Native-like Partially Folded Conformations and Folding Process Revealed in the N-terminal Large Fragments of Staphylococcal Nuclease: A Study by NMR Spectroscopy

Yingang Feng, Dongsheng Liu and Jinfeng Wang*

National Laboratory of Biomacromolecules, Center for Molecular Biology, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road Beijing 100101, People’s Republic of China

The N-terminal large fragments of staphylococcal nuclease (SNase), SNase110 (1–110 residues), SNase121 (1–121 residues), and SNase135 (1–135 residues), and the fragment mutants G88W110, G88W121, V66W110 and V66W121 were studied by heteronuclear multidimensional NMR spectroscopy. Ensembles of co-existent native-like partially folded and unfolded states were observed for fragments. The persistent native-like tertiary interaction drives fragments to be in partially folded states, which reveal native-like β-barrel conformations. G88W and V66W mutations modulate the extent of inherent native-like tertiary interaction in fragment molecules, and in consequence, fragment mutants fold into native-like β-subdomain conformations. In cooperation with the inherent tertiary interaction, 2 M TMAO (trimethylamine N-oxide) can promote the folding reaction of fragments through the changes of unfolding free energy, and a native-like β-subdomain conformation is observed when the chain length contains 135 residues. Heterogeneous partially folded conformations of 1–121 and 1–135 fragments due to cis and trans X-prolyl bond of Lys116–Pro117 make a non-unique folding pathway of fragments. The folding reaction of fragments can be characterized as a hierarchical process.

Introduction

Proteins generally fold into unique three-dimensional structures to convey their specific physiological functions. In a funnel-shaped energy landscape of protein folding, the unique, native state of protein is the state corresponding to the global free-energy minimum of the system. However, as has been pointed out, there is no unique pathway for protein folding toward the native state. The cis–trans isomerization of X-prolyl peptide bonds may cause heterogeneous conformational states in the folding process, which can be explained by the roughness of the energy landscape surface. One conformational state, which corresponds to a local energy minimum, may exchange with others at various rates according to the energy barrier height between them. Thus protein folding is a process driven by a free energy difference between states.

N-terminal fragments with different chain lengths of staphylococcal nuclease (SNase) molecules have long been used as a model system for studying protein folding and unfolding. As was first pointed out by Anfinsen, SNase is composed of two subdomains, the β and α-subdomains, divided along the active-site cleft.
Conformations and Folding of Nuclease Fragments

Results

Conformational states of wild-type SNase fragments

Figure 2 shows the 2D $^1$H–$^1$N heteronuclear single quantum coherence (HSQC) spectra of two wild-type SNase fragments, SNase110 (Figure 2a), and SNase121 (Figure 2b). The 2D $^1$H–$^1$N HSQC spectrum of SNase135 (spectrum not shown) was very similar to that of SNase121. Analysis of Figure 2 reveals that a great number of cross-peaks were squeezed into the narrow chemical shift region of 7.8–8.8 ppm and 108–128 ppm in the $^1$H and $^1$N dimensions, respectively, in the spectra of all three fragments. Obviously, SNase110, SNase121, and SNase135 presented as three SNase fragments in the largely unfolded state. However, there were a number of relatively weak resonance peaks dispersed in the whole 2D $^1$H–$^1$N HSQC spectral region (Figure 2a and b), which characterize the partially folded conformations of fragments. Most dispersed peaks appeared in the region of 8.8–10.2 ppm for $^1$H resonance and 120–131 ppm for $^1$N resonance, and the dispersion of these peaks was very similar to the distribution pattern of cross-peaks in the 2D $^1$H–$^1$N HSQC spectrum of native SNase and to the stabilization 1–103 fragment containing the G88W and V66L. Therefore, dispersed cross-peaks such as Ala17, Gly20, Met26, Tyr27, Gly29, Ala69, Lys70, Ile72, Val74, Asp95 and Gly96 can be assigned according to the similarity of chemical shifts. Figure 1 clearly shows that almost all the residues generating these assigned cross-peaks are located in the $\beta$-barrel of native SNase. Ala17, Met26, and Gly20 are located in the $\beta_1$, $\beta_2$, and the turn between $\beta_1$ and $\beta_2$, respectively. Ile72 and Val74 are located in the $\beta_5$ strand, while Ala69-Lys70 peptide link the $\alpha_1$ helix with the $\beta_5$ strand. Tyr27 and Gly29, and Asp95 and Gly96 are located in the $\beta_3$ strand, while Glu135. G88W and V66W mutations can modulate the extent of persistent tertiary interaction of fragments, and make the mutant fragments fold into a more stable, native-like $\beta$-subdomain. Heterogeneous conformational states, which are attributed to the cis and trans Lys116–Pro117 bond of fragments, were observed for fragments with longer chain lengths such as the 1–121 and 1–135 residues. The folding pathways of the fragments were analyzed.
the residues located in \( \beta \)-turns between \( \beta_2 \) and \( \beta_3 \), and \( \beta_6 \) and \( \alpha_2 \)-helix, respectively. On the basis of this spectral observation, an experimental fact can be deduced that a native-like \( \beta \)-barrel conformation may be formed in all three fragments.

In the 2D \(^1\text{H}–^{15}\text{N} \) HSQC spectra of SNase121 (Figure 2b) and SNase135, two sets of dispersed cross-peaks were observed for many residues, and one set was almost identical to the dispersed peaks in the spectrum of SNase110 (Figure 2a). The residues with assigned double peaks were Gly29, Ala69, Lys70, Asp95 and Gly96, which were distributed in different locations of the \( \beta \)-subdomain of SNase. This suggests that two kinds of partially folded conformations may be formed in the SNase121 and SNase135 fragments. The partially folded conformation of SNase110 was named PF1. Among two kinds of partially folded conformations of SNase121 and SNase135, the conformational states showing nearly identical chemical shifts of dispersed cross-peaks to that of SNase110 were named PF2C and PF3C, respectively, for SNase121 and SNase135.

*Figure 2* 2D \(^1\text{H}–^{15}\text{N} \) HSQC spectra of two wild-type SNase fragments: SNase110 (a), and SNase121 (b). c, Portion of 2D \(^1\text{H}–^{15}\text{N} \) exchange spectrum of SNase121 at mixing time of 402.1 ms. The symbols prime and double prime marked on the labels of cross-peaks denote the corresponding residues in the conformations PF2C and PF2T for SNase121, and in PF3C and PF3T for SNase135. Cross-peaks labeled with a star indicate the Gly96 from an unfolded state. Cross-peaks labeled with R denote the aliased peaks of arginine \( \varepsilon \)-amido. The chemical shifts of R peaks in \(^{15}\text{N} \) dimension can be estimated by subtraction of the spectral width (44 ppm) from the apparent chemical shift values.
other conformational states were named PF2T for SNase121 and PF3T for SNase135. The chemical shifts of assigned cross-peaks in the partially folded conformational states of fragments are listed in Table 1. The observed distinguishable sets of cross-peaks in Figure 2a and b indicate that the exchange between different conformational states of fragments must be slow in the NMR time scale. The exchange peaks between partially folded conformational states and the unfolded state can be observed clearly for Gly96 in the exchange spectrum of SNase121 (Figure 2c). The rate of exchange is slower than 10 s\(^{-1}\). However, the exchange peaks between heterogeneous partially folded conformations of SNase121 and SNase135 fragments are undetectable because of the weak dispersed peaks and low sensitivity of the slow exchange experiment\(^{24}\).

As previously reported, the effect of salts and the self-association of protein may influence the folding and stability of SNase.\(^{25,26}\) To exclude these effects in the experimental results for SNase fragments, two samples of SNase121, one with 0.1 mM of fragments and the other one without any salt in the buffer solution were prepared for the 2D \(\text{H}–\text{15N}\) HSQC experiments. No significant changes were observed in the spectra recorded with these two samples compared to the spectra of SNase taken in the designed experimental conditions described above. This indicates that the existence of partially folded conformations in SNase110, SNase121 and SNase135 fragments is not due to the effect of salt or of the self-association of SNase fragments.

### Native-like folded state of G88W mutant fragments

G88W110 and G88W121 showed very similar 2D \(\text{H}–\text{15N}\) HSQC spectra (Figure 3a and b), in which a single set of well-dispersed peaks was observed. Almost complete backbone resonance assignments of G88W110 and G88W121 were determined and all assignments were indicated in the corresponding spectra (Figure 3). The chemical shifts of most cross-peaks corresponding to the residues in the \(\beta\)-barrel for both G88W110 and G88W121 were similar to that of G88V136.\(^{37}\) Chemical shift indices (CSI) derived from \(\text{H}^2\) chemical shifts of G88W110, G88W121, G88V136,\(^{37}\) and native SNase\(^{27}\) showed similar histogram patterns for \(\beta_1–\beta_6\) and \(\alpha_1\) secondary structures of SNase (Figure 4). CSI derived from \(\text{H}^2\) chemical shifts provided similar results. The CSI data suggest that a stable native-like \(\beta\)-barrel consisting of \(\beta_1–\beta_6\) strands (Figure 1) is formed in the G88W110 and G88W121 fragments. Figure 5 shows the \(\text{H}^2\) chemical shift differences of G88W110 and G88W121 from that of SNase110 and SNase121 in PF1 and PF2C conformations, respectively. Clearly, the G88W fragment mutation causes slight changes of the \(\text{H}^2\) chemical shifts of residues Met26, Gly29, and Val74. Referring to the 3D structure (Figure 1) of native SNase,\(^{27}\) the observed chemical shift changes may result from the perturbation of the spatial packing of neighboring residues of Gly88 in the native state by the large side-chain of substituted Trp88. The \(\text{H}^2\) chemical shifts of corresponding residues indicated in Figure 5 showed no significant changes. It is possible that the G88W mutation makes fragments fold fully, and thereby tends to stabilize the conformation similar to the PF2C conformational state of SNase121.

Cross-peaks such as Leu37, Leu38, Val39, Glu57, Ala58, Ser59, Val66, Ala102, Val104, Arg105, Glu106, Leu108, Ala109, and Lys110 were clearly observable in the spectrum of G88W110 (Figure 3a), but became very weak or even disappeared in the spectrum of G88W121 (Figure 3b). Nevertheless, the cross-peaks of most residues in the elongation peptide segment from residue Lys110, i.e. Val111–His121 in G88W121, can be observed in Figure 3b, with the exception of the cross-peaks of Val111, Ala112 and Pro117. The 3D structure of native SNase (Figure 1) indicates that residues Glu57, Ala58, Ser59 and Val66 were located in the \(\alpha_1\) helix, whereas residues Ala102, Val104, Arg105, and Glu106 were in the \(\alpha_2\) helix. Moreover, the NMR-derived solution structure of SNase\(^{27}\) indicated explicitly that residue segments Ala109-Lys110-Val111 and Val39-Asp40-Thr41 form a short two-strand anti-parallel \(\beta\)-sheet (\(\beta\)III). Therefore, the observed signal broadening and disappearance of some corresponding residues may be due to the instability of these native-like secondary structural elements in G88W121.

### Table 1. \(\text{H}, \text{15N}\) chemical shifts (ppm) of assigned native-like peaks in 2D \(\text{H}–\text{15N}\) HSQC spectra of SNase fragments

<table>
<thead>
<tr>
<th>Residue</th>
<th>SNase110</th>
<th>SNase121(PF2C)</th>
<th>SNase121(PF2T)</th>
<th>SNase135(PF3C)</th>
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<td>9.59,122.6</td>
<td>8.48,103.1</td>
<td>8.43,103.2</td>
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<td>Gly29</td>
<td>8.49,103.1</td>
<td>8.48,103.1</td>
<td>9.42,103.2</td>
<td>10.07,125.5</td>
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<tr>
<td>Ala69</td>
<td>6.78,122.4</td>
<td>6.80,122.5</td>
<td>6.99,123.7</td>
<td>10.14,126.3</td>
<td>10.14,126.3</td>
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<tr>
<td>Lys70</td>
<td>10.14,126.3</td>
<td>10.13,126.3</td>
<td>10.07,125.5</td>
<td>10.07,125.5</td>
<td>10.07,125.5</td>
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<tr>
<td>Val74</td>
<td>9.61,118.8</td>
<td>9.61,118.8</td>
<td>9.42,119.6</td>
<td>9.43,103.8</td>
<td>9.48,103.8</td>
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<td>Asp95</td>
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<td>9.77,127.9</td>
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<tr>
<td>Gly96</td>
<td>9.41,103.9</td>
<td>9.42,103.8</td>
<td>9.42,103.8</td>
<td>9.43,103.8</td>
<td>9.48,103.8</td>
</tr>
</tbody>
</table>

Underlined values are those significantly different from SNase110. The errors of chemical shifts are: \(\text{H}\) dimension, \(\pm 0.01\) ppm; \(\text{15N}\) dimension, \(\pm 0.2\) ppm.
Figure 3. 2D $^1$H–$^{15}$N HSQC spectra of G88W110 (a) and G88W121 (b). The resonance assignments are indicated with the one-letter amino acid code and residue number. Underlined labels in (a) indicate the cross-peaks observed only for G88W110.

Figure 4. Chemical shift index of $^{13}$C$^\alpha$ for SNase fragments. Secondary structural elements of native SNase are labeled at the top of the Figure.

Figure 5. The $^1$H$_\alpha$ and $^{15}$N chemical shift differences of G88W110, V66W110, and G88W121 from that of SNase110 and SNase121 (PF2C) versus the residues corresponding to assigned cross-peaks of wild-type fragments. The filled and open bars indicate the $^1$H$_\alpha$ and $^{15}$N chemical shift differences ($\Delta\delta$), respectively.
Native-like folded states of V66W mutant fragments

The 2D \(^1\text{H}--15\text{N}\) HSQC spectrum of V66W110 showed a great quantity of dispersed cross-peaks (Figure 6a), and their distribution was quite unlike that for either SNase110 or for G88W110 (Figure 5). The V66W121 fragment provided two sets (M1 and M2) of cross-peaks in the 2D \(^1\text{H}--15\text{N}\) HSQC spectrum (Figure 6b); the M1 cross-peaks were identical to the cross-peaks of V66W110 shown in Figure 6a. Resonances of both V66W110 and V66W121 fragments displayed a crowded cross-peak region, and the range of \(^1\text{H}_\text{N}\) chemical shift of this region was about 8.0 – 8.8 ppm in the 2D \(^1\text{H}--15\text{N}\) HSQC spectra (Figure 6a and b). Comparing the spectra of SNase110 with SNase121 (Figure 2a and b), a weak cross-peak corresponding to residue Gly96 of an unfolded fragment (cross-peak designated with a star) could be observed in the 2D \(^1\text{H}--15\text{N}\) HSQC spectra of V66W110 and V66W121. Apparently, a small amount of V66W110 and V66W121 was still in the unfolded state.

The assignment of two sets of \(^1\text{H}_\text{N}\) and \(^15\text{N}\) resonances of V66W121 and the resonances of V66W110 are given in the 2D \(^1\text{H}--15\text{N}\) HSQC spectrum of Figure 6b. Clearly, most cross-peaks corresponding to the residues located in the \(\beta\)-barrel of native SNase were assigned. Strong resonance signals were observed for residues Thr2, Ser3, and Thr4 in the N-terminal and Val111, Ala112, Val114, Tyr115, Lys116, Asn118, Thr120, and His121 in the C-terminal of V66W121. However, the resonances of the \(\alpha2\) helix, most of the peaks for the pdTp-binding loop and the cross-peaks of N-terminal residues of \(\alpha1\) helix, and also the small \(\beta\)-sheet (\(\beta\text{III}\)) could not be assigned. Similar to that observed for G88W121, it could be due to the line broadening from the intermediate exchange between states with significant order in V66W mutants. Therefore, the conformational heterogeneity of V66W121, deduced from two sets of cross-peaks, is due mainly to the existence of two conformations of \(\beta\)-barrel in V66W121. CSI derived from \(^13\text{C}_\alpha\) chemical shifts of V66W121 in the M2 conformation was similar to the CSI of other fragments (Figure 5). This suggests that V66W mutations induce V66W121 to form native-like folded conformations. Undoubtedly, V66W110 can also form a native-like folded conformation, inferred from the spectral identity of V66W121 with V66W110.

The slow exchange experiments revealed that slow exchange exists in V66W121 between two folded conformations. Slow exchange cross-peaks were clearly observed for various residues, such as Thr13, Gly20, Lys24, Tyr27, Lys28, Met32, Phe34, Thr41, Thr44, Ser59, Ile72, Glu75, Phe76, Thr82, Tyr93 and Met98 (Figure 7). Longitudinal decay and exchange rates could be extracted accurately for residue Ile72: \(R_{M1} = 0.93(\pm 0.13)\) s\(^{-1}\), \(R_{M2} = 0.63(\pm 0.07)\) s\(^{-1}\), \(\kappa_{M1-M2} = 2.63(\pm 0.12)\) s\(^{-1}\).

Figure 6. 2D \(^1\text{H}--15\text{N}\) HSQC spectra of V66W110 (a), and V66W121 (b). Overlay of spectra of V66W110 (red) and V66W121 (blue) is presented in (b). The resonance assignments for V66W121 are indicated in (b) with the one-letter amino acid code and residue number.
The equilibrium constant was $K_{eq} = k_{M1!M2}/k_{M2!M1} = 1.87$. Residue Ile72 was located in the central $\beta$-strand of the $\beta$-barrel. Therefore, it can be considered that the M1 state of V66W121 changes its folded conformation more easily than does the M2 state, and the lifetime for V66W121 in the M2 state is a little bit longer than in the M1 state.

The $\varepsilon$-amido resonances of Trp66 indole ring gave two and three cross-peaks in the 2D $^1$H–$^{15}$N HSQC spectra of V66W110 and V66W121, respectively (Figure 6a and b). One of them was from the unfolded state, evidenced by the denaturation experiments with urea. Exchange peaks between two $\varepsilon$-amido signals of Trp66 in V66W121 spectrum were observed (Figure 7).

Conformations and Folding of Nuclease Fragments

Figure 7. 2D $^1$H–$^{15}$N exchange spectrum of V66W121 at mixing time 402.1 ms. Only the residues with observed exchange peaks are labeled. ex, indicates the cross-peaks of exchanging.

It has been reported that the solvophobic effect of TMAO on the protein backbone in osmolyte solution is to make the unfolded state of protein very unfavorable relative to the folded state. Thus, TMAO can increase the population of protein in a native state relative to an unfolded state by raising the free energy of the unfolded state. Indeed, the experiments with SNase110, SNase121, and SNase135 revealed that the population of native-like partially folded states of SNase fragments increases with the increase of TMAO concentration in solution (spectra not shown), and reaches a maximum at about 2 M TMAO. Figure 8a and b shows the 2D $^1$H–$^{15}$N HSQC spectra of SNase110 and SNase121 in the presence of 2 M TMAO, respectively. Both spectra were found to have dispersed cross-peaks that were similar to those in Figure 2a and b, but with higher intensity. This indicates that TMAO induces more SNase110 and SNase121 species to fold into the same folding motif than for fragments dissolved in aqueous buffer. For the G88W mutant fragments, the osmolyte force of 2 M TMAO drives G88W110 and G88W121 to fold further to a more stable $\beta$-barrel, and a more $\beta$-subdomain-like conformation. This was evidenced by the appearance of cross-peaks Val99, Asn100, and Glu101 in the 2D $^1$H–$^{15}$N HSQC spectra of G88W110 (Figure 8d) and G88W121 (spectrum not shown). The amino acid residues corresponding to cross-peaks Val99, Asn100, and Glu101 were located in the N-terminal of $\alpha_2$ helix connected to $\beta$6 strand (Figure 1).

Conformations of fragments stabilized by TMAO

As indicated above, SNase135 provides a 2D $^1$H–$^{15}$N HSQC spectrum very similar to that of SNase121. It seems that the elongation of the polypeptide from His121 to Glu135 will not affect the folding state of wild-type fragments. In
the presence of 1 M TMAO, SNase135 showed a spectrum that was identical to that of SNase121 at 2 M TMAO, except that the more crowded cross-peaks in the central spectrum region and a new cross-peak marked with A<sup>69</sup> (Figure 8b, inset) could be observed in addition. However, by the osmolyte force of 2 M TMAO, SNase135 showed new cross-peaks corresponding to residues Val39,
Lys78, Thr82, Val99, Asn100, Glu101, Val104, Ala112, and Thr120 in the 2D $^1$H–$^{15}$N HSQC spectrum (Figure 8c). The chemical shifts of these cross-peaks were very close to those provided by native SNase. According to the NMR-derived solution structure of native SNase, residues Val39 and Ala112 were located at the terminal of the small antiparallel $\beta$-sheet ($\beta$III) of Val39-Asp40-Thr41 and Val111-Lys110-Ala109; residues Lys78 and Thr82 were located in the pdTp-binding loop; residues Val99, Asn100, Glu101, and Val104 were located in the $\alpha2$ helix; and Thr120 was a residue in the loop Val111–His121 connecting the $\alpha3$ helix with the $\beta$-subdomain of SNase. Therefore, 2 M TMAO forced SNase135 to fold into the native-like $\beta$-subdomain, for which a native-like small $\beta$-sheet ($\beta$III), a pdTp-binding loop, and an $\alpha2$ helix are formed. Moreover, the observed new cross-peak marked as A$^{69}$ for SNase135 at 1 M TMAO (Figure 8b, inset) merged with the cross-peak marked as A$^{69}$ into a single peak for SNase135 at 2 M TMAO (Figure 8c). If A$^{69}$ indicates the appearance of a new local folded conformation of SNase135, then this new local conformation of SNase135 at 1 M TMAO must be very similar to the PF3C conformation of SNase135 at 2 M TMAO. Thus, the new conformation was simply named PF3C conformation of SNase135 at 2 M TMAO. Clearly, in the presence of 2 M TMAO, the PF3C conformation of SNase135 became dominant compared to the PF3T conformational state (Figure 8c).

Relative populations of heterogeneous conformations in fragments

Information about the populations of partially folded conformations for SNase fragments can be obtained by measuring the relative volumes of cross-peaks in the 2D $^1$H–$^{15}$N HSQC spectra of the fragments. Since G88W-mutant fragments, G88W110 and G88W121 fold fully in aqueous buffer, the populations of partially folded conformations of various fragments can be estimated on the basis of the signal volumes of G88W-mutant fragments. Fragments with various lengths of amino acid residues may have different dynamic properties; thus, the comparison of signal volumes was carried out between fragments with the same length of residues assuming the influence of residue substitutions on the internal motion of fragments is negligible. The populations of folded conformations for V66W mutant fragments can be obtained in the same way. The percentages of the signal volumes of fragments SNase110 and V66W110 were calculated in relation to the signal volumes of G88W110. Signal volume percentages for fragments SNase121, V66W121 and SNase135 were obtained relating to G88W121, since SNase135 shows a 2D $^1$H–$^{15}$N HSQC spectrum similar to that of SNase121. In the case of fragments SNase110, SNase121, and SNase135, the signal volumes can be determined only for the distinguished assigned cross-peaks in 2D $^1$H–$^{15}$N HSQC spectra. The majority of these dispersed peaks corresponded to the residues in the $\beta$-barrel of SNase. Therefore, the populations of fragments in the corresponding partially folded states were estimated according to the volume percentages of these dispersed cross-peaks. The averaged relative populations were taken principally by averaging the values of relative populations for residues Ala17, Gly20, Met26, Tyr27, Gly29, Ala69, Lys70, Ile72, Val74, Asp95 and Gly96.

Figure 9 shows the averaged relative populations of heterogeneous conformations of SNase fragments in the presence and absence of TMAO. In the absence of TMAO, the substitution of Trp for Val66 was unable to induce a folded state for whole molecule species of V66W110 or V66W121. The relative population of V66W110 in the folded state was about 78%, and the ratio of relative populations of conformations M1–M2 (M1/M2) of V66W121 was about 0.75. Similarly, the ratio of relative populations of conformations PF2C–PF2T (PF2C/PF2T) of SNase121 was 0.66. Clearly, the PF2T of SNase121 and M2 of V66W121 are more favorable conformational states for fragments with 121 residues. However, when the polypeptide

![Figure 9](https://example.com/figure9.png)
chain was elongated from Val111 to Glu135, the PF3C conformational state became more favorable, and the ratio of relative populations of conformation PF3C–PF3T (PF3C/PF3T) was about 1.24 for SNase135.

In the presence of 2 M TMAO, the denatured states of all the fragments almost vanished. TMAO at 2 M makes the M1 folded state of V66W121 even more unfavorable, and the M2 becomes the dominant conformation for V66W121. The ratio of relative populations of PF2C–PF2T (PF2C/PF2T) of SNase121 at 2 M TMAO was 0.76, which is at about the same quantitative level as for SNase121 as well as for M1/M2 of V66W121 in the absence of TMAO. However, the PF3C conformational state of SNase135 at 2 M TMAO became dominant. It seems that the ratios of relative populations of PF2C to PF2T for SNase121 and M1–M2 for V66W121 reflect some intrinsic properties of fragments consisting of 1–121 amino acid residues.

Discussion
Ensembles of conformations of SNase fragments

Large fragments of SNase have long been used as models of the denatured state of proteins. Denatured proteins may exist as an ensemble of conformations ranging from a compact native-like state to fully unfolded random coil-like states. A number of studies have been carried out with the wild-type SNase fragments Δ131Δ, 1–128 and 1–136 by various methods including NMR spectroscopy. The data have revealed some common properties: that all these fragments provide less NMR resonance dispersion and have much less residual structures. Similar properties have been explored for the wild-type SNase fragments SNase110, SNase121, and SNase135 (Figure 2).

The partially folded conformation of these fragments displayed as a molecular conformation consisting of β-barrel-like folding of relative chain segments and globally unstructured portions of fragment in respect to tertiary folding, and can be considered to be a native-like partially folded conformation.

Since the unfolded states and partially folded states of fragments co-exist physiologically at an equilibrium with a very slow exchange rate (< 10 s⁻¹), the conformations of large fragments of SNase molecules can be regarded as ensembles of conformations of native-like partially folded and unfolded states.

Heterogeneity of native-like partially folded states has been observed for SNase121 and SNase135. Two native-like partially folded conformations co-exist in SNase121 (PF2C and PF2T), and in SNase135 (PF3C and PF3T). The sum of the populations of the two partially folded conformations, PF2C and PF2T of SNase121 (27.3%), is larger than that of either SNase110 (16.6%) or the total population of SNase135 (19.5%). This can be seen in Figure 9. Nevertheless, in the presence of 2 M TMAO, the relative population of the native-like partially folded states for all fragments reached up to > 90%, and almost all the unfolded species of fragments in the ensembles converted to native-like partially folded conformations. Therefore, it can be inferred that the conformational ensembles of SNase121 and SNase135 at 2 M TMAO are composed of only two co-existent equilibrated native-like partially folded conformations, and SNase110 exists as a unitary native-like partially folded state.

As has already been established, SNase is a protein that can exist in the folded forms containing a predominant conformation of a cis or trans peptide bond between residues at the sequence positions of Lys116 and Pro117. It is presumed that about 85% of the SNase molecule has the cis Lys116–Pro117 peptide bond, whereas 15% of the molecule exhibits the trans Lys116–Pro117 peptide bond. Further NMR studies have identified the cis Lys116–Pro117 peptide bond in liganded and unliganded SNase. Moreover, SNase contains six proline residues, only the Lys116–Pro117 peptide bond resulted in the appearance of major and minor cross-peaks of remote residues.

In the present study, the heterogeneity of partially folded conformations was observed for fragments SNase121, V66W121, and SNase135, but not for SNase110, V66W110, G88W110 or G88W121. The elongation polypeptide sequences from Lys110 of 1–121 and 1–135 fragments contained a Lys116–Pro117 peptide bond. It seems that the observed conformational heterogeneity is linked with the prolyl cis–trans isomerization of the Lys116–Pro117 peptide bond.

The populations of two partially folded conformations in SNase121 and SNase135 molecules are not equal. According to the estimated values of relative populations (Figure 9), the PF2T conformation is favorable for the SNase121 molecule, and PF3C is favorable for SNase135 in the absence of TMAO. In fact, PF2T remains favorable for SNase121 also in the presence of 2 M TMAO. However, the PF3C partially folded conformation becomes dominant in SNase135 at 2 M TMAO. Since the cis-proline isomer of the Lys116–Pro117 peptide bond is dominant in native SNase, the PF3C native-like β-subdomain of SNase135 is attributed to the predominant cis conformation of the Lys116–Pro117 peptide bond. Because PF3C is a conformational continuation of PF2C, PF2C of SNase121 should have also the cis conformation of the Lys116–Pro117 peptide bond. Unquestionably, PF2T of SNase121 has a trans conformation of the Lys116–Pro117 peptide bond, and it is energetically favorable in the SNase121 fragment molecule.

As was observed, the V66W mutation has no potential to stabilize whole 121-residue molecules in a unique folding conformation. The slow exchange between the two heterogeneous conformations M1 and M2 of V66W121 was revealed. In
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the early studies by magnetization transfer experiments, the rates of cis–trans conversion were measured for SNase peptide corresponding to residues Tyr113 to Glu122 at 50 °C.7 The rate constant for the trans to cis isomerization, \( k_{\text{trans} \rightarrow \text{cis}} \), is 0.025(±0.013) s\(^{-1}\), and that for the cis to trans isomerization, \( k_{\text{cis} \rightarrow \text{trans}} \), lies between 0.42 s\(^{-1}\) and 1.2 s\(^{-1}\). The corresponding relaxation time \( (k_{\text{cis} \rightarrow \text{trans}} + k_{\text{trans} \rightarrow \text{cis}})^{-1} \) of 0.8–2.3 seconds is in agreement with those for unfolded nuclease of 0.3–1.2 seconds\(^{-1}\) and for folded protein of 0.6–1.5 seconds at 50 °C. These data indicate that the exchange data observed for V66W121 \( (k_{\text{M2} \rightarrow \text{M1}} = 1.41(±0.12) \text{ s}^{-1} \) and \( k_{\text{M1} \rightarrow \text{M2}} = 2.63(±0.12) \text{ s}^{-1} \); \( k_{\text{M1}} = 0.93(±0.13) \text{ s}^{-1} \) and \( R_{\text{M2}} = 0.63(±0.07) \text{ s}^{-1} \) may describe the cis–trans conversion that occurred in the Lys116–Pro117 peptide bond of V66W121. Moreover, the estimation of a higher population of M2 than M1 in V66W121 is consistent with the long lifetime of the M2 conformation indicated by slow exchange experiments. The ratio of M1/M2 (0.75) is almost identical to that of PF2C/PF2T (0.76) of SNase121, while PF2C and PF2T correspond to the cis and trans conformations of Lys116-Pro117, respectively, in the SNase121 fragment. Based on the above analysis, M1 and M2 folded conformations of V66W121 can be attributed to the cis and trans conformations of the Lys116-Pro117 peptide bond, respectively.

Driving forces for folding of SNase fragments

The folding process of SNase fragments 1–110, 1–121, and 1–135 involves the generation of a “β-barrel” hydrophobic core, and the overall architecture of the β-subdomain of SNase. For the problem of SNase fragment folding, an important aspect is to determine the specific interactions that influence the folding pathway and lead to the structure of folded species. As indicated by previous studies using fluorescence measurements,7 residues 1–110 of SNase molecules are essential for fragments to adopt the β-barrel fold. The present NMR data revealed that SNase110 can spontaneously form the partially folded species in aqueous solution, and the folded portion in the polypeptide chain of SNase110 is determined in correspondence with the β-barrel. Therefore, the SNase110 polypeptide chain’s intrinsic tendency to form a hydrophobic core drives fragments to be in a partially folded state in equilibrium with the unfolded state. The pattern of SNase110 residue type is crucial for this intrinsic tendency, and its native-like secondary structures in the β-barrel must result from the local interactions between the buried side-chains of residues in native-like contacts.

As has been proposed,14 the hydrophobic amino acid substitution at positions 88 or 66 can strengthen the hydrophobic core in the β-barrel. The 2D \( ^1\text{H}–^{15}\text{N} \) HSQC experiment for G88W110 provided a spectrum with well dispersed cross-peaks (Figure 3a); however, crowded cross-peaks from unfolded species of V66W110 molecules were observed in the central region of the 2D \( ^1\text{H}–^{15}\text{N} \) HSQC spectrum (Figure 6a). In the native SNase structure, the residue at position 88 is located at the end of the pdTp-binding loop, linking the β6 strand (Figure 1). The hydrophobic interaction of Trp88 with hydrophobic residues in the vicinity of β-strands may stabilize the spacial packing of the pdTp-binding loop and the β6 strand as well as the β5 strand relative to the first β-sheet (β1). However, residue Trp66 is at the C-terminal of α1 helix, and is remote from the N-terminal of the β5 strand. The buried indole ring of Trp66 may disturb the spatial tight packing of β-strands in the β-barrel, and the hydrophobic interaction of buried Trp66 with residues in the β-barrel is relatively weaker than that of Trp88. Early studies of SNase fragments with G88W and V66W mutations have indicated that the free energies for unfolding of G88W mutants are higher than those of V66W mutants.7 Clearly, a hydrophobic residue at position 88 influences the unfolding free energy more effectively than at position 66.

Portions of segments in the α1 and α2 helices and in the short two-strands β-sheet (βIII) of G88W121 were observed in intermediate chemical exchange between different native-like conformations as revealed by the 2D \( ^1\text{H}–^{15}\text{N} \) HSQC experiment with G88W121. However, G88W110 shows that the α1 and α2 helices are ordered to a certain degree. Apparently, this must correlate with the long-range interaction between the side-chains of the residues in segment Val111 ~ His121 and the residues in the main part of the fragment molecule. As determined by fluorescence measurements, the unfolding free energy for G88W121 is about 2.55 kcal/mol, and that one for G88W110 is about 2.74 kcal/mol.7 Therefore, the action of long-range interaction of the elongation polypeptide segment from Lys110 reduces the unfolding free energy for G88W121 as compared with G88W110. This was evidenced by the stabilizing C-terminal (Val99, Asn100, Glu101) portion of the α2 helix of G88W mutants in the presence of 2 M TMAO (Figure 8d). Nevertheless, the lower unfolding free energy of V66W110 (1.75 kcal/mol)7 makes only 78% of species of the fragment to be in a folded conformation. An increase of unfolding free energy of V66W121 (2.07 kcal/mol)7 by long-range interaction of Val111 ~ His121 still cannot stabilize V66W121 like that in G88W121, and only 2 M TMAO can facilitate V66W121 to take the more populous M2 partially folded conformation (Figure 9).

In cooperation with the intrinsic force for partial folding localized in the β-barrel of SNase110, the long-range interaction of segments Val111 ~ His121 of SNase121 and Val111 ~ Glu135 of SNase135 can change the populations of partially folded states only, but cannot drive the fragments for further folding relative to SNase110. 2 M TMAO as an external osmolyte force can raise the...
free energy of unfolded states of fragments SNase110, SNase121, and SNase135, and thus also raise the populations of the partially folded states of these fragments (Figure 9). However, having the aid of 2 M TMAO as a driving force, the long-range interaction of the polypeptide segment of Val111 ~ Glu135 can drive the SNase135 fragment to fold further into a native-like β-subdomain conformation. Residues of Glu122 ~ Glu135 as an elongation polypeptide chain from His121 of SNase121 construct the α3 helix in the native SNase. The long-range interaction of the residues in the α3 helix may be the most important factor for SNase135 to form a native-like β-subdomain conformation.

In consequence, the basic factor for the folding of SNase fragments SNase110, SNase121, and SNase135 is the intrinsic tendency to form a native-like conformation in aqueous solution. The intrinsic specific interactions consisting of the localized hydrophobic interaction in the β-barrel and the long-range interaction of buried side-chains between two subdomains in the SNase structure are the main driving force for native-like folding. The factors resulting in free energy changes of unfolded states of fragments, such as amino acid substitution and the presence of 2 M TMAO in osmolyte solution of protein, can promote the fragments to fold into more stable and more native-like tertiary folding states.

**Mechanism for folding process of SNase fragments**

As has been proposed, protein folding reactions may be classified as three different folding processes: hierarchical, hydrophobic collapse, and nucleation-growth. In the hierarchical mechanism, the marginal stable secondary structures form first, and then they interact to produce a growth tertiary structure. In hydrophobic collapse, a possible stable intermediate, the molten globule state of protein forms. The nucleation-growth mechanism is a hybrid of hierarchical and hydrophobic collapse.

Three SNase fragments, SNase110, SNase121, and SNase135, were taken as models for the investigation of the folding process of SNase in vitro. As revealed by CD measurements, all fragments contain relevant contents of secondary structures; the 110-residue fragment contains secondary structures similar to 121-residue and 135 residue fragments, although SNase110 has the least content of the three fragments. Moreover, it was deduced that the main driving force for fragment folding is intrinsic specific interactions in fragment molecules. The 2 M TMAO can only drive more SNase110 and SNase121 species to partially folded states, but not to fold further. This indicates that the interactions between side-chains in SNase110 and SNase121 are not combined well enough to play a role in native tertiary interaction. Only amino acid substitution can facilitate modulation of the extent of tertiary interaction in the molecule. G88W mutation of SNase110 provides evidence that the modulated tertiary interaction for G88W110 characterizes the tertiary structure of the native-like β-subdomain, and therefore makes the mutant to form native-like β-subdomain conformation (Figures 3a and 4). The same is true for G88W121 and V66W mutant fragments (Figures 4 and 6a,b). Explicit evidence of the inherent tertiary interaction existing in wild-type fragments is provided by SNase135. The C-terminal segment, Glu122 ~ Glu135, is almost the whole chain length of the third α-helix of the SNase molecule in the α-subdomain. The combination of side-chain interactions of this segment with those of the β-subdomain may construct a native-like β-subdomain-like tertiary interaction in the SNase135 molecule. Perhaps the free energy for tertiary folding contributed by the combined tertiary interaction cannot compete with the free energy of unfolding, and the spectral behavior of SNase135 is very similar to that of both SNase121 and SNase110. 2 M TMAO increases the free energy for an unfolded state, and the native-like tertiary interaction for folding becomes dominant in the folding process of SNase135. As a result, the native-like β-subdomain conformation can begin to form in the SNase135 molecule. In consequence, the extent of persistent native-like tertiary interactions differs for SNase fragment side-chains with different chain length and mutations.

The total population of heterogeneous partially folded SNase121 conformations (27.3%) was higher than populations of SNase110 (16.6%) or SNase135 (19.5%). Thus, the apparent content of secondary structures and exposed hydrophobic surface of SNase121 is supposed as greater than that of SNase110 and SNase135. Since the chain length of SNase121 is shorter than SNase135 by 14 amino acid residues, the apparent molecule size of SNase121 can be more compact than of SNase135. Therefore, the appearances of the SNase121 fragment cannot deduce a conclusion that the 121-residue fragment is in a molten globule state, which was concluded in the early report. In general, molten globules contain native-like secondary structures but lack the unique side-chain interaction characterizing the tertiary structure of native protein. Moreover, the molten globule conformation is generally taken to be a kinetic intermediate, and is supposed as being in rapid equilibrium with the unfolded state. However, the experimental observations for all three fragments, SNase110, SNase121, and SNase135, cannot be illustrated with these characterizations of molten globule states. Study of 1–136 nuclease fragments has also indicated that the conformational state of this fragment does not appear to correspond to the molten globule state. SNase135 has many features in common with 1–136 nuclease fragments. Both fragments can be driven to adopt a native conformation by binding of pTTP and Ca²⁺. Therefore, fragments SNase110,
SNase121, and SNase135 cannot be regarded as molten globules in aqueous solution.

Since all three fragment molecules represent nearly unique partially folded conformations in the solution under the interaction of 2 M TMAO (Figure 8a–c), folding reactions occurring in SNase110, SNase121, and SNase135 molecules can report the folding process of SNase fragments naturally with chain length elongation. The observed data in the present study suggest that the early folding step under the influence of persistent tertiary interaction would involve hydrophobic interactions localized within several individual chain segments corresponding to the β-barrel. The α-helix structural segments are very unstable, and clearly not related to the α-helices formed in native SNase. With chain length elongation from 110 residues to 121 and 135 residues, the local interactions within secondary structural elements merged with long-range interactions, and the native-like extent of tertiary interaction increased, leading to the formation of ordinary secondary structures and a native β-subdomain structure. Therefore, the folding of the fragments is most likely a hierarchical process.

**Folding pathway of fragments**

In native SNase, the β- and α-subdomains are linked by a loop consisting of the amino acid residues Val111 ~ His121, which is the difference between SNase110 and SNase121 fragments in chain length. The cis–trans isomerization of the Lys116–Pro117 peptide bond in this link loop brought about the major and minor conformational forms for SNase molecules. pdTp and Ca²⁺ binding can facilitate SNase stabilization in major conformational forms. Only by considering these structural properties of native SNase can the folding pathway of fragments be described rationally. Since the unfolded states of all fragments are diminished to nearly invisible at 2 M TMAO, molecules of all fragments can be considered to be in partially folded states only. The evolution of the features of the partially folded conformations with the elongation of fragment chain length at 2 M TMAO should depict the folding pathway of SNase fragments. Two pairs of the folding pathways of SNase fragments can be proposed as follows:

**Pathway A**

PF1 → PF2T → PF3C → major conformational form of SNase
PF1 → PF2C → PF3T → minor conformational form of SNase

**Pathway B**

PF1 → PF2C → PF3C → native conformational form of SNase
PF1 → PF2T → PF3T → native conformational form of SNase

The folding pathways in (A) involve the cis–trans isomerization and in (B) do not involve an isomerization during chain elongation.

As an essential amino acid segment for SNase fragments being in a partially folded state, SNase110 forms a PF1 partially folded conformation. Several individual SNase110 chain segments construct the β-barrel structure frame by the localized hydrophobic interaction in PF1. The other portions of the SNase110 polypeptide chain are not related to the folding state as in the native SNase, but retain the potential to fold into a native tertiary conformation as evidenced by the G88W mutation. The cis–trans isomerization of the Lys116–Pro117 peptide bond of SNase121 generates two heterogeneous partially folded conformations: PF2C and PF2T. The relative population of PF2T is greater than that of PF2C; however, the PF2C conformation of SNase121 is very similar to the PF1 conformation of SNase110. Therefore, the formation of a more populous PF2T conformation with relative low folding free energy in the SNase121 molecule may become the limiting step of the SNase folding process, when the SNase fragment chain length is elongated to residue His121. The rate of synthesis of the protein on the ribosome is estimated to be approximately 5 ~ 20 amino acid residues per second in prokaryotes. Therefore, the timescale for elongation of SNase fragments from 121 to 135 residues on the ribosome is comparable with that for isomerization occurring during the conformational evolution from PF2 to PF3. Two very native-like β-subdomain conformations are formed in the SNase135 molecule. The PF3C conformational state is dominant, but that of PF3T is minor in the SNase135 molecule. If the chain length of the SNase fragment is elongated to residue Trp140, and the α3-helix as well as the C-terminal loop, Lys136 ~ Glu142, in the α-subdomain of native SNase is nearly completed in the amino acid sequence, then the cross-peaks generated by the minor conformational state with a trans X-prolyl peptide bond for Lys116–Pro117 nearly vanishes in the 2D ¹H–¹⁵N HSQC spectrum of SNase140. The 2D ¹H–¹³N HSQC spectrum of SNase140 provides a single set of cross-peaks, the dispersion of which is very close to that of native SNase. Based on the above description, the pathways in (A) but not in (B) are considered to play a leading role in the folding process.
role in the folding of SNase fragments with elongation of the chain length. The upper path in (A) is the folding pathway energetically more favorable for SNase fragments. The lower path is at a disadvantage energetically. Therefore, with the elongation of fragment chain length the relative populations of folding conformations in the upper path increase. Since the folding conformations of fragments resemble the native SNase structural conformation during the evolution from PF1 to PF2 and to PF3, the enzyme function of SNase molecules should gradually be restored. This is evidenced by early reports that the obtained enzyme activities are 0.0004, 0.008, and 0.406 for SNR110, SNR121, and SNR135 fragments, respectively. Incidentally, the enzyme activity of 0.44 was obtained for SNase140 fragments.

**Conclusion**

Conformational states of the SNase fragments SNase110, SNase121, and SNase135 are represented as the ensembles of co-existent native-like partially folded and unfolded states in aqueous solution. The inherent native-like tertiary interaction persists in the fragments, and drives fragment molecules to be in partially folded states, in which a native-like β-barrel conformation is formed by several individual chain segments in molecules. Amino acid substitutions can modulate the extent of native-like tertiary interaction, and make the fragment mutants fold into a native-like β-subdomain. Cooperating with the inherent tertiary interaction, 2 M TMAO can also promote the fragment folding reaction, in which the native β-barrel-like partially folded conformation forms first for 1–110 fragments. Then two different β-barrel-like partially folded conformations form for 1–121 fragments. The prevailing one is a conformation with a cis Lys116–Pro117 bond, and the other is a conformation with a trans X-prolyl bond. Finally, a native-like β-subdomain may form and the cis X-prolyl bond of Lys116–Pro117 is dominant in the 1–135 fragment molecule. The folding reaction of fragments most closely resembles a hierarchical process.

**Materials and Methods**

**Sample preparation**

The SNase fragments 1–110 (SNase110), 1–121 (SNase121), and 1–135 (SNase135), and their G88W and V66W mutants, G88W110, G88W121, V66W110 and V66W121, were expressed and purified as described by Ye et al. Uniformly $^1$H and $^15$N/$^13$C-labeled samples were obtained as described in previous studies. The purity of proteins was checked by SDS-PAGE to ensure a single band.

Several kinds of NMR samples were prepared for different experiments. The samples used for 3D assignment experiments, 2D $^1$H–$^15$N HSQC experiments, and slow exchange experiments in the absence of TMAO were prepared by dissolving 1 mM protein in 90% H$_2$O/10% D$_2$O containing 50 mM deuterated acetate buffer (pH 5.0). Samples for experiments in the presence of TMAO contained an additional 2 M TMAO, and pH was adjusted to 6.5. 0.1 mM fragment proteins were used for experiments to exclude the self-association effect. For denaturation studies, the samples were prepared as those for experiments in the absence of TMAO, but with an additional 1 M, 2 M, and 3 M of urea. A sample for excluding the salt effect in buffers was prepared by dissolving 1 mM protein in 90% H$_2$O/10% D$_2$O directly without any buffer or pH adjustment. The protein concentration was calculated by the mass of the protein powder.

**NMR spectroscopy**

All NMR experiments were carried out on a Bruker DMX 600 spectrometer equipped with an inverse triple-resonance three-axis gradient probe. All 2D $^1$H–$^15$N HSQC experiments incorporated echo-antiecho gradient selection and sensitivity enhancement, which could minimize the proton resonance of water and TMAO. The spectra of 2D $^1$H–$^15$N HSQC experiments for comparing peak volumes were acquired with the same spectral width, data points, number of scans and receiver gains. Two pairs of three-dimensional experiments, HNCACB/CBCA(CO)NH and HBHA(CBCA)NH/ HBHA(CBCACO)NH, were used for the backbone assignments of G88W110, G88W121 and V66W121. The amino acid type-selective experiments for Pro, Lys, Ser, Ala, Gly and Asn were carried out to assist the backbone assignments. The exchange experiments for determination of slow exchange rates were carried out with mixing times of 12.1, 102.1, 402.1, 602.1, 802.1, 1002.1 and 1202.1 ms. Because the exchange experiment could not incorporate a sensitivity enhancement scheme, the original pulse sequence was modified by adding the WATGERATE scheme for better suppression of water proton resonance. All measurements were made at 298 K except for the thermal stability experiments. 2D $^1$H–$^15$N HSQC experiments for studying the thermal stability of V66W121 were performed at temperatures from 280 K to 316 K in 2 K increments for each experiment. The experiment was maintained for more than 30 minutes before each measurement. After the thermal stability experiments, a 2D $^1$H–$^15$N HSQC spectrum at 296 K was re-performed to ensure that the thermal denaturation was reversible.

All data were processed and analyzed using Felix 98 (MSI/Accelrys Inc.). The data points in each dimension were usually doubled by linear prediction and zero-filling. A 90° to 60° shifted square sine bell apodization was used for all dimensions prior to Fourier transformation. $^1$H chemical shifts were referenced to that of internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (at 0 ppm). $^13$C chemical shifts were referenced indirectly. The spectra for comparing peak volumes were processed with the same processing parameters. The peak volumes were calculated directly by Felix. A sequence-dependent CSI method, which is more suitable for studying partially folded and unfolded proteins than the general CSI method, was used for the CSI computation. The chemical shift index parameters were obtained from BMRB. An addition of 1.0 ppm for $^13$C

† http://www.bmrb.wisc.edu/ref_info/CSI.tar
chemical shifts of wild-type SNase was used to correct the referencing offsets.

**Slow exchange rate determination**

The exchange rate between two slow exchanging conformations can be obtained by fitting peak volumes in exchange spectra using the following equations:

\[ I_{11}(T) = I_1(0)(a_{11}e^{-\lambda_1T} + (\lambda_1 - a_{11})e^{-\lambda_2T})/(\lambda_1 - \lambda_2) \]

\[ I_{22}(T) = I_2(0)(a_{22}e^{-\lambda_1T} + (\lambda_1 - a_{22})e^{-\lambda_2T})/(\lambda_1 - \lambda_2) \]

\[ I_{12}(T) = I_1(0)(a_{12}e^{-\lambda_1T} - a_{21}e^{-\lambda_2T})/(\lambda_1 - \lambda_2) \]

where \( \lambda_{1,2} = (1/2)((a_{11} + a_{22}) \pm [(a_{11} - a_{22})^2 + 4k_1k_2]^{1/2}) \), \( a_{11} = R_1 + k_2 \), \( a_{12} = -k_2 \), \( a_{21} = -k_2 \), \( a_{22} = R_2 + k_1 \), \( R_1 \) and \( R_2 \) are the longitudinal relaxation rate constants for the conformations represented by 1 and 2. \( k_1 \) and \( k_2 \) are the exchange rate constants for the forward (1–2) and reverse (2–1) reaction between conformations 1 and 2. \( I_{11}(T) \) and \( I_{22}(T) \) are the volumes of the auto peaks for two conformations denoted by 1 and 2, while \( I_{12}(T) \) and \( I_{21}(T) \) are the volumes of the exchange peaks corresponding to the transfer of magnetization from 1 to 2 and from 2 to 1, respectively. \( I_1(0) \) and \( I_2(0) \) are the volumes of the auto peaks with zero mixing time and may be extracted from the fitting procedure.

A least-squares fitting procedure was employed to extract the longitudinal relaxation rate constants and the exchange rate constants. To estimate the errors, 100 simulated data sets were created, by adding the random errors to the simulated curves derived from the best-fit parameters. The simulated data sets were then subjected to the least-squares fitting procedure, and the fitting parameters were extracted. Standard deviations were obtained from the errors of the fitting parameters. The fitting procedure and error estimation were carried out using the program Octave.

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†http://www.octave.org

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