.A., Hinck, A.P., Loh, S.N., Prehoda, K.E., Peng, X., Jonas, J., and Markley, J.L. 1993. Effects of amino acid substitutions on the pressure denaturation of staphylococcal nuclease as monitered by fluorescence and nuclear magnetic resonance spectroscopy. *Biochemistry* **32:** 5222–5232.

Schmid, F.X. 1993. Prolyl isomerase: Enzymatic catalysis of slow protein folding reactions. *Annu. Rev. Biophys. Biomol. Struct.* **22:** 123–143.

Shortle, D. 1986. Guanidine hydrochloride denaturation studies of mutant forms of staphylococcal nuclease. *J. Cell. Biochem.* **30:** 281–289.

———. 1992. Mutational studies of protein structures and their stabilities. *Q. Rev. Biophys.* **25:** 205–250.

———. 1993. Denatured states of proteins and their roles in folding and stability. *Curr. Opin. Struct. Biol.* **3:** 66–74.

———. 1995. Staphylococcal nuclease: A showcase of *m*-value effects. *Adv. Prot. Chem.* **46:** 217–247.

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

Shortle, D. and Abeygunawardana, C. 1993. NMR Analysis of the residual structure in the denatured state of an unusual mutant of staphylococcal nuclease. *Curr. Biol.* **1:** 121–134.

Shortle, D., Stites, W.E., and Meeker, A.K. 1990. Contributions of the large hydrophobic amino acids to the stability of staphylococcal nuclease. *Biochemistry* **29:** 8033–8041.

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

Tuckses, D.M., Somoza, J.R., Prehoda, K.E., Miller, S.C., and Markley, J.L. 1996. Coupling between *trans*/*cis* proline isomerization and protein stability in staphylococcal nuclease. *Protein Sci.* **5:** 1907–1916.

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

Walkenhorst, W.F., Green, S.M., and Roder, H. 1997. Kinetic evidence for folding and unfolding intermediates in staphylococcal nuclease. *Biochemistry* **36:** 5795–5805.

Wang, Y. and Shortle, D. 1995. The equilibrium folding pathway of staphylococcal nuclease: Identification of the most stable chain-chain interactions by NMR and CD spectroscopy. *Biochemistry* **34:** 15895–15905.

———. 1996. A dynamic bundle of four adjacent hydrophobic segments in the denatured state of staphylococcal nuclease. *Protein Sci.* **5:** 1898–1906.

Wang, J., Hinck, A.P., Loh, S.N., and Markley, J.L. 1990a. Two-dimensional NMR studies of staphyloccocal nuclease. 2. Sequence-specific assignments of carbon-13 and nitrogen-15 signals from the nuclease H124L-thymidine 3′, 5′-bisphosphate-Ca2+ ternary complex. *Biochemistry* **29:** 102–113.

Wang, J., LeMaster, D.M., and Markley, J.L. 1990b. Two-dimensional NMR studies of staphylococcal nuclease. 1. Sequence-specific assignments of hydrogen-1 signals and solution structure of the nuclease H124L-thymidine 3′, 5′-bisphosphate-Ca^{2+} ternary complex. *Biochemistry* **29:** 88–101.

Wang, J., Truckses, D.M., Abildgaard, F., Dzakula, Z., Zolnai, Z., and Markley, J.L. 1997. Solution structures of staphylococcal nuclease from multidimensional, multinuclear NMR: Nuclease-H124L and its ternary complex with Ca^{2+} and thymidine-3′,5′-bisphosphate. *J. Biomol. NMR* **10:** 143–164.

Woodward, C. 1993. Is the slow exchange core the protein folding core? *Trends Biochem. Sci.* **18:** 359–360.

Zuiderweg, E.R.P. 1990. A proton-detected heteronuclear chemical-shift correlation experiment with improved resolution and sensitivity. *J. Magn. Reson. B* **86:** 346–357.