Min Wang
Lu Shan
Jinfeng Wang
National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Beijing 100101, People's Republic of China

Two Peptide Fragments G55–I72 and K97–A109 from Staphylococcal Nuclease Exhibit Different Behaviors in Conformational Preferences for Helix Formation

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Abstract: *Two synthetic peptides, SNaseα1 and SNaseα2, corresponding to residues G55–I72 and K97–A109, respectively, of staphylococcal nuclease (SNase), are adopted for detecting the role of helix α1 (E57–A69) and helix α2 (M98–Q106) in the initiation of folding of SNase. The helix-forming tendencies of the two SNase peptide fragments are investigated using circular dichroism (CD) and two-dimensional (2D) nuclear magnetic resonance (NMR) methods in water and 40% trifluoroethanol (TFE) solutions. The coil–helix conformational transitions of the two peptides in the TFE–H_2O mixture are different from each other. SNaseα1 adopts a low population of localized helical conformation in water, and shows a gradual transition to helical conformation with increasing concentrations of TFE. SNaseα2 is essentially unstructured in water, but undergoes a cooperative transition to a predominantly helical conformation at high TFE concentrations. Using the NMR data obtained in the presence of 40% TFE, an ensemble of α-helical structures has been calculated for both peptides in the absence of tertiary interactions. Analysis of all the experimental data available indicates that formation of ordered α-helical structures in the segments E57–A69 and M98–Q106 of SNase may require nonlocal interactions through transient contact with hydrophobic residues in other parts of the protein to stabilize the helical conformations in the folding. The folding of helix α1 is supposed to be effective in initiating protein folding. The formation of helix α2 depends strongly on the hydrophobic environment created in the protein folding, and is more important in the stabilization of the tertiary conformation of SNase.* © 2006 Wiley Periodicals, Inc. Biopolymers 83: 268–279, 2006

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Keywords: *helix-forming tendency; α-helical conformation; SNase peptide fragments; helix α1 and α2; coil–helix transition*

Correspondence to: Jinfeng Wang; e-mail: jfw@sun5.ibp.ac.cn
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INTRODUCTION

As well known, elucidating early folding events is difficult to detect in intact proteins because of the rapid and highly cooperative nature of the protein folding. One approach to this problem is to study fragments of proteins because they might provide clues about the formation of secondary structure in the absence of tertiary interactions. The conformational preferences of peptide fragments reflect at least to some extent the structural information of the corresponding sequences in the parent protein.[1,2]

Staphylococcal nuclease (SNase) has been extensively used as a model system in protein folding studies.[3] The tertiary structure of SNase consists of two antiparallel β-pleated sheets and three α-helices (Figure 1 in supplementary materials). The β_I-sheet is comprised of strands β1, β2, and β3, and the β_{II}-sheet consists of strands β4, β5, and β6. They form a six-strand "β-barrel" of the protein, which is the main hydrophobic core of the molecule. Studies of the large SNase fragments, such as Δ131Δ (residues 1–3 and 13–140), SNase135 (residues 1–135), and SNase136 (residues 1–136), reveal the partially folded conformations of these fragments.[4–6] Δ131Δ shows partially formed helices α1 and α2 and some reverse turns. The estimated fractional population of helical structure is of the order helix α2 (30%) > helix α1 (10%) > helix α3 (0%). Shortle's group has studied a synthetic 19-residue peptide corresponding to residues 92–110 in sequence of native SNase.[7] This synthetic 19-residue peptide includes a segment of helix α2, a segment of β-turn τ3, and partial strand β6 at its N-terminal, and a segment G107–K110. The nuclear Overhauser effect (NOE) data suggest that the helix-like conformation appears to be stable in this 19-residue peptide. The 1–110-residue SNase fragment consists of the "β-barrel" region and helices α1 and α2, and the corresponding part of the tertiary structure of native SNase is regarded as a β subdomain of the protein. In aqueous solution, SNase110 exists as an ensemble of coexistent native-like partially folded and unfolded states. The G88W- and V66W-mutant 1–110-residue SNase fragments, G88W110 and V66W110, provided a native-like tertiary conformation.[3] The tertiary structures of G88W110 and V66W110 have been calculated (PDB codes: 1RKN and 2F3V), which adopted a native-like β-subdomain conformation. In the structure of both G88W110 and V66W110, the native-like "β-barrel" and α-helical conformations were observed. The formation of a β-barrel structural region is a subsequent event to the local nucleation of the β-turn regions I18–D21 and Y27–Q30 in SNase.[8,9] However, the role of the relevant segments for helix α1 and helix α2 in initiation of folding of G88W110 and V66W110 is unclear.

A key goal of the present study is to elucidate the folding behaviors of helix α1 (E57–A69) and helix α2 (M98–Q106) and the role played by these two helices in the folding of SNase. Two synthetic peptides, SNaseα1 and SNaseα2, spanning the sequences G55-PEASAFTKKMVENAKK-I72 and K97-MVNEAL-VRQGL-A109 of native SNase, respectively, are used to dissect the folding behaviors of helices α1 and α2 of SNase. The conformational states of these two SNase peptide fragments have been studied by circular dichroism (CD) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy in both aqueous and 2,2,2-trifluoroethanol (TFE)-H_2O solutions. The implication of the conformational propensities of the two synthetic peptides in the folding of helices α1 and α2 in native SNase is discussed.

MATERIALS AND METHODS

Peptide Synthesis

Peptides SNaseα1 and SNaseα2 were synthesized by Genemed Biotechnologies, Inc. (San Francisco, CA, USA). Both N- and C-terminals of the two SNase peptide fragments were linked with no protective groups to ensure the solubility of the peptides. The homogeneities of the two synthesized peptides were assessed by analytical high performance liquid chromatography (HPLC) to be 100%, and the compositions were confirmed by mass spectrometry and amino acid analysis.

CD Measurements

Far-ultraviolet (UV) CD spectra of the two peptides were recorded over the wavelength range of 190–260 nm on a Jasco-720 spectropolarimeter at 298 K using a 1-mm pathlength quartz cell. At least four scans were averaged for each measurement. The peptides of concentration of 0.5 mg/mL were dissolved in 50 m*M* NaAc/HAc buffer (pH 5.0), or in a TFE–H_2O mixture at different TFE concentrations of 10, 20, 30, 40, 50, and 60% (v/v). The baseline correction of the CD spectra was performed for the samples in neat and mixture solvents. The quantitative estimations of the secondary structure contents of the two peptides were obtained using the programs in the software package CDPro (http://lamar.colostate.edu/~sreeram/CDPro). The CD data from 195–250 nm of the two peptides were used for the CDPro analysis.

NMR Spectroscopy

NMR samples were prepared by dissolving 2.0 m*M* peptide in 90% H_2O/10% D_2O containing 50 m*M* NaAc/HAc buffer (pH 5.0) for experiments in the aqueous solution and by dissolving 2.0 m*M* peptides in 40% (v/v) trifluoroethanol-*d*4

(TFE-*d*4)–H_2O mixture for experiments in the TFE–H_2O mixtures (pH 5.0).

The 2D ^1H double quantum filtered–correlated spectroscopy (DQF-COSY), total correlated spectroscopy (TOCSY), and rotating frame nuclear Overhauser (ROESY) experiments were performed on a Bruker Avance 600 spectrometer at 283 K and at 298 K for the peptides in H_2O and TFE–H_2O mixtures, respectively. The 2D ^1H-TOCSY data were collected at the mixing times of 50, 80, and 100 ms, and the mixing times for 2D ^1H-ROESY experiments were 150, 250, and 300 ms. The 2D ^1H–^{13}C heteronuclear single quantum correlation (HSQC) and HSQC-TOCSY experiments were carried out with the natural abundance of ^{13}C in the peptides SNaseα1 and SNaseα2 in TFE–D_2O mixtures on a Bruker DMX 600 spectrometer equipped with a triple-resonance cryo-probe. All NMR data were processed and analyzed using FELIX 98 (MSI/Accelrys, Inc.). ^1H chemical shifts were referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) at zero ppm. Assignment of resonance cross-peaks in the 2D ^1H-NMR spectra of each peptide was made using the standard methods.[10] The $^{13}C_\alpha$ resonances were assigned through the 2D ^1H–^{13}C HSQC and HSQC-TOCSY spectra by using the assigned chemical shifts for $^1H_\alpha$ resonances.

Structure Calculation

The solution conformations of SNaseα1 and SNaseα2 in the TFE–H_2O mixtures were calculated on the basis of obtained distance restraints. The NOE distance constraints were extracted from the assigned NOE cross-peaks in the 2D ^1H-ROESY spectra recorded at the mixing time of 250 ms. The intensities of all NOE cross-peaks were classified as strong, medium, and weak corresponding to 2.7, 3.5, and 5.3 Å, respectively, for upper bound of distance restraints. Standard pseudoatom corrections were applied to the nonstereospecifically assigned $^1H_\beta$ and methyl resonances.[11] Structure calculation was carried out using the program CNS1.1. A total of 80 structures were calculated for both peptides, and 15 conformers were used for statistical analysis of the ensemble structures for each peptide.

Ascertain the Monomeric State of Peptides

For ascertainment of the monomeric state of the two peptides in solution, the CD measurements were performed with the samples in aqueous solution at different peptide concentrations range from 0.1 to 3.0 mg/mL, and one-dimensional (1D) ^1H-NMR spectra were taken for the samples containing 0.3–3.0 m*M* synthetic peptides. No significant changes in CD and NMR spectra were observed. The chemical shifts and linewidths of cross-peaks in the NMR spectra of 0.3 m*M* peptide samples showed no differences with those of the 3.0 m*M* peptide samples. These observations can affirm that the peptide is in the monomeric state under the experimental conditions. Thus, the possibility of self-association effect of peptides on the experimental results can be excluded.

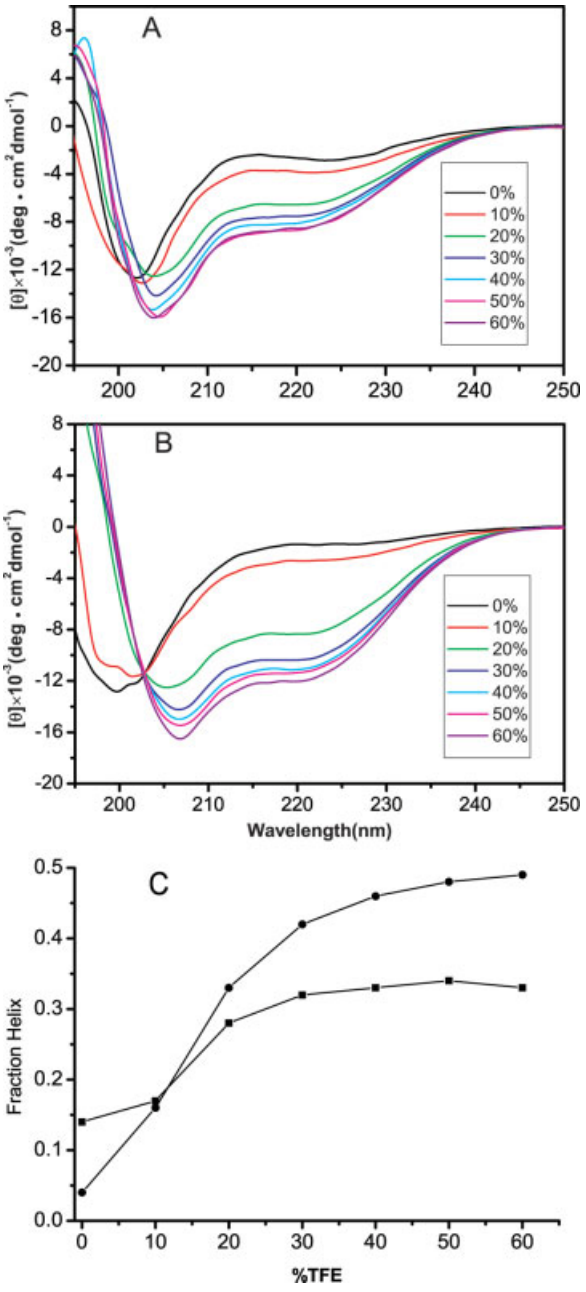

FIGURE 1 Far-UV CD spectra (195–250 nm) of SNaseα1 (A) and SNaseα2 (B) recorded at 298 K in H_2O and in TFE–H_2O mixtures containing different TFE concentrations. (C) The amount of helix induced in SNaseα1 (■) and SNaseα2 (●) as a function of TFE concentration.

RESULTS

Secondary Structures of Two Peptides in H_2O and TFE–H_2O Mixtures

The Far-UV CD measurements were used to provide the information of secondary structures of the two peptides in H_2O and TFE–H_2O mixtures. Figure 1A

and B show the CD spectra of SNaseα1 and SNaseα2 from 195–250 nm, respectively, in aqueous solution and in the TFE–H_2O mixture. The CD spectrum of SNaseα1 in H_2O shows a strong negative absorbance at about 202 nm and a broad, shallow shoulder centered at 220 nm. This indicates that the conformation of SNaseα1 is a mixture of random-coil and helix states. As the TFE concentration increased up to 20% TFE, the peak at 202 nm shifted to about 205 nm and the peak at 220 nm remained essentially unchanged, aside from an explicit increase in ellipticities (Figure 1A). At TFE concentrations above 30%, the strong negative band at 205 nm and a minimum at 220 nm indicate that the helical structure is in dynamic equilibrium with a disordered conformation in SNaseα1. SNaseα2 in H_2O provide CD spectra (Figure 1B) different from those shown by SNaseα1. A pronounced minimum around 200 nm and lack of the ellipticity at about 220 nm suggest that SNaseα2 is typical of a peptide without a dominant folded conformation in H_2O. Increasing the TFE concentration from 10 to 20% increased largely the ellipticity at 220 nm and shifted the negative peak at about 200 to about 205 nm. At the TFE concentrations above 30%, the spectra exhibited strong negative ellipticities at 207 and 222 nm, characterizing the formation of α-helical structure in SNaseα2. An isodichroic point was observed at 204 nm in the TFE titration of SNaseα2, suggesting a TFE-induced cooperative coil to helix transition of this peptide.[12]

The secondary structure content was estimated using CDPro for SNaseα1 and SNaseα2 in the presence and absence of TFE (Table 1 in supplementary materials). The helix content of SNaseα1 and SNaseα2 in the TFE–H_2O mixture is shown in Figure 1C as a function of TFE concentration. SNaseα2 shows a dramatic increase in helical content between 0 and 20% (v/v) TFE, and a significant change in ellipticity at the TFE concentrations above 30% (v/v). However, the increase in helix content of SNaseα1 is less steep, between 0 and 20% TFE, because it already contains about 14% helix by CD at 0% TFE. At TFE concentrations above 30%, the amount of helix structure in SNaseα1 remained essentially unchanged. Comparison of the CD spectra of SNaseα2 with those of SNaseα1 indicates that SNaseα2 can form more ordered helix structure than SNaseα1 in the presence of TFE.

Conformation of SNaseα1 in H_2O

Almost all ^{1}H resonances of SNaseα1 in aqueous solution were assigned (Table 2 in supplementary materials). The strong sequential $d_{\alpha N}(i, i+1)$ NOEs were

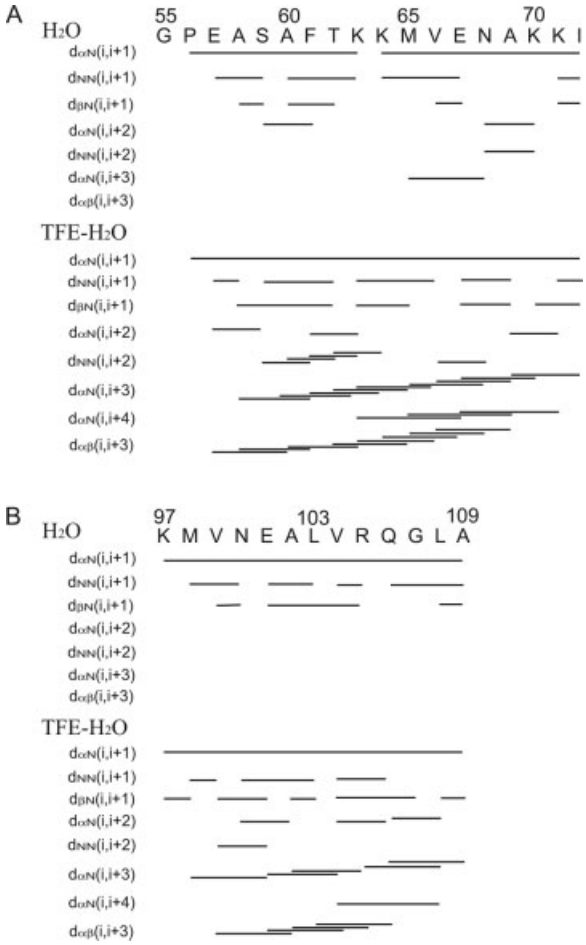

FIGURE 2 Summary of sequential and medium-range NOEs identified for SNaseα1 (A) and SNaseα2 (B) in H_2O and TFE-—H_2O mixtures.

observed for segments P56–K63 and K64–I72, and the relatively weak $d_{NN}(i, i+1)$ NOEs were obtained for residues E57–S59, A60–K63, and K64–E67, and for a pair of residues K71–I72 (Figure 2 in supplementary materials). A number of sequential $d_{\beta N}(i, i+1)$ NOE connectivities were observed for pairs of residues A58–S59, V66–E67, and K71–I72, and for pairs of residues in segment A60–T62 (spectrum not shown). Two medium-range $d_{\alpha N}(i, i+3)$ and $d_{NN}(i, i+2)$ NOE connectivities were identified for pairs of residues M65–N68 and N68–K70, respectively, and two $d_{\alpha N}(i, i+2)$ NOEs were assigned to pairs of residues S59–F61 and N68–K70. The determined NOE connectivities are summarized in Figure 2A. The medium-range $d_{\alpha N}(i, i+2)$, $d_{\alpha N}(i, i+3)$ and $d_{NN}(i, i+2)$ NOE connectivities are diagnostics of turn and helix formation in folded proteins. Thus, the localized helix- and turn-like conformations may transiently populate in SNaseα1. Nevertheless, the sequential $d_{\alpha N}(i, i+1)$, $d_{NN}(i, i+1)$, and $d_{\beta N}(i, i+1)$ NOEs obtained for

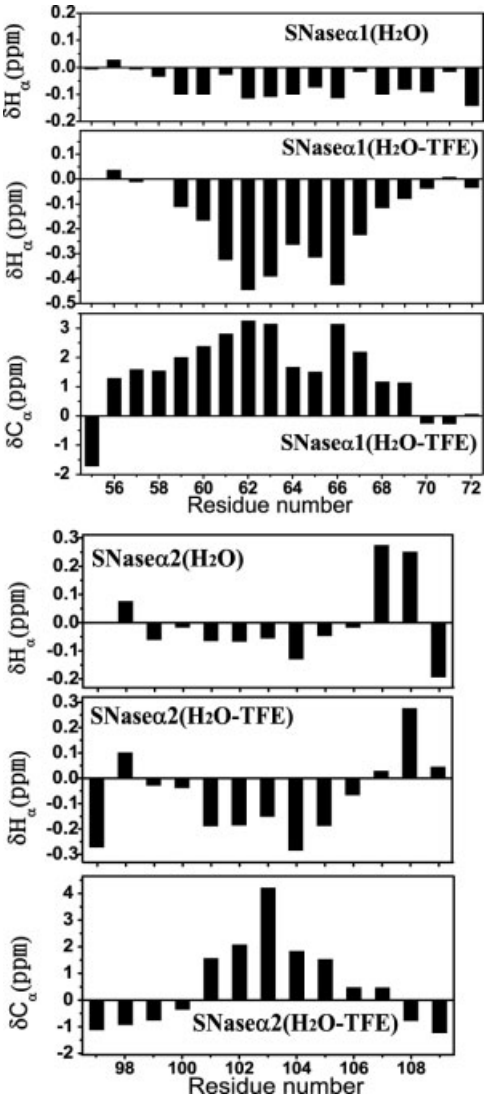

FIGURE 3 Secondary chemical shifts (δH_α and δC_α) for SNaseα1 and SNaseα2 in H_2O and TFE–H_2O mixtures.

most of the residues in the peptide indicate the presence of conformational averaging in a random-coil ensemble.[13]

As well known, $^1H_\alpha$ resonances experience upfield shifts in a α-helix structure. The secondary chemical shifts of $^1H\alpha$ resonances (δH_α) can give an indication of α-helix formation in a peptide.[14,15] Most residues in the sequence region S59–N68 of SNaseα1 in H_2O have negative δH_α values of 0.1 ppm or $>$0.1 ppm (Figure 3), indicating a helix-forming tendency of this region. Therefore, the identified NOE connectivities and secondary chemical shifts (δH_α) indicate that SNaseα1 exhibits a low level of propensity for helix formation, and the peptide is in a transient equilibrium involving a number of interconverting helix- or turn-containing species and unfolded

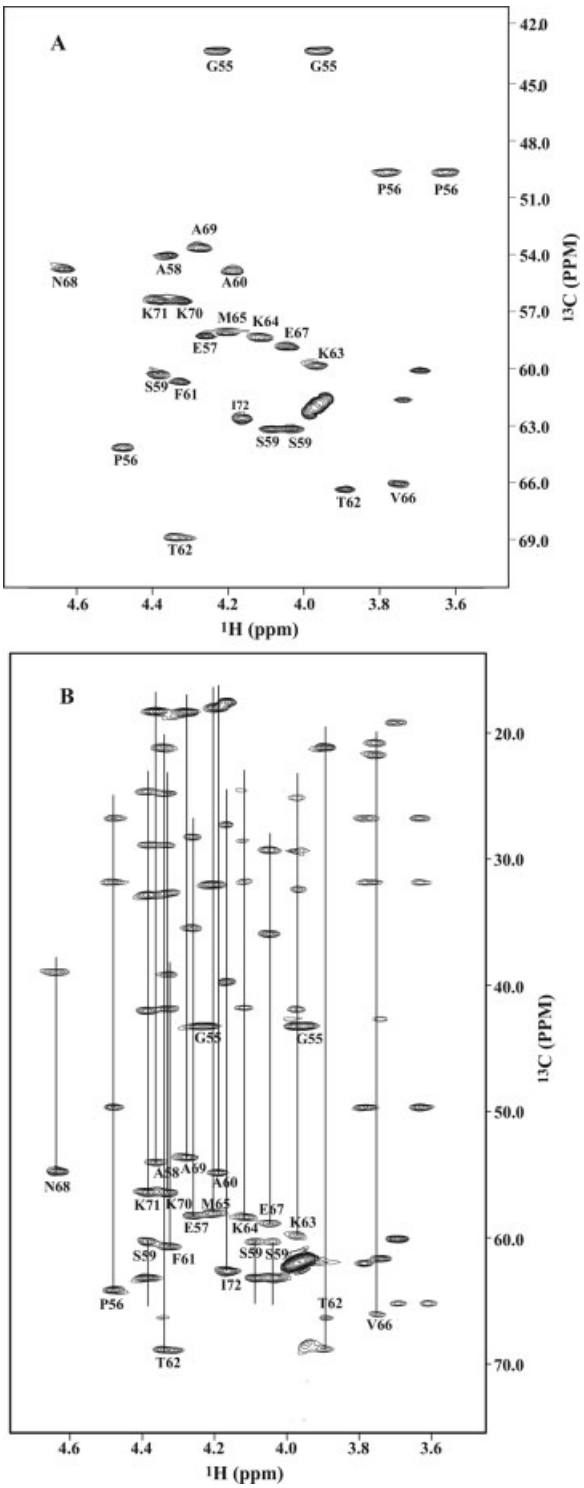

FIGURE 4 2D ^1H–^{13}C HSQC (A) and HSQC-TOCSY (B) spectra for the natural abundance of ^{13}C in the peptide SNaseα1 in TFE–D_2O mixtures. The assigned resonances are indicated by the amino acid code and sequence position of corresponding residues.

states. Therefore, the conformational ensemble of SNaseα1 in H_2O exhibits a coil–helix transition.

Conformation of SNaseα1 in TFE–H_2O Mixtures

Nearly complete ^1H resonances of SNaseα1 in a 40% (v/v) TFE–H_2O mixture were assigned (Table 3 in supplementary materials). The assignments of $^{13}C_\alpha$ resonances were obtained for all residues of SNaseα1 in a TFE–H_2O mixture using 2D ^1H–^{13}C HSQC (Figure 4A) and HSQC-TOCSY spectra (Figure 4B). The identified NOE connectivities for SNaseα1 in the TFE–H_2O mixture are shown in Figure 5 and summarized in Figure 2A. As shown by Figures 2A and 5, the presence of 40% TFE generated a great number of the sequential $d_{\alpha N}(i, i+1)$, $d_{NN}(i, i+1)$, and $d_{\beta N}(i, i+1)$ NOEs between pairs of residues throughout the sequence of SNaseα1. A large number of medium-range NOEs were induced by TFE. Dense $d_{\alpha N}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$ NOE connectivities were identified for pairs of residues in sequence region E57–A69 of the peptide. A series of $d_{NN}(i, i+2)$ and two $d_{\alpha N}(i, i+2)$ NOEs were observed in the sequence region E57–K64 while a series of $d_{\alpha N}(i, i+4)$ NOEs and a $d_{NN}(i, i+2)$ NOE were obtained for the region K63–A69. The medium-range NOE connectivities suggest that the C-terminal half of the peptide can form a α-helical conformation while the N-terminal half of the peptide may sample helix and turn conformations, which have been termed "nascent helix formation."[16]

The secondary chemical shifts of $^1H_\alpha$ and $^{13}C_\alpha$ resonances (δH_α and δC_α) were obtained for SNaseα1 in a 40% (v/v) TFE–H_2O mixture. As was indicated, $^{13}C_\alpha$ resonances of residues located in α-helix usually shift on average of 2.6 ppm down field from the random-coil value.[17] The δC_α values of residues P56–A69 are >1 ppm, and residues in sequence region S59–N68 have negative δH_α values >0.1 ppm (Figure 3). Both δH_α and δC_α predict the formation of a helical conformation in the sequence region S59–N68 of SNaseα1 in the presence of 40% TFE. Therefore, the NOE connectivities and secondary chemical shifts indicate that a coil to helix conformational transition in SNaseα1 is induced by TFE and the peptide has a largely populated α-helical conformation in 40% the TFE–H_2O mixture.

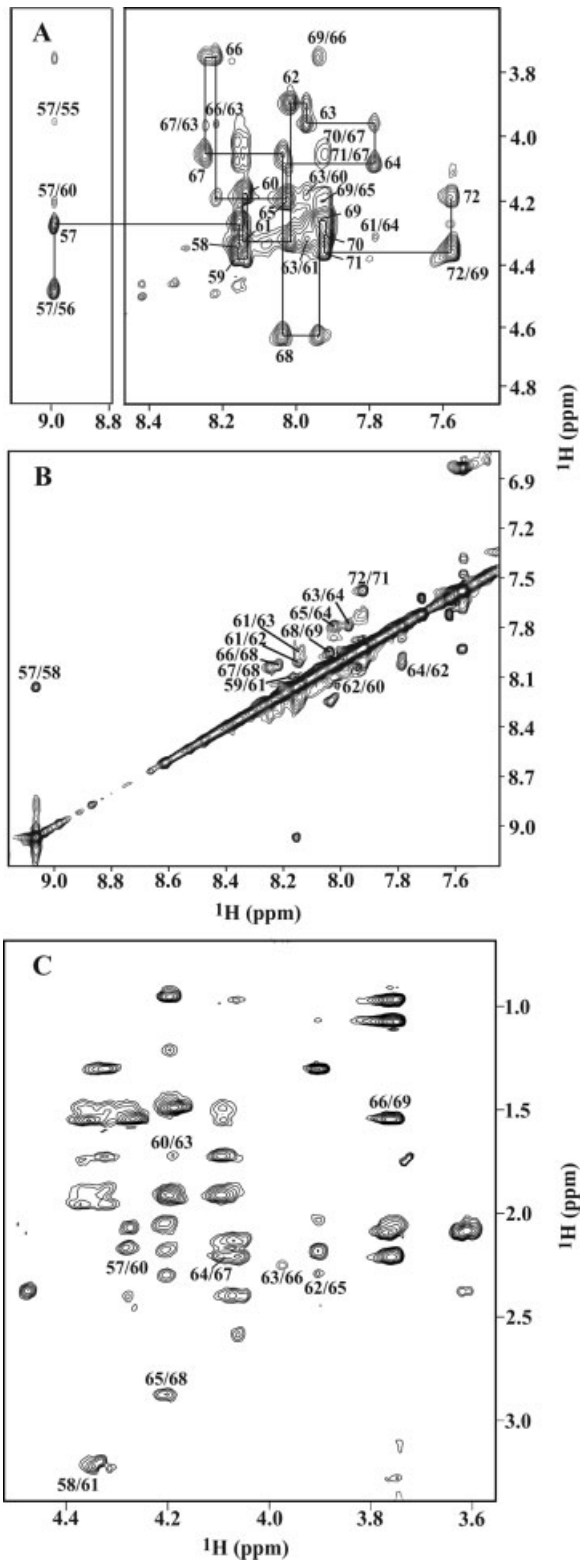

FIGURE 5 2D ^1H ROESY spectrum of SNaseα1 in 40% (v/v) TFE–H_2O mixtures (pH 5.0) at 298 K. The mixing time used for experiment was 250 ms. (A) NOEs in the fingerprint resonance region. (B) NOEs in the amide proton resonance region. (C) NOEs in the aliphatic proton resonance region. The $d_{\alpha N}(i, i+1)$ NOE connectivities are indicated by continuous lines. All the NOEs are indicated by corresponding pairs of residues. The medium range d_{NN}, $d_{\alpha N}$, and $d_{\alpha\beta}$ NOEs are indicated by arrows and labeled by a pair of residues.

Conformation of SNaseα2 in H_2O

The ^1H resonances were assigned for almost all residues of SNaseα2 in H_2O, except the N-terminal residue K97 (Table 4 in supplementary materials). The secondary chemical shifts were obtained for all assigned residues. Residues V99–Q106 and residue A109 show the negative δH_α values in Figure 3. However, only residues V104 and A109 have negative values >0.1 ppm. The features of secondary chemical shifts reveal that SNaseα2 gives no evidence of a tendency to form helical conformation in aqueous buffer. The strong sequential $d_{\alpha N}(i, i+1)$ NOEs for residues K97–A109, and the $d_{NN}(i, i+1)$ connectivities for pairs of residues V99–N100 and V104–R105 and for pairs of residues in segments E101–L103 and Q106–A109 of SNaseα2, were identified (Figure 3 in supplementary materials). The sequential $d_{\beta N}(i, i+1)$ NOEs were identified for pairs of residues V99–N100 and L108–A109, and for pairs of residues in segment E101–A102–L103–V104–R105 (spectrum not shown). No medium-range NOE connectivities were detected. The pattern of NOE connectivities of SNaseα2 in H_2O (Figure 2B) characterizes mainly the random-coil conformation. Therefore, the NOE data, particularly the secondary chemical shifts, indicate that peptide SNaseα2 adopts largely an unstructured conformation in aqueous solution.

Conformation of SNaseα2 in TFE–H_2O Mixtures

The standard 2D ^1H-NMR spectra of SNaseα2 in TFE–H_2O mixtures provided nearly complete ^1H resonance assignments (Table 5 in supplementary materials). The $^{13}C_\alpha$ resonances were assigned for all residues of SNaseα2 in TFE–H_2O mixtures using 2D ^1H–^{13}C HSQC and HSQC-TOCSY spectra (spectra not shown). The secondary chemical shifts δH_α and δC_α (Figure 3) suggest that residues E101–R105 of the peptide can form a α-helix structure in the presence of TFE.

The NOE assignments for SNaseα2 in TFE–H_2O mixtures are indicated in Figure 6. The $d_{\alpha N}(i, i+1)$ NOE connectivities were determined for whole sequence K97–A109, and $d_{NN}(i, i+1)$ NOEs were identified for M98–V99 and G107–L108, and for pairs of residues in segments N100–L103 and V104–Q106. A great number of sequential $d_{\beta N}(i, i+1)$ NOEs were

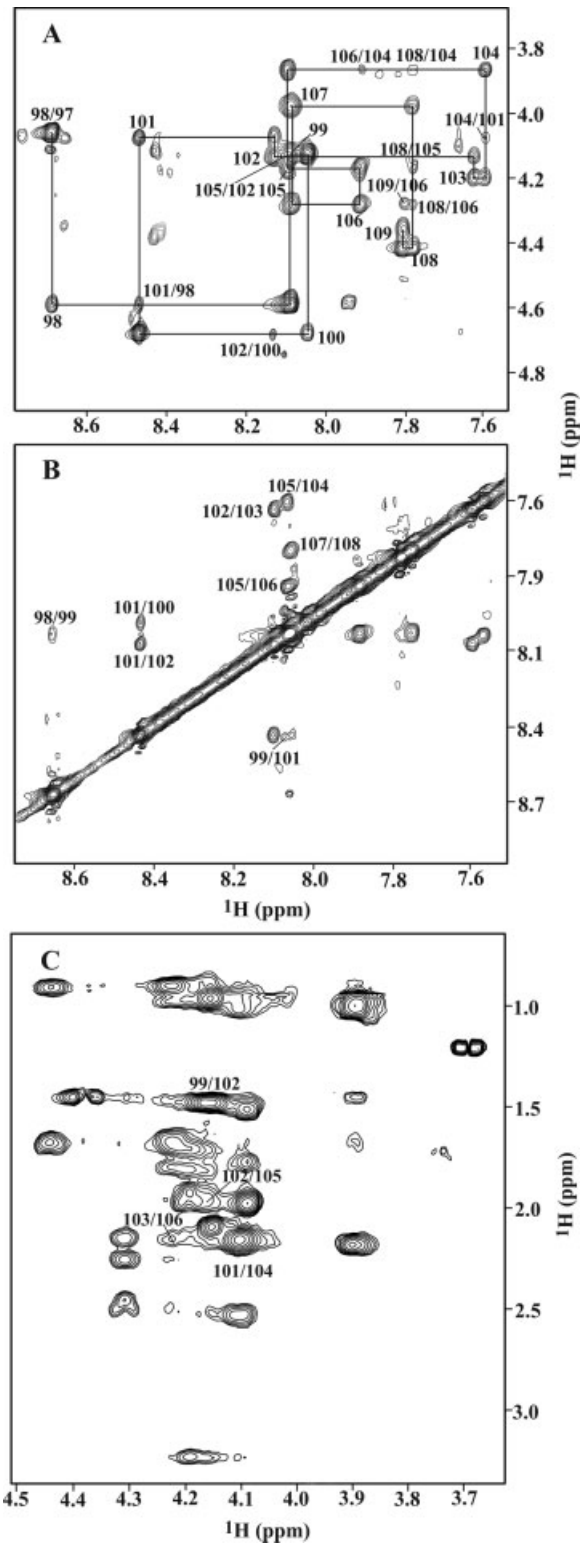

FIGURE 6 2D ^1H-ROESY spectrum of SNaseα2 in 40% (v/v) TFE–H_2O mixtures (pH 5.0) at 298 K. The mixing time used for the experiment was 250 ms. (A) NOEs in the fingerprint region. The sequential $d_{\alpha N}(i, i+1)$ NOE connectivities are indicated by continuous lines. (B) NOEs in the amide proton resonance region. (C) NOEs in the aliphatic proton resonance region. The assigned sequential and medium-range NOEs are labeled.

also obtained (spectra not shown). The presence of TFE in the sample solution caused the generation of medium-range NOEs. The $d_{NN}(i, i+2)$ and $d_{\alpha N}(i, i+4)$ NOEs were assigned to pairs of residues V99–E101 and V104–L108, respectively. A continuous network of medium-range $d_{\alpha N}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$ NOEs was observed between pairs of residues in sequence region M98–A109, as indicated in Figures 2B and 6. The $d_{\alpha N}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$ NOE connectivity patterns indicated the formation of α-helical structure in the segment V99–Q106 of SNaseα2 in TFE solution. Nevertheless, addition of TFE resulted in the appearance of $d_{\alpha N}(i, i+2)$ NOEs for pairs of residues V99–E101, V104–Q106, and Q106–L108, which indicated the formation of turn-like conformations in both N- and C-termini of the helical region V99–Q106 in SNaseα2. Therefore, SNaseα2 exhibits a high propensity towards helix formation, and has a predominantly helical conformation at 40% TFE concentration.

NMR-Derived Tertiary Structures in the Presence of TFE

A family of 80 structures was calculated for both SNaseα1 and SNaseα2. Among 80 structures obtained for each of the SNaseα1 and SNaseα2, 15 structures were converged with relatively low backbone root mean square deviation (RMSD) and low target function energy. Structural statistics for the final ensemble of 15 structures of the two SNase peptide fragments are given in Table I. The stereochemical quality of the backbone coordinates of each structural family was analyzed using the program PROCHECK_NMR.[18] Results of the Ramanchandran map analysis are given in Table I. Best-fit superpositions of the backbone heavy atom coordinates of the 15 structures for SNaseα1 and SNaseα2 by MOLMOL[19] are shown in Figure 7A.

In the calculation of tertiary structures for the two SNase peptide fragments in TFE–H_2O mixtures, only the NOE connectivities identified for residues in the sequences of SNaseα1 and SNaseα2 are used. The calculated structures of these two peptides, spanning residues in the sequences of helices α1 and α2 of native SNase, illustrate two well formed α-helices.

Table I Distance and Dihedral Angle Restraints Used for the Structure Calculations and Structural Statistics for SNaseα1 and SNaseα2 in TFE–H_2O Mixtures

	SNaseα1	SNaseα2
Number of distance restraints		
Total NOEs	221	175
Intraresidue	101	92
Sequential: $(i - j) = 1$	67	49
Medium range: $1 < (i - j) < 5$	53	33
Long range: $(i - j) \geq 5$	0	1
CNS energies (kcal/mol)		
E_{total}	13.62 ± 0.67	18.05 ± 0.51
E_{Bond}	0.69 ± 0.04	0.58 ± 0.11
E_{Angle}	6.93 ± 0.11	16.90 ± 0.80
E_{Improp}	0.41 ± 0.03	1.84 ± 0.24
E_{VDW}	4.18 ± 0.43	8.66 ± 1.34
E_{NOE}	1.40 ± 0.05	7.45 ± 2.11
PROCHECK NMR Ramachandran map analysis		
Most favored regions	72.0%	83.3%
Additional allowed regions	28.0%	16.7%
Generously allowed regions	0%	0%
Disallowed regions	0%	0%
Atomic RMSD (Å)		
Backbone	0.197	0.146
Heavy atoms	0.941	0.975

DISCUSSION

Comparison with Two Helices in the Native State

It is interesting to compare the conformations adopted by the two SNase peptide fragments in the TFE–H_2O mixture with the corresponding α-helices in native SNase. Helix α1 contains six hydrophobic and seven polar residues, and is likely to be an amphipathic helix in native SNase.[20] Residues A58, F61, M65, V66, and A69 of six hydrophobic residues in helix α1 form a hydrophobic face of this amphipathic helix (Figure 7B). The side chains of polar residues E57, K63, K64, and E67 are exposed to the solvent-accessible region in the overall structure. The amphipathic nature of helix α1 determines its location in the tertiary structure that its hydrophobic surface faces to the interior of protein and the polar surface prefers facing the solvent. Therefore, packing of helix α1 onto the β-strands of the β-barrel in native SNase is dominated by the hydrophobic interactions of residues on its hydrophobic face with the residues in the β-barrel region. The side chain of residue M65 makes a hydrophobic contact (distance between methyl protons of two residues is <5 Å) with A94 from the β-turn τ3. Residue V66 has the hydrophobic contacts with L14 and A17 from strand β1, V23 from strand β2, and I92 from strand β6. The interactions associated with the hydrophobic side chains of these residues may stabi-

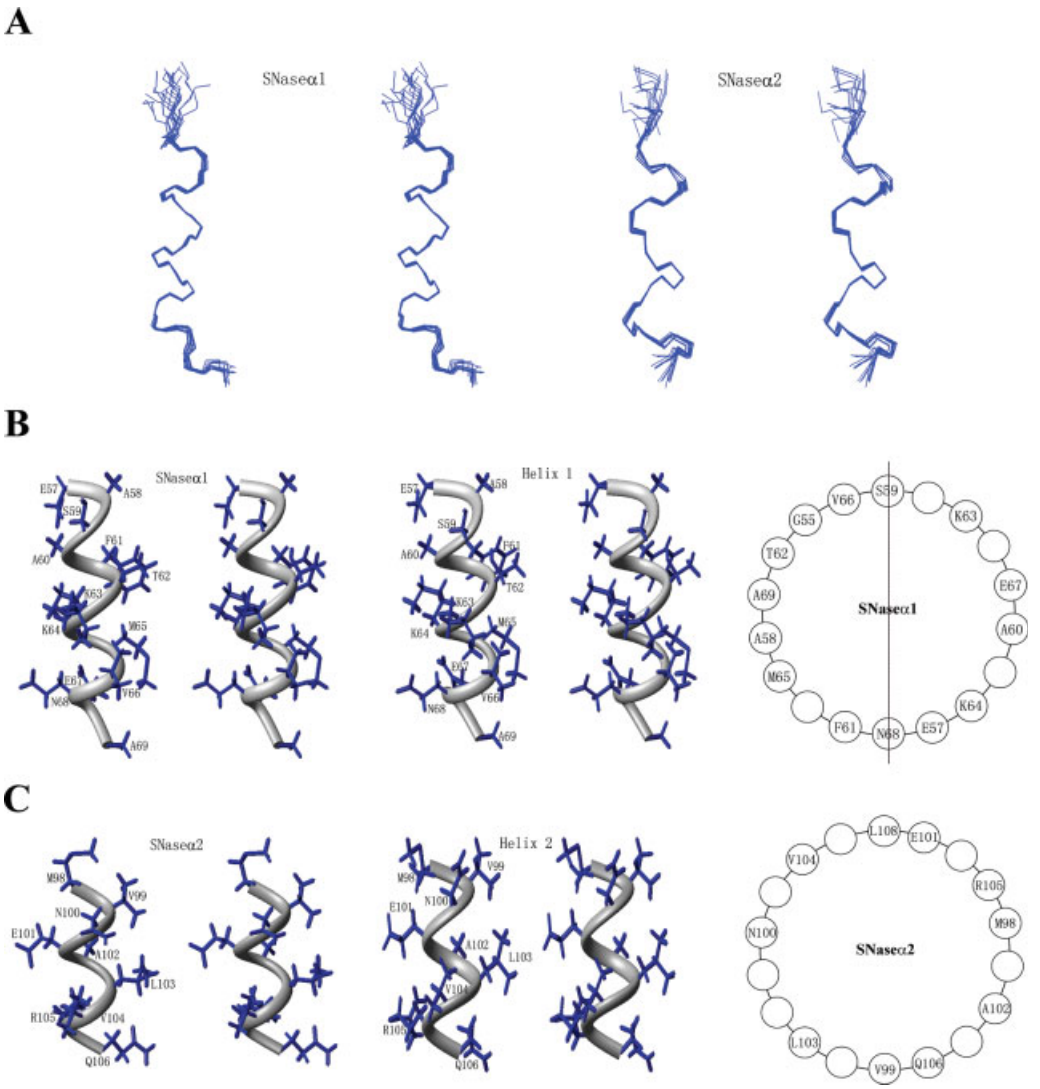

FIGURE 7 Superposition of the 15 conformers of SNaseα1 and SNaseα2 in 40% (v/v) TFE–H_2O mixtures obtained using the program MOLMOL (A). The left panels of (B) and (C) show the stereo view of α-helical conformations for SNaseα1 and SNaseα2. The heavy atoms of the side chains are presented as sticks. The middle panels of (B) and (C) provide the stereo view for corresponding helices α1 and α2 in native SNase. The right panels of (B) and (C) show the helical wheel models of residues E57–A69 and M98–Q106 in helices α1 and α2 of native SNase, respectively.

lize the structure of helix α1 in native SNase. Helix α2 is located in the interior of native SNase. It contains 5 nonpolar (M98, V99, A102, L103, and V104) and 4 polar residues. Almost all of the hydrophobic residues except A102 in helix α2 have hydrophobic contacts with hydrophobic residues in other parts of the native protein. Residues M98 and V99 form a hydrophobic cluster with residues V23 from strand β2, I92 and Y93 from strand β6, A94 from β-turn τ3, and L36 from loop connecting strands β3 and β7. Another hydrophobic cluster is formed by residues L103 and V104 from helix α2 with residues V39 from strands β7, A109 from strands β8, L125 from helix α3, and L36 in the loop connecting strands β3 and β7. The hydrophobic interactions between helix α2 and other parts of native SNase involve M98–Y93, V99–V23, V99–L36, V99–I92, V99–A94, L103–L36, L103–V39, L103–L108, V104–V39, V104–A109, and V104–L125. In addition, the hydrophobic contacts between side chains of residues A58–L103 and V66–V99 of helices α1 and α2 are obtained in native SNase. These tertiary hydrophobic interactions determine the structure of helix α2.

Figure 7B and C show the calculated α-helical structures of SNaseα1 and SNaseα2 and the structures of helices α1 and α2 from native SNase.[20] The determined conformations for the two SNase peptide fragments in 40% TFE are similar to those they have in

the folded protein. In the α-helical conformations of both SNaseα1 and helix α1, most of the hydrophobic residues are on the same side of the helix (Figure 7B). The local hydrophobic contacts between M65–V66 and M65-A69 found along the hydrophobic face of SNaseα1 are similar to those between residues M65–A69 and V66–A69 obtained for helix α1 in native SNase. The possible formation of an intrahelix ion pair between residues K63 and E67 in helix α1 on the hydrophilic side also can be found in SNaseα1. The hydrophobic residues in helix α2 of the native structure are distributed along the helix (Figure 7C). Both helix α2 and SNaseα2 provide the same hydrophobic contacts among residues V99-A102, V99-L103, and A102-L103. In addition, SNaseα2 shows hydrophobic contacts between residues V104–L108 and V104–A109, which are similar to those among L103–L108, L103–A109, and V104–A109 in helix α2 of native SNase. The similarity of the hydrophobic contacts implies that 40% TFE increases the solvent hydrophobicity and creates a hydrophobic environment for SNaseα1 and SNaseα2, which is supposed to be similar to the hydrophobic environment of two helices in native SNase. The above analysis of two α-helices in native SNase has revealed the hydrophobic clusters formed between residues in helix α2 and other parts of native SNase, which suggests that the tertiary hydrophobic interactions in native SNase may play an important role in stabilization of the structure of helix α2 compared to the case of helix α1. Therefore, it can be proposed that the formation of α-helical structure in SNaseα2 may depend more strongly on the "hydrophobic media" TFE[21,22] compared to SNaseα1.

Conformational Preferences in SNaseα1 and SNaseα2

The NMR and CD data show that SNaseα1 can sample localized turn- or helical-like conformations, and adopts an extended conformation in aqueous solution. In contrast, SNaseα2 adopts largely an unstructured conformation in the same buffer solution. Particularly, the secondary chemical shifts of the two peptide fragments reveal the different tendencies towards the helical structure in aqueous solution. SNaseα1 exhibits its low propensity for helix formation in water, but the conformational preference for helical structure cannot be observed for SNaseα2 in aqueous solution. In general, the conformational preference of peptide in H_2O arises from the amino acid sequence of the peptide. Thus, the different conformations of the two SNase peptide fragments in H_2O may be dominated by their intrinsic stability, which is correlated to the local amino acid interactions in the peptide.

Table II Percentage of α-Helical Structure Obtained for the Two SNase Peptide Fragments in H_2O and 40% TFE–H_2O Mixtures by Analysis of CD and NMR Data

	SNaseα1		SNaseα2	
	CD	NMR[a]	CD	NMR[a]
H_2O	14	14	4	5
TFE–H_2O	33	37	46	44

[a] The estimation was based on the average secondary chemical shifts. Only upfield shifts in the region assumed to be helical were considered. The average secondary chemical shifts were divided by 0.35 ppm, which was assigned as an average for 100% helicity.

However, secondary structure of peptide is known to also depend on the solvent environment. TFE is known as a water cosolvent that can facilitate the rearrangement of non-native tertiary contacts in peptide by increasing the solvent hydrophobicity. The TFE–H_2O mixture is most likely to be a "hydrophobic" media interacting with amino acids of the peptide.[23,24] Both CD and NMR data obtained in the presence of TFE indicate that a coil to helix conformational transition occurs in the two SNase peptide fragments. The amount of helix increases in SNaseα1 and SNaseα2 on increasing of TFE concentration. However, the coil–helix transition in SNaseα2 is more likely to be a cooperative process. Upon addition of TFE, the helix content increased dramatically in SNaseα2. At TFE concentrations above 20%, the amount of helix in SNaseα2 is higher than those in SNaseα1 (Figure 1C). These phenomena indicate that the helix content of SNaseα2 is more strongly affected by the solvent environment compared with those of SNaseα1. Thus, the local amino acid interactions are not sufficient to account for the formation of ordered helical conformations in these peptides.

The overall helical content in the absence and presence of 40% TFE was estimated based on the average chemical shift deviation[25] provided by NMR data and the mean ellipticity at 222 nm of CD spectra (Table II). The helix content of SNaseα1 is approximately 3 times as much as those of SNaseα2 in H_2O, because only SNaseα1 exhibits a measurable inherent helix-forming tendency. As is well known, TFE can promote helix formation in peptide sequences that have inherent helix-forming tendency. However, SNaseα2, in turn, has about 1.3-fold of helix content of SNaseα1 in the presence of 40% TFE. Therefore, the helix-forming tendency of the two peptide fragments determined from their response to increasing

concentrations of TFE is relatively high in SNaseα2, but is comparatively low in SNaseα1. The observed different conformational preferences of the two peptides in the absence and presence of TFE suggest that the hydrophobic environment, rather than local amino acid interactions, can largely determine the helical conformations sampled in the two SNase peptide fragments.

Implication in the Folding of Helices α1 and α2 in SNase

Comparison of the helical conformations of SNaseα1 and SNaseα2 in 40% TFE with those of helix α1 and helix α2 in the native state indicates that the interactions between solvent molecules and nearby hydrophobic residues of the two peptides in TFE–H_2O mixtures are similar to the interactions between the hydrophobic residues from two α-helices and other parts of the SNase structure. Thus, it is likely that the conformational features of SNaseα1 and SNaseα2 in the absence and presence of TFE may elucidate the behaviors of helices α1 and α2 in initiation of folding of single-mutant 110-residue SNase fragments G88W110 and V66W110. As is known, the behavior of peptide in aqueous solution is relevant to the early stage of protein folding. Thus, the observed propensity for helix formation of SNaseα1 in H_2O implies that helix α1 may form at the early stage in the folding process of SNase. The early formed helix α1 may be unstable and incomplete, because SNaseα1 adopts only a low population of helical conformations and exhibits as a mixture of unstructured and helix components in H_2O. In the native structure, helix α1, packing onto the β-strands in the β-barrel, connects to the Ω-loop (residues P42–P56) at its N-terminus and to strand β5 of the β-barrel region at the C-terminus. Therefore, the packing of early formed helix α1 to the β-barrel structural region can facilitate the formation of native-like long-range hydrophobic interactions between W66 from helix α1 and residues from the β-barrel in the folding of V66W110. In consequence, the single mutation at sequence position 66 can cause the 110-residue SNase fragment to form a native-like conformation. Nevertheless, the hydrophobic interaction of W66 with residues in the β-barrel region is relatively weak,[3] and is unable to stabilize the folded conformation of the β-barrel. Thus, the unstable β-barrel and partially folded helix α1 in V66W110 are unable to provide the native-like tertiary hydrophobic interactions for the segment of helix α2. Therefore, helix α2 cannot be observed in the structure of V66W110 because the formation of α-helical structure in SNaseα2 depends more strongly on the "hydrophobic media" TFE.

As discussed above, the N-terminal residues M98 and V99 and C-terminal residues such as L103 and V104 of helix α2 have hydrophobic contacts with residues from strand β6, β-turn τ3, and strand β8 in native SNase. The hydrophobic interactions between these residues may largely determine the structure and stability of helix α2 in native SNase. This can be seen in the folding behavior of peptide SNaseα2 and the 19-residue (92–110) peptide from SNase. In the 19-residue peptide, helix α2 remains approximately 30% helical in aqueous solution.[7] It is likely that the local hydrophobic interactions among residues M98–Y93, V99–A94, and V104–A109 in the sequence I92–K110 of native SNase may exist in this 19-residue peptide, stabilizing the intrinsic tendency to helical conformation of the peptide. In this study, the 13-residue peptide spanning over the sequence K97–A109—namely, SNaseα2—was used for detecting the intrinsic tendency towards the helical structure. However, no conformational preference for helical structure can be observed for SNaseα2 in aqueous solution due to absence of the above-indicated interactions. In G88W110, the hydrophobic interactions of W88 with hydrophobic residues located in the vicinal β-strands can stabilize the packing of the β-strands in the β-barrel region.[3] In consequence, the well-formed β-barrel and helix α1 can be obtained for G88W110, which create the native-like hydrophobic environment for the segment of helix α2. This facilitates residues M98, V99, and L103 in the segment of helix α2 to form native-like hydrophobic clusters with residues V23, L36, I92, Y93, and A94 from the relevant structural regions in G88W110. Therefore, the folded helix α2 in G88W110 is the result of the formation of stable β-barrel and helix α1 structural regions in this fragment. As discussed above, helix α2 can form a hydrophobic cluster not only with residues in the β-barrel structural region, but also with residues in other parts of the intact protein, such as the residues from strands β7 and β8 and helix α3. Therefore, the folding of helix α2 is supposed to play an important role in stabilization of the tertiary conformation of SNase. The above-described behaviors of helices α1 and α2 in initiation of folding of single mutant 110-residue SNase fragments G88W110 and V66W110 elucidate the fact that formation of these two α-helices in SNase requires nonlocal interactions through transient contact with hydrophobic residues in other parts of the protein for stabilizing the helical conformation in the folding. The folding behaviors of helices α1 and α2 and their roles in the folding of the protein may be unique for

the folding of SNase. However, the requirement of nonlocal interactions for stabilizing the helical conformation in the folding process is supposed to be common to the folding of other proteins.

CONCLUSIONS

The above analysis indicates that the folding of helix α1 is supposed to be effective in initiating protein folding. However, formation of helix α2 may be more important in stabilization of the tertiary conformation of SNase in the folding process. Folding of helix α2 depends to a large extent on the interactions between the hydrophobic residues from the segment of helix α2 and other parts of native SNase. The formation of ordered α-helical structure in the two helix segments E57–A69 and M98–Q106 of SNase may require nonlocal interactions through transient contact with hydrophobic residues in other parts of the protein to stabilize the helical conformations in the folding.

Coordinates: The atomic coordinates of SNaseα1 and SNaseα2 in 40% TFE have been deposited in the Protein Databank (accession code 2FXY and 2FXZ, respectively).

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REFERENCES

1. Morikis, D.; Roy, M.; Sahu, A.; Troganis, J.; Jennings, P. A.; Tsokos, G. A.; Lambris, J. D. J Biol Chem 2002, 277, 14942–14953.
2. Tsomaia, N.; Pellegrini, M.; Hyde, K.; Gardellaand, T. J.; Mierke, D. F. Biochemistry 2004, 43, 690–699.
3. Feng, Y.; Liu, D.; Wang, J. J Mol Biol 2003, 330, 821–837.
4. Shortle, D.; Abeygunawardana, C. 1993, 1, 121–134.
5. Ermacora, M. R.; Ledman, D. W.; Fox, R. O. Nat Struct Biol 1996, 3, 59–66.
6. Alexandrescu, A. T.; Abeygunawardana, C.; Shortle, D. Biochemistry 1994, 33, 1063–1072.
7. Wang, Y.; Shortle, D. Folding Design 1997, 2, 93–100.
8. Dai, J.; Wang, X.; Feng, Y.; Fan, G.; Wang, J. Biopolymers 2004, 75, 229–241.
9. Wang, X.; Tong, Y. F.; Wang, J. F. Biochem Biophys Res Commun 2005, 329, 495–501.
10. Wüthrich, K. NMR of Proteins and Nucleic Acids; John Wiley & Sons: New York, 1986.
11. Wüthrich, K.; Billeter, M.; Brown, W. J Mol Biol 1995, 169, 949–961.
12. Callihan, D. E.; Logan, T. M. J Mol Biol 1999, 285, 2161–2175.
13. Fiebig, K. M.; Schwalbe, H.; Buck, M.; Smith, L. J.; Dobson, C. M. J Phys Chem 1996, 100, 2661–2666.
14. Wishart, D. S.; Sykes, B. D.; Richards, F. M. J Mol Biol 1991, 222, 311–333.
15. Wishart, D. S.; Sykes, B. D. J Biomol NMR 1994, 4, 171–180.
16. Dyson, H. J.; Rance, M.; Houghten, R. A.; Wright, P. E.; Lerner, R. A. J Mol Biol 1988, 201, 201–217.
17. Wishart, D. S.; Sykes, B. D. Methods Enzymol 1994, 239, 363–392.
18. Laskowski, R. A.; Rullmannn, J. A.; MacArthur, M. W.; Kaptein, R.; Thornton, J. M. J Biomol NMR 1996, 8, 477–486.
19. Koradi, R.; Billeter, M.; Wüthrich, K. J Mol Graph 1996, 14, 51–55.
20. Wang, J.; Truckses, D. M.; Abildgaard, F.; Dzakula, Z.; Zolnai, Z.; Markley, J. L. J Biomol NMR 1997, 10, 143–164.
21. Lu, H.; Buck, M.; Radford, S. E.; Dobson, C. M. J Mol Biol 1997, 265, 112–117.
22. Kentsis, A.; Sosnick, T. R. Biochemistry 1998, 37, 14613–14622.
23. Munoz, V.; Serrano, L. Biopolymers 1997, 41, 495–509.
24. Lacroix, E.; Viguera, A. R.; Serrano, L. J Mol Biol 1997, 284, 173–191.
25. Rizo, J.; Blanco, F. J.; Kobe, B.; Bruch, M. D.; Gierasch, L. M. Biochemistry 1993, 32, 4881–4894.

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