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Tryptophan 140 Is Important, but Serine 141 Is Essential for the Formation of the Integrated Conformation of Staphylococcal Nuclease[1]

Jinhu Yin and Guozhong Jing[2]

National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, Beijing 100101, People's Republic of China

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A series of N-terminal fragments of staphylococcal nuclease with different chain lengths has been taken as an *in vitro* nascent peptide folding model. Previous studies have shown that nascent peptide folding of the nuclease may begin early in the synthetic process with the content of ordered secondary structure increases with increasing peptide chain length, and that conformational adjustments are observed at certain stages during nascent peptide folding. Here, we focus attention on the conformational changes in the later stage of nascent peptide folding of the nuclease when the N-terminal fragment elongates nearly to the C-terminus of the nuclease in order to determine the role of the C-terminal region of the nuclease in the formation of the integrated conformation of the nuclease. We compared the conformational features of SNase R and its larger N-terminal fragments SNR135, SNR139, SNR140, and SNR141 using circular dichroism spectra, ANS-binding fluorescence and intrinsic fluorescence spectra. The results show that Trp140 is important for the enrichment of ordered secondary structure and for producing a greater ability to fold into a native-like conformation, but Ser141 is essential for the formation of the integrated conformation of the nuclease with a tightly packed tertiary structure. Note that the addition of only one residue to the C-terminus of elongating peptide chain can cause a dramatic change in conformation. The data also show the occurrence of continuous adjustments in conformation during peptide elongation, even after a rigid tertiary structure has formed, suggesting that the last eight residues (residues 142–149), which are disordered at the C-terminus of the nuclease, also possess a structural role, forming the native tertiary structure to provide a framework for the active site, even though they are remote from the active site in both sequence and spatial structure.

Key words: N-terminal fragment, peptide folding, staphylococcal nuclease.

Staphylococcal nuclease (SNase A, EC 3.1.4.7) is a small globular protein of 149 amino acid residues containing no disulfide bonds or cysteines, and its crystal structure has been determined to high resolution (*1, 2*). The nuclease has been widely used as a model for studies of protein folding (*3–12*). Staphylococcal nuclease R (SNase R) is an analogue of SNase A, in which a hexapeptide (DPTVYS) is appended to the alanine of SNase A. The additional residues of SNase R are disordered in solution and have no discernible effect on the remainder of the molecule or its activity (*6, 13*). Since the C-terminal truncated fragments of the N-terminal extended enzyme are somehow more easily isolated and purified from host cells than their wild type counterparts, the C-terminal truncated fragments of the enzyme have been used as a model to study the nascent peptide folding process by different physico-chemical methods (*14–18*). Recently, the conformational features of seven N-terminal fragments of SNase R, *i.e.*, SNR52, SNR79, SNR102, SNR110, SNR121, SNR135, and SNR141, which extend from residues –6–52, –6–79, –6–102, –6–110, –6–121, –6–135, and –6–141, respectively, have been compared. The results indicate that the ordered secondary structures of the fragments increase with increasing peptide length, and there are apparent conformation adjustments during peptide elongation (*16–18*). We have found that although the conformation of SNR135 is partially unfolded, it is able to fold into a native-like state in the presence of ligands, pdTp and Ca^{2+}. There are drastic changes in conformation when the polypeptide grows from SNR135 to SNR141, *i.e.*, the further addition of six residues (residues 136–141) to the C-terminus of SNR135 transforms the more flexible conformation into a stable and integrated conformation, which is almost the same as that of the native enzyme. Hu *et al.* pointed that Trp140, which is the only tryptophan residue and is located near the C-terminus buried in the hydrophobic contact surfaces of the three helices of the nuclease,

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[2] To whom correspondence should be addressed Tel. +86-10-64888493, Fax: +86-10-64872026, E-mail: Jingenx@public.east.cn.net

Abbreviations: SNase, *Staphylococcal* nuclease; CD, circular dichroism; UV, ultraviolet; GdnHCl, guanidine hydrochloride; pdTp, thymidine-3′,5′-bisphosphate; ANS, 1-anilinonaphthalene-8-sulfate.

plays a key role in maintaining the structural integrity of the helices and active sites (*19*). To address what happens during peptide elongation from residue 135 to 141, and which amino acid residues in the C-terminal region are important for the formation of the integrated conformation of the nuclease, and whether the C-terminal eight amino acid residues (residues 142–149) are still involved in the formation of the native conformation, two additional N-terminal fragments of SNase R, SNR139 and SNR140, which extend from residues –6–139 and –6–140, respectively, were constructed and expressed. The conformational features of SNR135, SNR139, SNR140, SNR141, and SNase R were studied by circular dichroism, intrinsic tryptophan fluorescence, ANS-binding fluorescence and the unfolding-refolding behaviors of the polypeptides. The results indicate that the tryptophan 140 is important, but that serine 141 is essential for the formation of the integrated conformation of staphylococcal nuclease, and that conformational adjustments occur even after the rigid tertiary structure of the nuclease has formed during peptide elongation.

MATERIALS AND METHODS

Expression and Purification of Protein Samples—Two ochre mutations (TAA) at positions 140, 141 along SNase R gene were created by polymerase chain reaction (PCR), and verified by DNA sequencing. The mutant gene encoding each of the N-terminal fragment sequences was then inserted into an expression vector, pBV221, as described previously (*13*). The SNase R and its N-terminal fragments were expressed in *Escherichia coli* DH5α cells harboring the appropriate recombinant plasmid, and purified as described by Jing *et al.* (*18*). The purified protein samples appeared as single bands on SDS–15%-polyacrylamide gel. Protein concentration was determined by the methods described by Zhou *et al* (*20*).

Anilinonaphthalence-8-sulfonate (1,8-ANS, Sigma) was used as a hydrophobic fluorescence probe to monitor the surface hydrophobicity of the proteins. Thymidine-3′,5′-bisphosphate (pdTp), a competitive inhibitor of the nuclease, was purchased from Pharmacia. pdTp binds at the active site of the nuclease forming a ternary complex of the nuclease with Ca^{2+} (*1, 2*). Guanidine hydrochloride (GdnHCl, ultra pure) was purchased from Life Technologies. All other reagents were of analytical grade.

Enzyme Activity—The enzyme activity of SNase R and its fragments for the hydrolysis of single-stranded DNA was measured at 25°C in a reaction mixture containing 20 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$, and 50 $\mu g \cdot ml^{-1}$ denatured salmon sperm DNA monitored with a Shimadzu UV-250 spectrophotometer according to the method described by Cuatrecasas (*21*).

Circular Dichroism (CD) Measurements—CD were made with a Jasco J-720 spectropolarimeter. The samples were scanned from 250–190 nm in a quartz cuvette with a 1.0 mm path length. The concentration of each sample was 25 μM in 20 mM Tris-HCl buffer, pH 7.4. The same conditions were also used to study the effect of pdTp on the CD spectra of the N-terminal fragments. The concentration of pdTp was the same as that of the proteins, and the Ca^{2+} concentration was 10 mM. The CD spectra data were reported as mean residue ellipticity by taking 112 Da as the mean residue mass.

Fluorescence Measurements—Fluorescence measurements were made with a Hitachi F4010 spectro-fluorimeter at an excitation wavelength of 295 nm for tryptophan fluorescence at 25°C. The slit width was 5 nm.

Unfolding Studies—SNase R and its N-terminal fragments were incubated with the required concentration of GdnHCl at 4°C overnight in 20 mM Tris-HCl buffer, pH 7.4, for the reaction to reach completion as indicated by no further CD changes. The concentration of each sample was 25 μM and 2.5 μM for CD and fluorescence determinations, respectively.

Refolding Studies—Each of the proteins was first denatured in 6 M GdnHCl at 4°C overnight, then diluted into different concentrations of GdnHCl in 20 mM Tris-HCl, pH 7.4, and kept 4°C until reaction reached completion. The final concentration of each sample was also 25 μM and 2.5 μM for CD and fluorescence determinations, respectively. Changes in the ellipticity at 222 nm, and in the emission intensity at 335 nm for tryptophan, and the shift in the emission maximum of the intrinsic fluorescence were used to indicate protein unfolding and refolding, respectively. All measurements were taken at 25°C.

ANS-Binding Fluorescence—ANS-binding fluorescence was used to study the changes in the surface hydrophobicity of SNase R and its fragments. Each sample contained 50 μM 1,8-ANS and 5 μM of protein in 20 mM Tris-HCl buffer, pH 7.4. Fluorescence spectra were obtained with a Hitachi F4010 spectro-fluorimeter at 25°C. The excitation wavelength was 345 nm, and the slit width was 10 nm.

RESULTS

Activity—The specific activities of the N-terminal fragments and the effect of NaCl concentration on their activities are shown in Fig. 1. The activity of SNase R in 20 mM Tris-HCl (pH 7.4) is 732 $unit \cdot mg^{-1}$, and the activity values of SNR135, SNR139, SNR140, SNR141 are 28.4, 42.6, 45.9, and 76% of SNase R, respectively, indicating that the activity increases with increasing peptide chain length. It is worth noting that like SNase R, SNR140, and SNR141 are activated with increasing concentrations of NaCl, and their

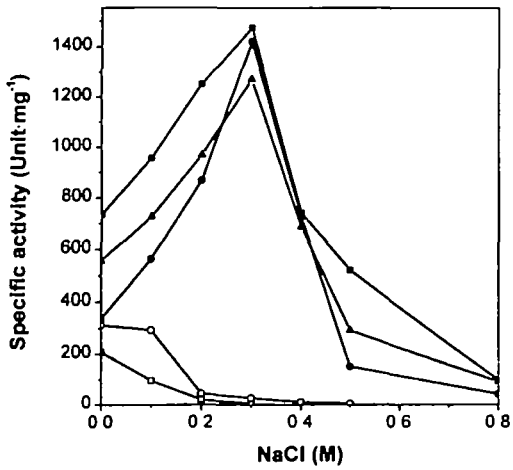

Fig. 1. **Effects of different concentrations of NaCl on the activity of SNase R and its N-terminal fragments.** The activity was measured under the conditions described in the text. SNR135 (□), SNR139 (○), SNR140 (●), SNR141 (▲), SNase R (■)

activities reach a maximum at 0.3 M NaCl. However, such activation is not observed for SNR139 and SNR135, and their activities are dramatically inhibited with increasing concentrations of NaCl, suggesting that the conformational state of SNR140 and SNR141 is much closer to that of SNase R than that of SNR135 and SNR139 in the presence of substrate. Note that the addition of Trp140 to SNR139 causes the activation of SNR140 by NaCl, suggesting that Trp140 is important for the formation of the native conformation of the nuclease.

Shortle's experiment showed that the wild type nuclease (1–149) is also activated by increasing concentrations of NaCl, but the maximal activation occurred at 0.15 M NaCl in Tris-HCl buffer, pH 8.8 (*9*), instead of 0.3 M NaCl at pH 7.4 as described in our experiment. The difference in the maximal activation concentration of NaCl shows that it may be related to the pH value of the reaction buffer.

Circular Dichroism—The ellipticity in the far-UV region, and especially at 222 nm, is usually considered as an index of the content of α-helix in a protein (*22*). The far-UV CD spectra of SNase R and its N-terminal fragments are shown in Fig. 2. The $[\theta]_{222\ nm}$ values of SNR135, SNR139, SNR140, and SNR141 are 44.5, 54.2, 75, and 99% of SNase R, respectively, indicating that the ordered secondary structures increase with the growth of the peptide chain. It is worth noting that there are larger changes in the contents of secondary structure from SNR139 to SNR140 to SNR141, and the spectrum of SNR141 is almost the same as that of SNase R, in which there are two apparent negative peaks at 208 nm and 222 nm. The results show that Trp140 is important for the enrichment of the ordered secondary structure of the polypeptide, but that Ser141 is essential for the polypeptide to gain its secondary structural integrity, especially for the structural integrity of the helices.

Thymidine 3′,5′-bisphosphate (pdTp) is a competitive inhibitor of SNase R. As a binding ligand at the active site of the nuclease, it is involved in the formation of the ternary complex of the nuclease with Ca^{2+} (*23*). pdTp binding can make larger N-terminal fragments of the nuclease fold into a native-like conformation in the presence of Ca^{2+} (*9*, *18*). Previous results showed that $[\theta]_{222\ nm}$ of SNR135 increases with increasing pdTp concentration, and that a sufficient amount of pdTp (molar ratio of pdTp to SNR135 ≥ 1) can make the CD spectra of SNR135 similar to that of SNase R with two negative peaks at 208 and 222 nm. The ellipticity values at 208 and 222 nm of SNR135 reach maxima when the molar ratio of pdTp to SNR135 is greater than 4, but never reach the magnitude of the values of SNase R (*14*). Here, pdTp binding is used as an indicator of the adjustable ability of the conformations of SNR135, SNR139, and SNR140 fragments. As shown in Fig. 3, pdTp binding (molar ratio of pdTp to each fragment = 1:1) can make the CD spectra of SNR135, SNR139, and SNR140 like that of SNase R with negative peaks at 208 and 222 nm. The $[\theta]_{222\ nm}$ values for the fragments increase from SNR135 to SNR140; however, $[\theta]_{222\ nm}$ of SNR140 is almost as the same as that of SNase R. This suggests that the ability to fold into a native-like state in the presence of pdTp and Ca^{2+} increases from SNR135 to SNR140, and that the addition of Trp140 to SNR139 leads to SNR140 having a much greater ability to fold into a native-like state, confirming the important effect of Trp140 on the formation of the secondary structure of the nuclease.

ANS Fluorescence Spectra in the Presence of N-Terminal Fragments of SNase R—The difference in surface hydrophobicity reflects changes in the exposure of hydrophobic protein side-chains, and changes in the extent of exposure of hydrophobic side-chains reflect the folding of a protein molecule (*24*). Differences in surface hydrophobicity among the N-terminal fragments of SNase R were studied using

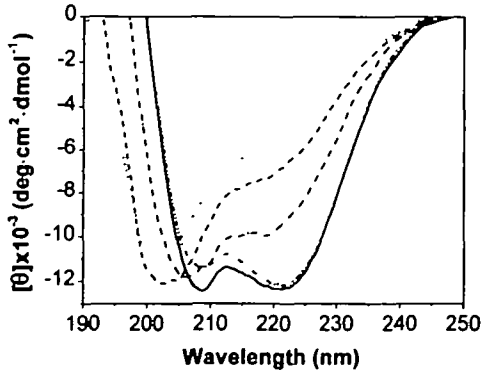

Fig. 2 **Far-UV CD spectra of SNase R and its N-terminal fragments.** The samples were treated as described in the text. SNR135 (····), SNR139 (- - -), SNR140 (——), SNR141 (—·—), and SNase R (—)

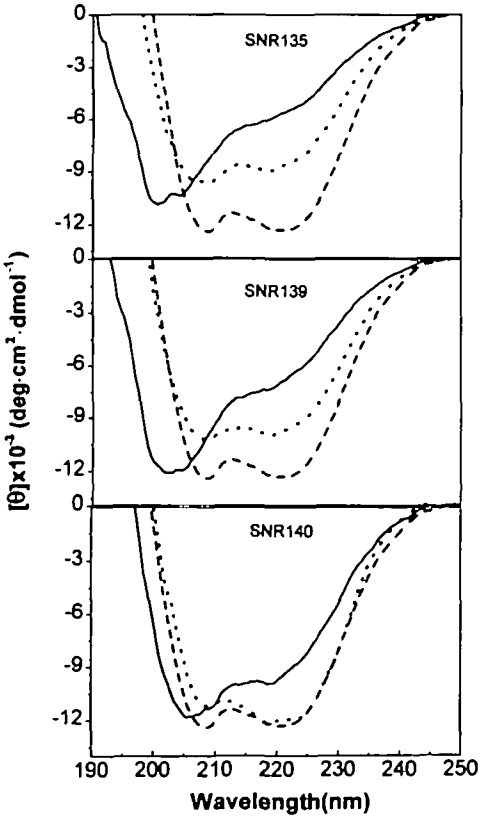

Fig. 3 **Far-UV CD spectra of SNase R and its N-terminal fragments in the presence of 10 mM Ca^{2+} and pdTp.** The experimental conditions are described in the text Protein concentration: 25 μM, the molecular ratio of pdTp to each fragment is 1:1. CD spectra of the fragments (—), CD spectra of the fragments in the presence of pdTp and Ca^{2+} (····), CD spectra of SNase R (- - -).

ANS fluorescence. Figure 4 shows the ANS fluorescence emission spectra in the presence of SNase R and its N-terminal fragments. There is an apparent decrease in fluorescence intensity with a red shift in the fluorescence maximum from SNR135 to SNase R. The ANS fluorescence spectrum with SNR135 is very similar to that of ANS with SNR139, which corresponds well with their activity changes at 0.3 M NaCl. The ANS fluorescence spectrum with SNR141 is almost the same as that of ANS with SNase R. The fluorescence intensity and shift in the fluorescence maximum for ANS in the presence of SNR140 are between those for SNR135/SNR139 and SNR141/SNase R. All these results indicate that the surface hydrophobicity of the fragments decreases with increasing peptide chain length. In other words, the folding extent of the fragments increases with increasing peptide chain length. It is worth noting that compared with the surface hydrophobicity of SNR139, the surface hydrophobicity of SNR140, and especially SNR141, undergoes an apparent change, showing that there is a greater conformation adjustment from SNR139 to SNR140, and especially from SNR140 to SNR141. The results indicate that residue Trp140 and especially, Ser141 are important for the formation of the integrated conformation of the nuclease.

Change in Ellipticity at 222 nm of SNase R and Its N-Terminal Fragments during Unfolding and Refolding in GdnHCl—The conformational changes of SNase R and its N-terminal fragments during unfolding and refolding in different concentrations of GdnHCl were monitored by measuring the changes in mean residue ellipticity at 222 nm ($[\theta]_{222\ nm}$). As shown in Fig. 5, like SNase R, there is no discernible change in the $[\theta]_{222\ nm}$ for SNR141 up to 0.6 M GdnHCl, and then the $[\theta]_{222\ nm}$ of SNR141 decreases sharply at GdnHCl concentrations from 0.6 to 1.0 M. The unfolding curves are very cooperative for both SNase R and SNR141. However, a marked decrease in $[\theta]_{222\ nm}$ has already taken place for SNR140, SNR139, and SNR135 at GdnHCl concentrations above 0.1 M, and the $[\theta]_{222\ nm}$ of SNR140, SNR139, and SNR135 decrease gradually with increasing GdnHCl concentration with no distinct transition region, Fig. 5. These results show that although the addition of Trp140 makes SNR140 more stable than SNR139, SNR140 is still much less stable than SNR141. By comparing the unfolding curve of SNR140 with that of SNR141, it can be seen that the addition of only one amino acid residue (Ser141) to the C-terminus of SNR140 transforms the unfolding pattern from uncooperative for SNR-140 to fully cooperative as the full length enzyme for SNR-141, indicating that Ser141 is very important for the conformational stability and integrity of the nuclease.

The refolding of SNase R and its N-terminal fragments was also studied. All of them can refold to their initial states. The refolding curves coincide with the unfolding curves (data not shown), which are also cooperative for SNase R and SNR141, and uncooperative for SNR140, SNR139, and SNR135.

Changes in the Intrinsic Fluorescence of SNase R and Its N-Terminal Fragments during Unfolding and Refolding in GdnHCl—SNase R contains a single tryptophan residue at position 140 (Trp140), buried in a hydrophobic environment (*1*, *2*). Chen and Tsong pointed out that the fluorescence changes of Trp140 may reflect changes in the overall conformation of the nuclease in denaturants (*25*). To investigate the effect of Trp140 on the conformational formation and integrity of the nuclease during peptide elongation, the intrinsic tryptophan fluorescence of SNase R and its N-terminal fragments, as well as changes during unfolding and refolding in GdnHCl, were compared. The fluorescence

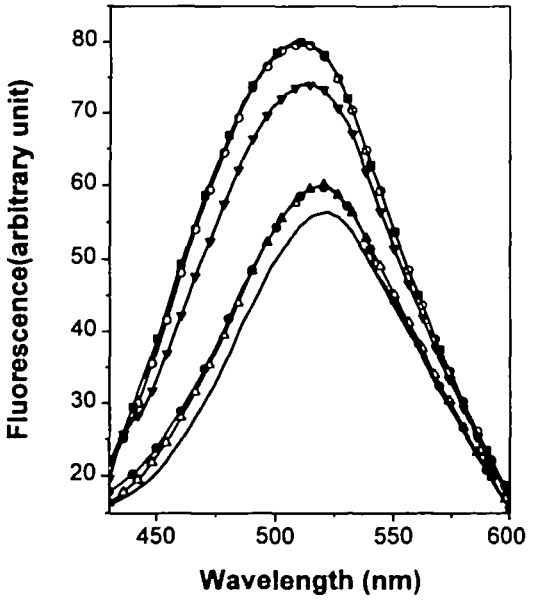

Fig. 4. **ANS fluorescence emission spectra in the presence of SNase R and its N-terminal fragments.** Each sample contained 50 μM of ANS and 5 μM of each protein in 20 mM Tris-HCl (pH 7.4) The fluorescence spectra were measured after incubation of the samples for 20 min at 25°C, at an excitation wavelength of 345 nm, and a slit width of 10 nm. Spectra for ANS with SNR135 (○), SNR139 (■), SNR140 (▼), SNR141 (△), SNase R (●), and 1,8-ANS itself (—)

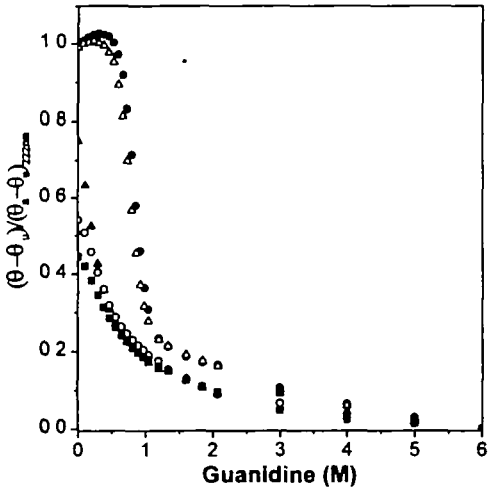

Fig. 5. **Changes in ellipticity at 222 nm ($[\theta]_{222\ nm}$) of SNase R and its N-terminal fragments in different concentrations of GdnHCl.** Protein concentration was 25 μM. Changes in ellipticity at 222 nm during unfolding were measured under the conditions described in the text. The ordinate indicates relative changes in ellipticity at 222 nm, and the point of SNase R at 0 M GdnHCl (12.2 × 10^3 deg cm^2 $dmol^{-1}$) was regarded as 1.0. Other $[\theta]_{222\ nm}$ values on the unfolding curves were compared with that of SNase R at 0 M GdnHCl. SNR135 (■), SNR139 (○), SNR140 (▲), SNR141 (△), SNase R (●).

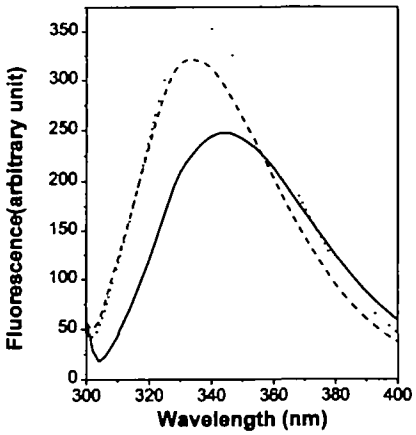

Fig 6. **Tryptophan fluorescence emission spectra of SNase R (---), SNR141 (—), and SNR140 (—).** Each sample contained 3 μM of each protein in 20 mM Tris-HCl buffer (pH 7.4) The experimental conditions are described in the text; the excitation wavelength was 295 nm, and the slit width was 5 nm.

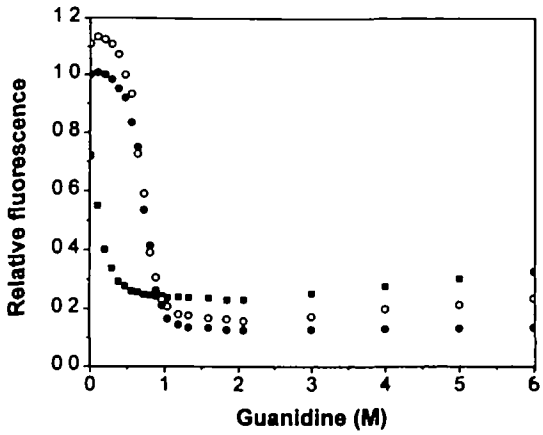

Fig. 7. **Changes in tryptophan fluorescence of SNase R (•), SNR141 (○), and SNR140 (■) in different concentrations of GdnHCl.** Experimental conditions are described in the text. Changes in the intrinsic fluorescence at 335 nm for tryptophan were taken to indicate protein unfolding, The refolding of the proteins is reversible (data not shown). The ordinate indicates relative fluorescence, and the point of SNase R at 0 M GdnHCl was regarded as 1.0. Other fluorescence values on the unfolding curves were compared with that of SNase R at 0 M GdnHCl. The excitation wavelength was 295 nm, and the slit width was 5 nm.

emission spectra of SNase R, SNR141, and SNR140 are shown in Fig. 6. Compared with the spectrum of SNase R, the fluorescence intensity of SNR140 apparently decreases with a red shift in the fluorescence maximum, but the intensity of SNR141 is greater than that of SNase R with little red shift. The results indicate that the conformation of SNR140 is much looser than that of SNase R, but that the conformation of SNR141 seems more compact than that of SNase R, consistent with the observation that SNR141 has a smaller Stokes radius than SNase R (*15*). The difference in their fluorescence spectra at least reflect the change in the environment tryptophan residue 140 in these molecules, showing that the conformation from SNR140 to SNR141 and further to SNase R should pass through apparent stages of conformational adjustments. The changes in the intrinsic fluorescence of SNase R and its N-terminal fragments during unfolding and refolding in GdnHCl are shown in Fig. 7. Like the unfolding and refolding curves monitored by $[\theta]_{222\ nm}$, both SNase R and SNR141 have the same unfolding and refolding patterns with a highly cooperative transition region, indicating that a rigid tertiary structure has already formed when the peptide chain elongates to SNR141, although there are still some differences in conformation between SNR141 and the full length enzyme as described above. However, the fluorescence of SNR140 decreases gradually with increasing GdnHCl concentration, and no distinct transition region appears. Another difference in their fluorescence changes during unfolding is that the red shift in the emission maximum for SNR140 appears at a much lower GdnHCl concentration compared with either SNase R or SNR141, indicating a less stable conformation of SNR140.

DISCUSSION

One important approach to exploring the mechanism of nascent peptide folding is to study the conformational features of a series of N-terminal fragments of a protein with different chain lengths. This will reveal the dynamic process of nascent peptide folding during peptide chain elongation and provide further understanding of the pathway of nascent peptide folding (*26*). Shortle *et al.* used a series of four fragments of staphylococcal nuclease extending over residues 1 to 103, 1 to 112, 1 to 128, and 1 to 136, as a model for the denatured state of the nuclease to examine residual structure in the truncated fragments at equilibrium. The far-UV CD spectra of all four fragments suggested the presence of small to moderate amounts of residual structure (*9*). Furthermore, the authers used nuclear magnetic resonance (NMR), and small angle X-ray scattering measurements to characterize the structure and dynamics of some partially folded larger fragments of the nuclease, such as $SNase_{1\text{-}136}$ and $SNase_{\Delta 131\Delta}$. Their experiments demonstrated that the most persistent elements of structure in the fragments are native-like, suggesting that the nuclease may fold by a hierarchical mechanism (*10, 27, 28*). We use a family of N-terminal fragments of SNase R with different peptide chain lengths as an *in vitro* nascent peptide folding model to study the relationship between the formation of the native conformation and the functional expression of polypeptides during elongation of the peptide chain. Previous experiments have demonstrated that the nascent peptide folding of staphylococcal nuclease may begin early in the synthetic process, that the content of ordered secondary structures increases with increasing peptide chain length, and that conformational adjustments are observed at certain stages during nascent peptide folding (*14, 17, 18*). That the content of ordered secondary structure increases with increasing peptide chain length agrees closely with Shortle's report (*9*). In the present report, we focus attention on the conformational changes that occur at later stages of nascent peptide folding of the nuclease when the N-terminal fragment elongates nearly to the C-terminus of the nuclease to find which amino acid residues are important for the formation of an integrated conformation. For this, we compared the conformational features of SNase R and its N-terminal fragments, SNR135, SNR139, SNR140, and SNR141.

Trp140 Is Important, but Ser141 Is Essential for the Formation of the Integrated Conformation of the Nuclease—As described above, the activity assay in the presence of different concentrations of NaCl, Fig. 1, divides the proteins into two groups: SNR135/SNR139, which are inhibited by NaCl, and SNR140/SNR141/SNase R which are activated at 0.3 M NaCl. These phenomena indicate that the conformational state of SNR140 is much closer to that of SNase R in the presence of substrate, suggesting that Trp140 is important for the formation of the active conformation of the nuclease, although the activation level is less than that of SNase R. This result corresponds well with the finding that SNR140 has much a greater ability to fold into a native-like state in the presence of ligands, Fig. 3, as compared with SNR135 and SNR139.

The CD spectra and ANS-fluorescence spectra clearly divide the proteins into three conformational groups. The conformations of SNR135/SNR139 are in a partially unfolded state with more disordered structure, Fig. 2, and more hydrophobic residues exposure, Fig 4, although they are able to fold into the native-like state in the presence of ligands, Fig. 3. The conformation of SNR141 in both secondary structure content and in surface hydrophobicity is the same as the full-length nuclease. The conformation of SNR140 is between SNR135/SNR139 and SNR141/SNase R; adding Trp140 to the C-terminus of SNR139 causes the polypeptide SNR140 to gain more ordered secondary structure. The far-UV CD spectrum of SNR140 seems to have two negative peaks at 208 and 222 nm which represent typical α-helix conformation. The conformational features of SNR140 indicate the roles played by Trp140 in the integrity of the helices during peptide folding, Fig. 2. The addition of Trp140 also results in the exposure of some hydrophobic side chains buried in the interior of the polypeptide SNR140, although the surface hydrophobicity does not reach the level of SNR141 or the full-length enzyme SNase R.

Although the addition of Trp140 to the C-terminus of SNR139 largely enriches the amount of ordered secondary structure of SNR140 and decreases the exposure of its hydrophobic side chains, giving SNR140 a much greater ability to fold into a native-like active conformation, the unfolding and refolding behaviors of SNase R and its N-terminal fragments, SNR140 and SNR141, in GdnHCl as monitored by both CD and intrinsic fluorescence spectra show that SNase R and SNR141 have the same unfolding and refolding patterns with high cooperativity. In contrast, there is no distinct transition region in the conformation changes of SNR140, just like SNR135 and SNR139. The unfolding and refolding cooperativity clearly indicates that a highly packed tertiary structure does not appear until the N-terminal fragment of the nuclease elongates to SNR141. Taken together, the above results show that Trp140 plays an important role in the conformation, especially in the enrichment of the ordered secondary structure during peptide elongation, but that Ser141 is essential for the formation of the integrated conformation of the nuclease.

The crystal structure of SNase shows that different hydrogen bonds play important roles in maintaining the integrated conformation (*1*, *2*). As described by Loll and Lattman, the side chain of Asn138 hydrogen bonds to the carbonyl oxygen of residue Gln106, helping to "tack down" α-helix 3 to the main body of the nuclease (*1*). In the view of the fact that the addition of Ser141 to the C-terminus of SNR140 transforms the partially unfolded polypeptide SNR140 to such a conformational state with a tightly packed tertiary structure, SNR141, hydrogen bonds between Ser141 and other residues in the nuclease should be key factors in the conformational integrity of the nuclease. There are three hydrogen bonds between Ser141 and other residues, *i.e.*, the main chain of Ser141 to the main chain of Asn138, the side chain of Ser141 to the main chain of Asn138, and the side chain of Ser141 to the side chain of Tyr54, which may play a stabilizing role in the formation of the hydrogen bond between the side chain of Asn138 and the carbonyl oxygen of Gln106 and in maintaining the integrated conformation of the nuclease It is obvious that the deletion of residue Ser141 in the case of SNR140, eliminates the hydrogen bonds between Ser141 and the other residues indicated above, which severely affects the formation of the integrated conformation of SNR140. The sole tryptophan residue in the nuclease, Trp140, involves two reverse turn formations, *i.e.*, a type II′ turn (137–140) and a type I turn (138–141), and is located near the C-terminus, buried in the hydrophobic contact surfaces of the three helices of the nuclease. Such hydrophobic interaction also plays a key role in enriching the ordered secondary structure of the nuclease. As compared with the intrinsic fluorescence spectrum of SNase R, Fig. 6, the fluorescence intensity of SNR140 apparently decreases with a red shift in the fluorescence maximum, showing a change in the hydrophobic environment of Trp140 in SNR140. This phenomenon indicates that the hydrogen bonds between Ser141 and other residues may also play important roles in maintaining Trp140 in a favorably hydrophobic environment

Continuous Conformational Adjustments Are Necessary for the Formation of the Native Conformation during Peptide Elongation, Even after the Rigid Tertiary Structure Has Formed—Previous experiments have demonstrated that conformation adjustments are necessary for the formation of the native conformation during nascent peptide folding (*16–18*). Here we show the conformational changes as the N-terminal fragment elongates nearly to the C-terminus of the nuclease. As described above, there is no apparent conformational change when the peptide elongates from SNR135 to SNR139, both of which are in a partially unfolded state with more disordered secondary structures as compared with larger N-terminal fragments. However, there are larger changes in conformation when the peptide elongates from SNR139 to SNR140, and even more during elongation from SNR140 to SNR141 as described above. Note that the addition of only one residue to the C-terminus of the elongating peptide chain can cause dramatic changes in the conformation of the peptide. For instance, the addition of Ser141 to the C-terminus of SNR140 transforms the partially unfolded polypeptide SNR140 to a conformational state with a tightly packed tertiary structure, SNR141. As shown above, the tightly packed tertiary structure of the nuclease appears when the peptide elongates to SNR141. A question is whether there is a conformational adjustment when the peptide elongates from SNR141 to the full length nuclease. The differences between SNR141 and SNase R in both the tryptophan fluorescence emission spectrum and the unfolding and refolding pattern, Fig. 6 and Fig. 7, provide evidence in addition to the indirect evidence deduced from the activity changes from SNR141 to

SNase R (*16–18*). Besides the above evidence, the crystal structure of SNR141 also shows some differences between SNR141 and the full length nuclease (*29*). For example, the side chain of Tyr113 turns toward the inside of the active pocket of the nuclease in the case of SNR141, instead of toward outside in the case of the full length enzyme. All the results indicate that there are continuous adjustments in conformation during nascent peptide folding, even after the native-like rigid tertiary structure has formed, suggesting that the last eight residues (residues 142–149) at the C-terminus of the nuclease have structure roles, forming the native tertiary structure to provide a framework for the active site, even though they are remote from the active site in both sequence and spatial structure, from which it appears that the C-terminal region is involved in the full active expression of the nuclease.

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