J. Biochem. 120, 881-888 (1996)

Unfolding and Refolding of the N-Terminal Fragments of Staphylococcal Nuclease R in Guanidine Hydrochloride[1]

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Received for publication, April 8, 1996

The conformational and activity changes of a family of peptide fragments of staphylococcal nuclease R, which extend from residues −6 to 102, −6 to 110, −6 to 121, −6 to 135, and −6 to 141, during unfolding and refolding in different concentrations of guanidine hydrochloride have been studied. The studies indicate that the conformational stability in guanidine hydrochloride solution of the N-terminal fragment increases with increasing chain length, and that interaction and recognition between amino acid residues which are related to formation of the native conformation also increase with growth of the peptide chain, but such interaction becomes effective only when the polypeptide chain reaches a certain length. The changes in conformation and catalytic activity of the N-terminal fragments during unfolding and refolding demonstrate that conformational adjustments are necessary during chain elongation to generate the native conformation of a biologically active protein.

Key words: N-terminal fragment, nuclease, peptide folding, unfolding and refolding, *Staphylococcus*.

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It is now generally accepted that the one-dimensional amino acid sequence of a protein determines the corresponding three-dimensional structure of the protein. However, little is known about how a nascent peptide chain folds into its native conformation so as to generate a functional protein. Although the refolding of denatured intact proteins provides a lot of information, it is not a valid model, as the refolding starts from the complete chain, while the folding of the nascent peptide might begin early during synthesis of the peptide chain from its N-terminal to C-terminal end on the ribosome (*1*). Thus, studies of unfolding and refolding of a family of peptide fragments of different chain lengths starting from the N-terminal end of a protein should provide a better model for the process of protein folding than study of the refolding of the denatured intact protein.

Staphylococcal nuclease (SNase A, EC 3.1.4.7) is a small globular protein of 149 residues containing no disulfide bonds or cysteines. The protein reversibly folds to yield the active enzyme *in vitro*, and its crystal structure is known to high resolution (*2, 3*). The developments in the cloning and expression of SNase A and its mutants in *Escherichia coli* have made this protein particularly attractive for studies of protein folding (*4-10*), and C-terminal truncated fragments of the enzyme have also been used as models to characterize the structure of folding intermediates and to study the early stages of the folding process by using different physiochemical methods (*11-14*).

Staphylococcal nuclease R (SNase R) is an analogue of SNase A in which a hexapeptide (DPTVYS) is appended to the N-terminal alanine of SNase A. The additional residues of SNase R have no discernible effect on the remainder of the molecule or its activity (*9, 15*). Recently, five N-terminal fragments of SNase R of different chain lengths starting from the N-terminal end have been overproduced in *E. coli* and their conformations in solution have been comparatively studied (*16*). The studies have shown that the peptide fragments of SNase R have certain amounts of residual structure and enzyme activity, and the contents of secondary structure and enzyme activity increase with increasing peptide chain length, suggesting that these fragments may be a reasonable model for the study of nascent peptide folding *in vitro*. Therefore, the unfolding and refolding of the N-terminal fragments of staphylococcal nuclease R and the changes in conformation and activity of the fragments during denaturation with guanidine hydrochloride (GdnHCl) of different concentrations were studied by using far-UV circular dichroism spectra and fluorescence spectra. The results may help us to understand the relationship between the folding of the peptide chain and the functional expression during chain elongation.

MATERIALS AND METHODS

Staphylococcal nuclease R and its five N-terminal fragments were purified from *E. coli* DH5α cells harboring an appropriate recombinant plasmid as described by Jing *et al.* (*16*). The five N-terminal fragments were named SNR102, SNR110, SNR121, SNR135, and SNR141, which extend from residues −6 to 102, −6 to 110, −6 to 121, −6 to 135, and −6 to 141, respectively. Since the crystal

[1] This work was supported by a Pandeng grant from the China National Natural Science Foundation.

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Abbreviations: SNase, staphylococcal nuclease; CD, circular dichroism; FTIR, Fourier-transform infrared spectrum; GdnHCl, guanidine hydrochloride; pdTp, thymidine 3',5'-bis-phosphate; 1,8-ANS, 1-anilinonaphthalene-8-sulfate.

structure of the nuclease shows that the overall tertiary fold of the nuclease is composed of a highly twisted, five-stranded β-barrel (residue ranges: 10-19, 22-27, 30-36, 71-76, and 88-95) and three α-helices (residue ranges: 54-68, 98-106, and 121-135) (*3*), the above N-terminal fragments contain the residues which contribute to formation of β-strands. SNR141 contains the residues required for formation of all the secondary structure in the native nuclease. SNR135 contains all the residues forming the three α-helices. Compared with SNR135, the residues forming the third α-helix are removed from SNR121 and SNR110, and the difference between SNR121 and SNR110 is that 11 more residues (residue range: 111-121) which form the left side of the nucleotide binding pocket (*3*) are deleted in the case of SNR110. SNR102 just contains the residues for formation of the first α-helix. The mutant SNase R (W140F) was obtained by replacing tryptophan (W) at position 140 with phenylalanine (F), which is considered as essentially the same as the wild-type enzyme since it has almost the same CD spectrum and activity as SNase R (*17*). The purified SNase R and its N-terminal fragments each showed a single band during electrophoresis. GdnHCl (ultra pure) was purchased from Life Technologies. All other reagents were of analytical grade. Protein concentrations of the N-terminal fragments were determined by Goodwin and Morton's method (*18*).

Enzyme activity for 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 with a Shimadzu UV-250 spectrophotometer according to the method described by Cuatrecasas (*19*). For the measurement of enzyme activity after denaturation or renaturation in GdnHCl, the reaction mixture was always adjusted to contain the same concentration of GdnHCl during activity assay.

For unfolding studies, SNase R and its N-terminal fragments were first incubated with the required concentration of GdnHCl at 4°C overnight in 20 mM Tris·HCl, pH 7.4, to allow the reaction to reach completion before CD and fluorescence measurements and activity assay. The concentration of each sample was 0.4 $mg \cdot ml^{-1}$ and 40 $\mu g \cdot ml^{-1}$ for CD and fluorescence determinations, respectively. Without GdnHCl the proteins are stable as judged from the CD and fluorescence spectra under identical conditions.

For refolding studies, each of the proteins was first denatured in 6 M GdnHCl at 4°C overnight, then it was diluted into GdnHCl of different concentrations in 20 mM Tris·HCl, pH 7.4 and stored at 4°C for 24 h to allow the reaction to reach complete equilibrium before CD and fluorescence measurements and activity assay. The final concentration of each sample was 0.4 $mg \cdot ml^{-1}$ and 40 $\mu g \cdot ml^{-1}$ for CD and fluorescence determinations, respectively.

CD spectra were obtained on a Jasco J-500A spectropolarimeter, and the sample was scanned from 250 to 200 nm in a quartz cuvette with 1 mm pathlength. Fluorescence measurements were made with a Hitachi F4010 spectrofluorimeter with excitation at 277 nm for tyrosine fluorescence and 295 nm for tryptophan fluorescence. Changes in the ellipticity at 222 nm, and in intrinsic fluorescence at 335 nm for tryptophan and at 305 nm for tyrosine were taken to indicate the protein unfolding and refolding, respectively. All the measurements were taken at 25°C.

For the study of the changes of surface hydrophobicity of SNR121 at 0.1 M GdnHCl in 20 mM Tris·HCl buffer (pH 7.4), 1-anilinonaphthalene-8-sulfate (1,8-ANS, Sigma) was used as a hydrophobic fluorescence probe. Each sample contained 80 μM 1,8-ANS and 8 μM SNR121. The concentration of pdTp was 4 times that of SNR121, and the Ca^{2+} concentration was 10 mM (*16*). Fluorescence spectra were measured by using a Hitachi F4010 spectrofluorometer at 25°C. The wavelength of excitation was 345 nm, and the slit width was 10 nm. The presence of pdTp and Ca^{2+} had no effect on the fluorescence spectrum of 1,8-ANS itself.

RESULTS

Changes in Ellipticity at 222 nm and Activity of the N-Terminal Fragments of SNase R during Unfolding in GdnHCl—The conformational changes of the N-terminal fragments of SNase R during unfolding in GdnHCl were monitored by measuring the far-UV CD spectra. The ellipticity at 222 nm of SNase R in buffer without GdnHCl is 11.9×10^3 $deg \cdot cm^2 \cdot dmol^{-1}$, and the values of θ_{222nm} of SNR141, SNR135, SNR121, SNR110, and SNR102 are 99, 47, 57, 43, and 27% of that of SNase R, respectively. Their far-UV CD spectra are shown in Fig. 1. The changes in ellipticity at 222 nm (θ_{222nm}) of the N-terminal fragments in GdnHCl solution of different concentrations are shown in Fig. 2. As in the case of SNase R, there is no discernible change of θ_{222nm} for SNR141 up to 0.7 M GdnHCl concentration, whereas at GdnHCl concentrations higher than 0.1 M for SNR135 and SNR121 and at 0.1 M GdnHCl for SNR110 and SNR102, marked decreases of θ_{222nm} take place. These phenomena indicate that the conformational stability of the N-terminal fragments in GdnHCl decreases with increasing deletion of amino acid residues from the C-terminal end. In other words, the conformational stability of the N-terminal fragments in GdnHCl solution increases with increasing length of the peptide chain.

It is worth noting that the unfolding curve of SNR141 is

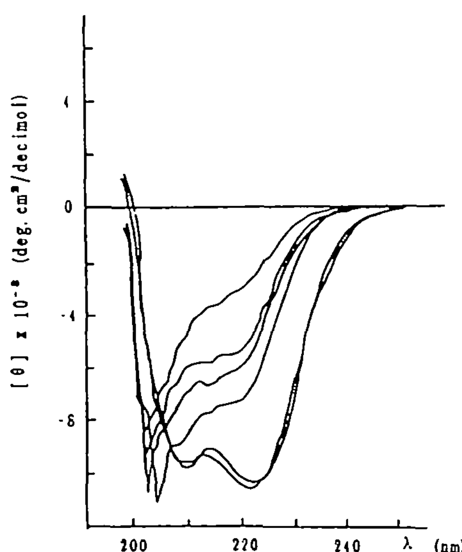

Fig. 1. **Far-UV CD spectra of SNase R and its N-terminal fragments.** Samples were scanned by using a quartz cuvette with a 1 nm path length at 25°C. The concentration of each sample was 0.4 $mg \cdot ml^{-1}$ in 20 mM Tris·HCl buffer (pH 7.4). From top to bottom: SNR102, SNR110, SNR135, SNR121, SNR141, and SNase R.

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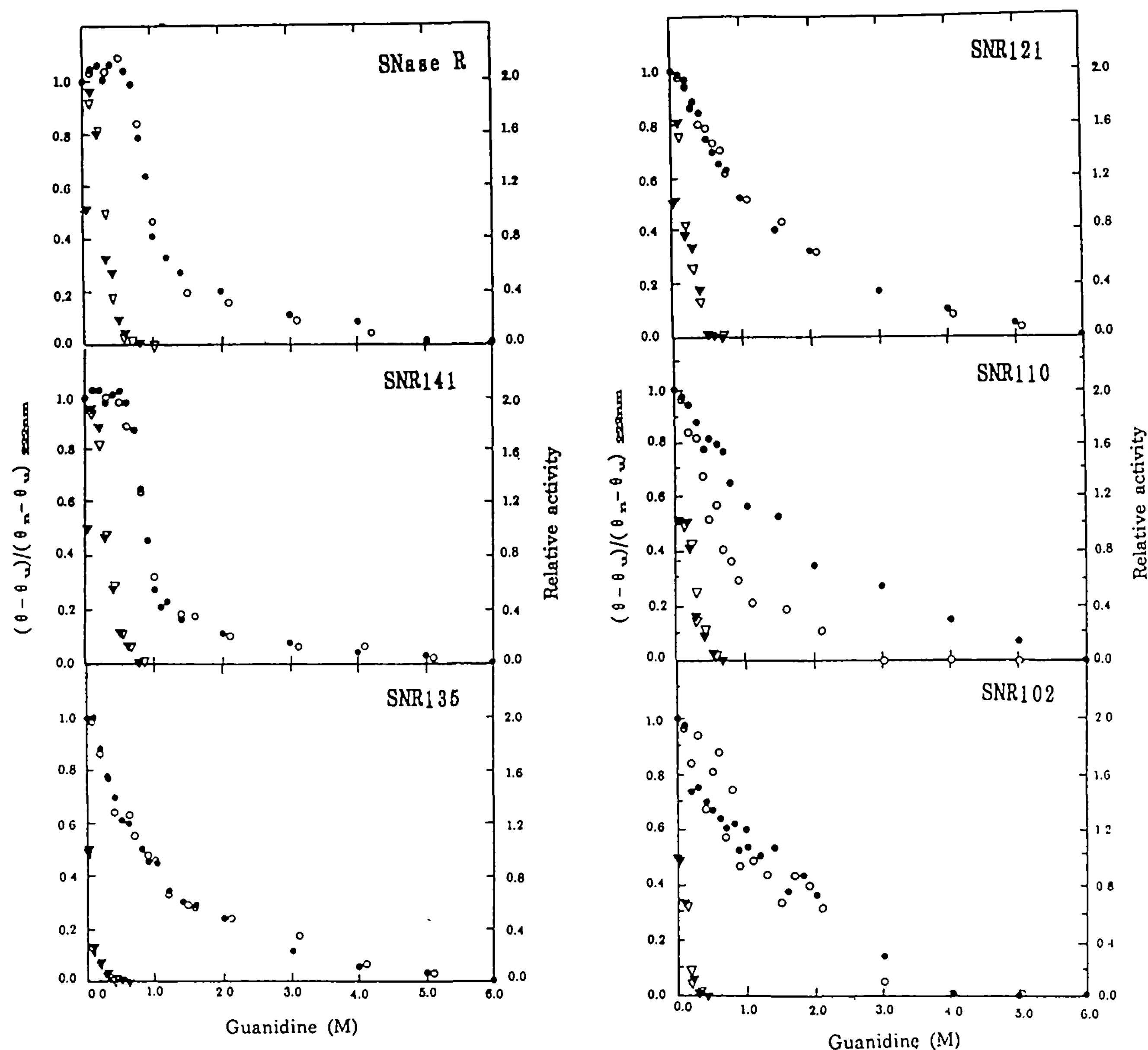

Fig. 2. **Changes in ellipticity at 222 nm and activity of SNase R and its N-terminal fragments in GdnHCl solutions of different concentrations.** Changes in ellipticity at 222 nm during unfolding (●) and refolding (○) were measured under the conditions described in the text. Changes in activity during unfolding (▼) and refolding (▽) were measured under the same conditions. In this figure, the changes in ellipticity at 222 nm and activity in GdnHCl of different concentrations were compared with the values in the buffer without GdnHCl for each fragment. The left ordinate indicates relative changes in ellipticity at 222 nm, and the right ordinate indicates relative changes in activity.

different from those of the other fragments, as shown in Fig. 2. The θ_{222nm} of SNR141 sharply decreases at GdnHCl concentrations from 0.7 to 1.0 M, just like that of SNase R. However, the θ_{222nm} of the other fragments decreases gradually with increasing GdnHCl concentrations and no distinct transition region of conformational change appears. The crystal structure of the enzyme shows that the residues 137-141 can form hydrogen bonds with the residues that form the third α-helix (residue range: 121-136) and the left side of the nucleotide binding pocket (residue range: 111-121) (*3*). These hydrogen bonds will be disrupted by the deletion of residues 136-141, and the NMR analysis of the SNase A 1-136 fragment provided no evidence that the third α-helix, formed by residues 121-136, is present (*14*).

Taking account of these results, the features of the unfolding curves of SNR141 and the other fragments indicate that SNR141 has a more compact structure and a more stable conformation than other fragments do, and thus residues 136-141 are important to maintain the integrity and stability of the enzyme conformation. The similar shape of transition curves between SNase R and SNR141 means that the deletion of 8 amino acid residues from the C-terminal end does not markedly affect the conformational integrity and stability of the enzyme, which is consistent with the CD spectra of SNase R and SNR141 without GdnHCl (*16*).

Activity changes of SNase R and its N-terminal fragments during unfolding in GdnHCl have been studied (also

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shown in Fig. 2). The specific activity of SNase R in 20 mM Tris·HCl buffer (pH 7.4) is 680 units·mg^{-1}, and the enzyme activities of SNR141, SNR135, SNR121, SNR110, and SNR102 under the same conditions are 79, 41, 0.8, 0.04, and 0.25% of that of SNase R, respectively. It is interesting that SNase R, SNR141, and SNR121 are activated at 0.1 M GdnHCl [their enzyme activities are about 190, 190, and 140% of that without GdnHCl, respectively (Fig. 2)], but the activation is not accompanied with any significant conformational change as judged from the far-UV CD spectra. However, such activation is not observed for SNR135, SNR110, and SNR102. In particular, SNR135 loses its activity sharply and only about 30% of original activity is retained at 0.1 M GdnHCl, though SNR135 has 14 more amino acid residues than SNR121 consisting of the third α-helix of the nuclease. The activation of SNR121 by a low concentration of GdnHCl implies that SNR121 has some special conformation, which is consistent with the results that SNR121 has its own unique surface hydrophobicity and higher "apparent" ordered secondary structure (*16*). The fluorescence spectra for 1,8-ANS with SNR121 under various conditions are shown in Fig. 3. At 0.1 M GdnHCl, the surface hydrophobicity of SNR121 decreases, and the fluorescence of 1,8-ANS with SNR121 has a clear blue shift in the presence of pdTp and Ca^{2+}. The blue shift of the emission maximum is not observed in the fluorescence spectra for 1,8-ANS with other N-terminal fragments under the same conditions (data not shown). Although the mechanism of the effects of 0.1 M GdnHCl on SNR121 is not clear, the changes of intensity and emission maximum of 1,8-ANS fluorescence shown in Fig. 3 indicate that the conformation of SNR121 and its ability to combine with pdTp and Ca^{2+} do alter at 0.1 M GdnHCl, though the changes can not be observed in the CD spectrum at 0.1 M GdnHCl (Fig. 4). Perhaps the activation by a low concentration of GdnHCl could be caused by transforming a more rigid conformation to a flexible one at the active site, allowing the full expression of the activity (*20*).

Changes in Ellipticity at 222 nm and Activity of the N-Terminal Fragments of SNase R during Refolding in GdnHCl—The refolding of the N-terminal fragments of SNase R was also studied (Fig. 2). Like SNase R, all five N-terminal fragments can refold their conformation to the initial state, respectively, because the ellipticity and activity of the fragments are restored to the initial values at the lowest concentration of GdnHCl. But the transition curves of the denaturation reaction for SNR110 and SNR102 do not coincide with the unfolding curves. The phenomena are different from those of the other longer fragments, of which the refolding curves coincide with the unfolding ones, indicating that the interaction and recognition between amino acid residues in the shorter fragments, such as SNR110 and SNR102, are more readily disrupted by GdnHCl, meaning that the interaction and recognition

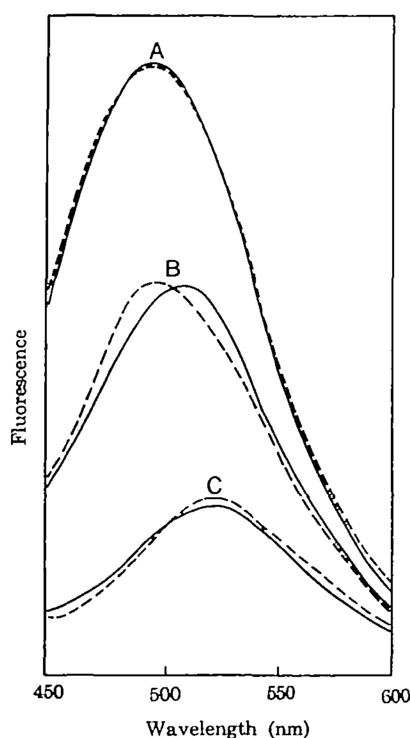

Fig. 3. **Fluorescence emission spectra of 1,8-ANS with SNR121 under different conditions.** The experimental conditions are described in the text. (A) Fluorescence spectra of 1,8-ANS with SNR121 in 20 mM Tris·HCl, pH 7.4 (solid line) and in the presence of pdTp and Ca^{2+} (broken line). (B) Fluorescence spectra of 1,8-ANS with SNR121 in 20 mM Tris·HCl, pH 7.4, containing 0.1 M GdnHCl (solid line) and in the precence of pdTp and Ca^{2+} (broken line). (C) Fluorescence spectra of 1,8-ANS itself in 20 mM Tris·HCl, pH 7.4 (solid line) and in 20 mM Tris·HCl, pH 7.4 containing 0.1 M GdnHCl (broken line).

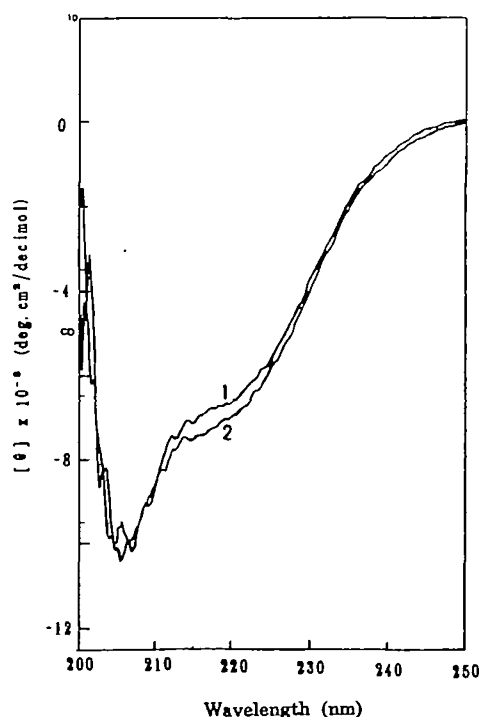

Fig. 4. **Far UV-CD spectra of SNR121 in the presence of pdTp and Ca^{2+} in 20 mM Tris·HCl buffer (pH 7.4) containing 0.1 M GdnHCl.** Samples were scanned in a quartz cuvette with 1 nm path length at 25°C. Each sample contained 0.4 mg·ml^{-1} SNR121. The concentration of pdTp was 4 times that of SNR121, and the Ca^{2+} concentration was 10 mM. 1, CD spectrum of SNR121; 2, CD spectrum of SNR121 in the precence of pdTp and Ca^{2+}.

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between the amino acid residues, which are related to restoration of the initial conformations, increase with increasing peptide chain length. It is also worth noting that there is a sharp conformational transition region for SNase R and SNR141 during refolding at GdnHCl concentration from 1.0 to 0.7 M, which is very different from the other fragments. The above phenomena indicate that the interaction and recognition between amino acid residues for formation of the native conformation of the enzyme molecule can be more effective only when the polypeptide chain reaches a certain length, and again show the important role of the residues from 136 to 141 for maintaining the conformational integrity of the enzyme (*16*).

Activity changes of SNase R and its N-terminal fragments during refolding in GdnHCl are also shown in Fig. 2. The enzyme activity can be reversibly restored with decreasing GdnHCl concentration. It should be noted that in contrast with the unfolding, the conformational restoration both for SNase R and for its N-terminal fragments precedes their activity restoration during refolding in GdnHCl. This means that conformational integrity is very important to regain functional expression both for the whole enzyme molecule and for its N-terminal fragments.

Intrinsic Fluorescence Changes of the N-Terminal Fragments of SNase R during Unfolding and Refolding in GdnHCl—SNase R has a single tryptophan residue at position 140 (W140), buried in a hydrophobic environment (*2, 3*). Recently Chen and Tsong pointed out that the fluorescence changes of W140 can reflect the changes of the whole conformation of the molecule in denaturants (*21*). This makes fluorescence measurement an excellent means of following major changes in the protein conformation. Changes in the emission intensity at 335 nm and the position of the emission maximum of the intrinsic fluorescence, as well as in the activity of SNase R and SNR141, during unfolding and refolding in GdnHCl solutions of different concentrations are shown in Fig. 5. The curves of relative fluorescence *versus* GdnHCl concentration have very similar features to those obtained from CD spectra. The enzyme activity for both SNase R and SNR141 is completely inactivated at 0.8 M GdnHCl, at which a marked red-shift of the emission maximum of their intrinsic fluorescence occurs. Together with the results obtained from the CD spectra, all the studies indicate that SNR141 has almost the same secondary structure and overall conformation as SNase R; the W140 in SNR141 is still

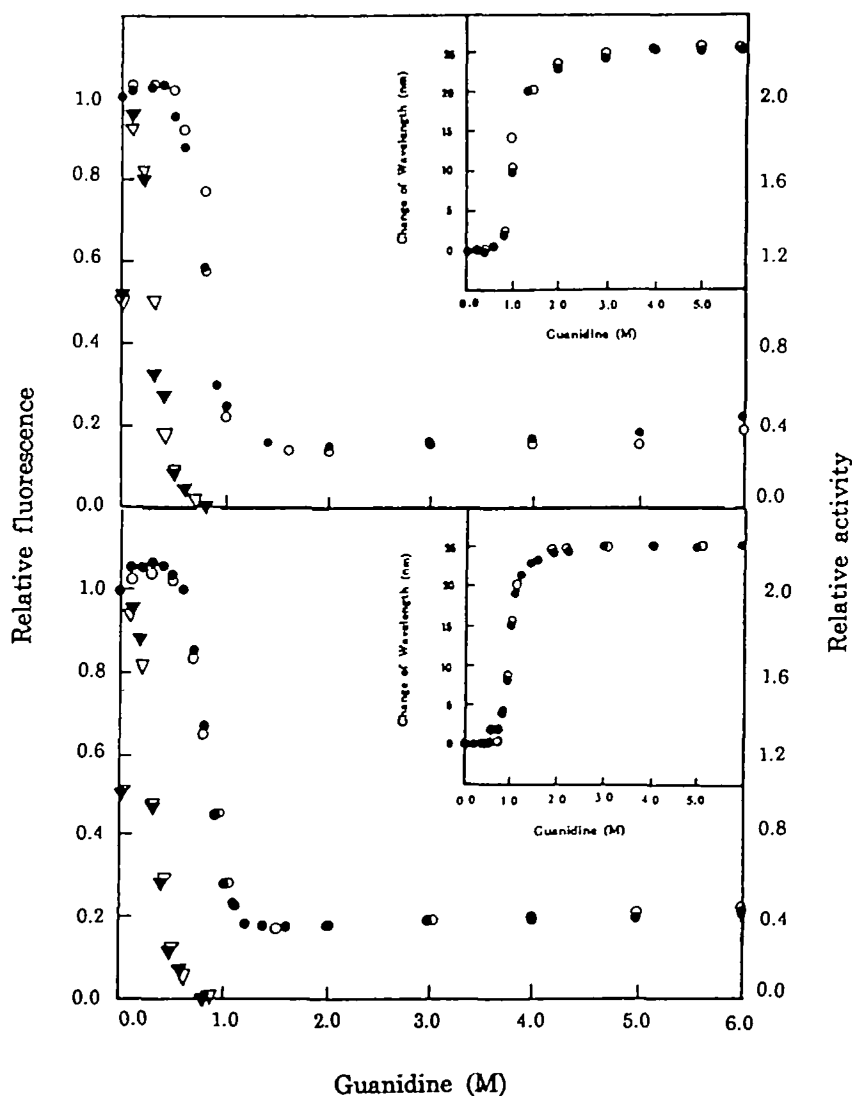

Fig. 5. **Changes in tryptophan fluorescence and activity of SNase R (upper) and SNR141 (lower) in GdnHCl solution of different concentrations.** Experimental conditions were described in the text. Changes in intrinsic fluorescence at 335 nm for tryptophan were taken to indicate the protein unfolding (●) and refolding (○). The wavelength of excitation was 295 nm, and the slit width was 5 nm. Changes in activity during unfolding (▼) and refolding (▽) were measured under the same conditions. The insets show the shift in emission maximum during unfolding (●) and refolding (○). Emission intensity (left ordinate) and relative activity (right ordinate).

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buried in a hydrophobic environment, and the deletion of residues 142–149 from the C-terminal end does not affect the integrity of the molecular conformation. But comparison of the second-derivative FTIR spectra of SNR141 and SNase R indicated that the conformation of SNR141 is more compact than that of SNase R, because its bands representing β-strands and α-helices have lower wave numbers than those of SNase R, and the enzyme activity of SNR141 is only about 80% of that SNase R (*16*), suggesting that the deletion of the 8 residues from the C-terminal end of SNase R may perturb the active site and decrease the flexibility of the enzyme, as the active site is more fragile than the molecule as a whole (*22*).

As the N-terminal fragments of SNase R, except SNR141, have no tryptophan residue, tyrosyl intrinsic fluorescence of the fragments was measured during unfolding and refolding in GdnHCl. The curves in Fig. 6 show that the emission intensity at 305 nm increases with increasing GdnHCl concentration. Compared with the fluorescence of a model compound (Gly-L-Tyr amino acid salt) under the same conditions, the increases in tyrosyl fluorescence with GdnHCl concentration do reflect the conformational changes during denaturation, which causes the exposure of buried tyrosyl residues (*23*). It can be seen from Fig. 6 that most of the tyrosyl residues are exposed at 1.0 M GdnHCl, at which the N-terminal fragments are completely inactivated. In contrast to the CD spectra, the transition curves of refolding monitored by measuring tyrosyl intrinsic fluorescence coincide with the unfolding ones for all the fragments. A reasonable explanation is that the intrinsic fluorescence reflects the conformational changes related to the environment of tyrosyl residues in protein, but not only to α-helix. The results clearly show that even in a shorter fragment (*e.g.* SNR102), the tyrosyl residues are not fully exposed, and some residual structure is retained.

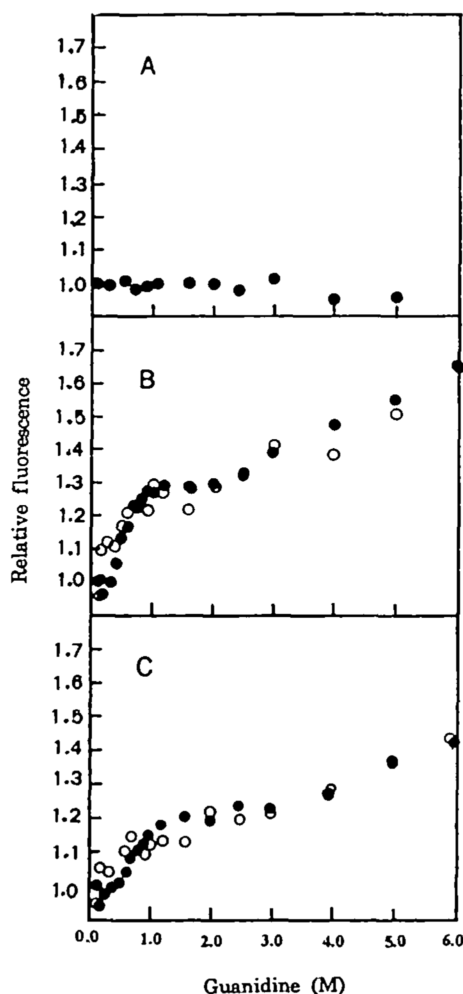

Fig. 6. **Changes in tyrosyl fluorescence of SNase R (W140F) and its N-terminal fragments in GdnHCl solutions of different concentrations.** Because the features of the changes in tyrosyl fluorescence are the same among the fragments, the relative fluorescence of SNR102 was taken as representative of the fragments. Experimental conditions were described in the text. Changes in intrinsic fluorescence at 305 nm for tyrosine were taken to indicate the protein unfolding (●) and refolding (○). The wavelength of excitation was 277 nm, the slit width was 10 nm. (A) The relative fluorescence of a model compound, Gly-L-Tyr amide acetate salt. (B) The relative fluorescence of SNase R (W140F). (C) The relative fluorescence of SNR102.

DISCUSSION

The previous studies in our laboratory on the conformation of the N-terminal fragments of SNase R in solution showed that all five N-terminal fragments examined have certain amounts of residual structure. Although the ordered secondary structure does not always increase with increasing length of the peptide chain, growth of the peptide chain could have important effects on the conformation of the peptide fragment already synthesized, suggesting that the N-terminal part of a nascent peptide could begin to fold during the course of translation, and some structural adjustments should be necessary for the new synthesized polypeptide to attain its final native conformation (*16*). In the present report, studies on the changes in conformation and activity of the peptide fragments during unfolding and refolding in different concentrations of GdnHCl further confirm the above observations. The changes in ellipticity at 222 nm of the N-terminal fragments during unfolding clearly indicate that the conformational stability in GdnHCl of the N-terminal fragments increases with increasing chain length. The refolding studies show that the effective interaction between the amino acid residues which are related to formation of the native conformation in the peptide fragments increases with growth of the peptide chain. The above result corresponds well with the finding that the ordered secondary structures of the fragments increase with increasing length of the peptide chain described previously (*16*).

The CD and FT-IR spectra indicated that SNR121 may have more secondary structure (α-helix, β-sheet) than SNR135 (*16*), but the "apparent" ordered secondary structure may not represent a favorable conformational state for expression of the activity, because the enzyme activity of SNR121 is only about 20% of that of SNR135. From this study, the very interesting phenomenon was found that SNR121 is activated at 0.1 M GdnHCl, but SNR135 is inactivated dramatically under the same condition. Although no significant conformational change was apparent from the CD spectra, the 1,8-ANS fluorescence data indicate that the conformation of SNR121 and its ability to combine with competitive inhibitor do change at

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0.1 M GdnHCl, implying that the activation of SNR121 by 0.1 M GdnHCl could be caused by transforming a more rigid active site to a more flexible one required for full expression of the activity, whereas the active site of SNR135 was more fragile and more greatly perturbed at 0.1 M GdnHCl. So the difference in catalytical behavior between them reflects the difference in their conformational state at 0.1 M GdnHCl. Years ago, Tsou proposed that the folding of a nascent peptide chain begins early during synthesis, but constant adjustments may be necessary during and after completion of peptide synthesis (*1*). The changes of conformation and activity in going from SNR121 to SNR135 and further to SNR141 confirm the necessity of conformational adjustments. As described before, SNR135 has less secondary structure than SNR121 as judged from CD and FTIR spectral analyses, but SNR135 has much higher activity than SNR121. This paradox is resolved by the fact that SNR135 has a greater ability to form a conformation close to the native state of the enzyme in the presence of pdTp and Ca^{2+}, while the presence of pdTp and Ca^{2+} has only a slight effect on the CD spectra of the other N-terminal fragments (*16*). However, the fact that SNR135 loses its activity dramatically during unfolding in a low concentration of GdnHCl indicates that the active site in the fragment is more fragile and prone to be perturbed by GdnHCl, implying that SNR135 has a more relaxed conformation than SNR121. Furthermore, from the unfolding and refolding curves monitored both by CD and by tryptophan fluorescence, it can be seen that further addition of six residues (residues 136-141) at the C-terminal end of SNR135 transforms the more relaxed and fragile conformation into a more stable and integrated conformation which is almost the same as that of the native enzyme, indicating that the elongation of the peptide from residue 136 to residue 141 is very important for finally establishing the conformational integrity and stability of the enzyme. From these results, we suppose that the formation of the enzyme conformation from residue 121 to residue 141 goes through at least three stages during chain elongation, *i.e.* from a more rigid and exposed conformation to a more fragile and relaxed conformation, and further to a more stable and integrated conformation. This supports Tsou's hypothesis that nascent peptide folds during elongation of the peptide chain, and the conformation of that part of the molecule already synthesized should be continuously modified by the newly synthesized segments and undergo constant adjustments (*1*).

Unfolding and refolding of protein in denaturants is a general method to study protein stability and the forces causing spontaneous folding and acquisition of native structure. In this study, we used this approach to explore nascent peptide folding and to examine what happens with the growth of the peptide chain. Of course, nascent peptide folding is a higly complex process in the cell, where it may be directed by chaperones or foldases. Nevertheless, it is clear that all the information required to define the tertiary folding is encoded in the amino acid sequence of a protein, so at the present stage, peptide fragments of different chain lengths starting from the N-terminal end should be a reasonable model for revealing the folding forces. It is worth noting that for the enzyme the residues at 122-135 and 135-141, which are at the C-terminal end, remote from the active site in sequence and spatial structure, play important roles in conformational formation and adjustments, as well as expression of the activity. These findings demonstrate that cooperative interactions between amino acids which are distant in a protein sequence are crucial to the structural stability and integrity of the protein. This is consistent with the viewpoint that the main information in the amino acid sequence is not primarily encoded in the relationship between each amino acid and its next neighbor in the sequence, but rather in the potential relationships of all possible nonlocal pairings (*24*).

We thank Professor C.L. Tsou for his advice and a critical reading of the manuscript.

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