Local Structural Features around the C-Terminal Segment of *Streptomyces* Subtilisin Inhibitor Studied by Carbonyl Carbon Nuclear Magnetic Resonances of Three Phenylalanyl Residues†

Masatsune Kainosho* and Hiromasa Nagao
Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Fukazawa, Setagaya-ku, Tokyo, 158 Japan
Takashi Tsuji
Central Research Laboratories, Ajinomoto Company Inc., Suzuki-cho, Kawasaki, 221 Japan
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ABSTRACT: The carbonyl carbon NMR signals of the Phe residues in *Streptomyces* subtilisin inhibitor (SSI) were selectively observed for [F]SSI, in which all phenylalanines were uniformly labeled with [1-^{13}C]Phe. The three enhanced resonances in the spectrum of [F]SSI were unambiguously assigned to the specific sites in the amino acid sequence by means of ^{15}N,^{13}C double-labeling techniques. Namely, the resonances at 174.9 and 172.6 ppm (in D_2O, pH 7.3, 50 °C) showed the satellite peaks due to ^{13}C–^{15}N spin coupling in the spectra of [F,GS]SSI and [F,A]SSI, in which Ser/Gly and Ala residues were labeled with [^{15}N]Gly/Ser and [^{15}N]Ala, respectively, together with [1-^{13}C]Phe. The carbonyl groups of Phe-97 and Phe-111 are involved in peptide bonds with the amino nitrogens of Ser-98 and Ala-112, respectively. These results clearly indicate that the signals at 174.5 and 172.6 ppm are due to Phe-97 and Phe-111, respectively. The signal at the lowest field (177.1 ppm) was thus assigned to the carboxyl carbon of the C-terminal Phe-113. The lifetimes of the amide hydrogens of the three Phe residues and their C-terminal-side neighbors (Ser-98 and Ala-112) were investigated by using the effect of deuterium–hydrogen exchange of amide on the line shapes (DEALS) for the Phe carbonyl carbon resonances. In this method, the NMR spectra of [F]SSI dissolved in 50% D_2O (pH 7.3) were measured at various temperatures, and the line shape changes caused by deuteriation isotope shifts were analyzed. At 50 °C, the line shape of the Phe-97 carbonyl carbon appeared to be composed of four poorly separated lines, but they coalesced into a rather broad single line above 60 °C. It was thus concluded that the amide hydrogen of Ser-98 is rapidly exchangeable with the solvent hydrogen (or deuterium) at elevated temperatures. The Phe-111 and Phe-113 signals in 50% D_2O appeared as double peaks up to 80 °C, although the separations of the double peaks were quite different from each other and were 5.0 and 1.7 Hz, respectively. The larger separation observed for Phe-111 was caused by the partial deuteriation (half-deuteriation) of Ala-112 amide nitrogen (the β-shift). A small separation observed for Phe-113 was induced by the partial deuteriation of Phe-113 amide nitrogen (the γ-shift). Since these values of separation were essentially unchanged up to 80 °C, we suggest that the local environment around the C-terminus of SSI was hydrophobic and therefore the amide nitrogens of Ala-112 and Phe-113 are inaccessible to the solvent even at elevated temperatures. The results are consistent with the relative solvent accessibilities of these amide nitrogens calculated by using the atomic coordinates obtained by X-ray crystallography. Temperature dependence of the carbonyl chemical shifts indicates that Phe-111 and Phe-113, both of which exist in the hydrophobic interior of SSI, have a considerable degree of motional freedom. The local structure around Phe-97, which exists in the outermost part of the 5-fold β-pleated sheet, seems to be less flexible at lower temperatures but becomes fairly mobile above 60 °C. Comparisons of the chemical shifts between native and thermally denatured [F]SSI were found to be useful to study the structural characteristics and local environments of the C-terminal segment of the native SSI.

Streptomyces subtilisin inhibitor (SSI)[1] is a microbial protein that specifically inhibits alkaline serine proteinases such as subtilisin BPN′ (Murao et al., 1972; Sato & Murao, 1973). The molecular weight of SSI is 23 000 as a dimer, which is composed of two identical subunits having 113 amino acid residues. Information that has been obtained so far concerning the solution conformation is confined to the vicinities of the residues that can easily be identified by conventional methodologies (Hiromi et al., 1985). SSI does have a number of advantages for NMR investigations. First, the tertiary structures, in both the free and complexed states with subtilisin BPN′, have been well characterized by X-ray analyses (Mitsui et al., 1979; Hirono et al., 1984). Second, there have been intensive collaborative studies focused on various aspects of SSI, which have been summarized in a recent monograph (Hiromi et al., 1985). Third, since SSI can be produced with a very high efficiency by microorganisms in the amino acid

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[1] Abbreviations: SSI, *Streptomyces* subtilisin inhibitor; NMR, nuclear magnetic resonance; Me_4Si, tetramethylsilane; [F]SSI, SSI having all phenylalanines labeled with [1-^{13}C]Phe; [F,A]SSI, doubly labeled SSI containing [1-^{13}C]Phe and [^{15}N]Ala; [F,GS]SSI, labeled SSI containing [1-^{13}C]Phe, [^{15}N]Gly, and [^{15}N]Ser; R_a, relative solvent accessibility (%); ppm, parts per million; J_{CN}, spin coupling constant between directly bonded ^{13}C and ^{15}N in a peptide bond; T_2, spin–spin relaxation time; DEALS, effect of deuterium–hydrogen exchange of amide on the line shapes of the carbonyl carbon resonances; β-shift, isotope shift of the ith carbonyl carbon resonance of a peptide caused by deuteriation of the $(i + 1)$th amide nitrogen; γ-shift, isotope shift of the ith carbonyl caused by deuteriation of the ith residue; [des-110–113]SSI or SSI-A, SSI without four residues in the C-terminus; D and N forms, thermally denatured and native SSI, respectively.

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mixture medium (Sato, 1975), various isotope labeling techniques may potentially by fully applicable. We have already explored such an approach as the ^{15}N,^{13}C double isotope labeling method which led us to assign the three methionyl carbonyl carbon NMR resonances unambiguously (Kainosho & Tsuji, 1982). Use of the definitively assigned Met carbonyl carbon signals played a crucial role in the characterization of the structure of the SSI–subtilisin complex having a molecular weight of about 78 000 [Kainosho et al., 1983; Tsuji, 1983; see also pp 322–337 in Hiromi et al. (1985)].

The double-labeling method relies solely on knowledge of the amino acid sequence and can be applied, in principle, for the assignments of carbonyl carbons of any residues in the polypeptide backbone of proteins. Since one can expect a better spectral resolution in ^{13}C NMR spectroscopy than in the ^{1}H NMR counterpart, various approaches stemming from the assigned carbonyl carbon signals are essential for the studies of larger proteins for which most of recently developed sophisticated NMR techniques including two-dimensional Fourier transform spectroscopy (Wagner & Wüthrich, 1982) fail to be applied.

We have been intensively exploring the possibility of using the carbonyl carbon NMR resonances for the determination of the solution conformation of proteins with larger molecular weights. For this purpose, we have studied the carbonyl carbon NMR of SSI using various isotope labeling techniques (Kainosho & Tsuji, 1982; Kainosho et al., 1985a,b). One of the main objects of our studies has been focused on the establishment of the network of structural probes throughout the backbone polypeptide chain of SSI. This can be achieved by unambiguously assigning a large number of the backbone carbonyl resonances of SSI. By establishing such network, one would be able, for instance, to determine the sites of conformational changes induced by complex formation with subtilisin. As suggested by Mitsui and co-workers on the basis of their X-ray studies, such a global conformational change of SSI seems to be essential for SSI to act as a proteinase inhibitor (Hiromi et al., 1985).

Since all of the three Phe residues are localized in the C-terminal segment of the SSI subunit, we could delineate the structural characteristics of the C-terminal region using the Phe carbonyl resonances as conformational probes. In the crystalline state, the C-terminal segment of SSI seems to maintain the hydrophobic core of the subunit through van der Waals contacts among the side chains of numerous hydrophobic residues (Mitsui et al., 1979). The carboxyl group of the C-terminal Phe-113 seems to interact with the guanidinium cation of Arg-29 to stabilize the subunit structure (Mitsui et al., 1979). In fact, [des-110–113]SSI prepared by limited proteolysis using carboxypeptidase A (SSI-A) turned out to be a temporary inhibitor (Sakai et al., 1980). The fact that SSI-A, unlike intact SSI, is hydrolyzable in the presence of an excessive amount of subtilisin strongly suggests the importance of these few C-terminal residues for maintaining the overall structure of SSI.

In this paper, assignments of the carbonyl carbon signals of the three Phe residues and the local structural characteristics around the Phe residues will be described. The results obtained here, together with future experimental data on a subtilisin complex, will undoubtedly contribute to the understanding of the structural role of the C-terminal segment in the inhibitory activity of SSI.

Materials and Methods

Isotopically Labeled Amino Acids. DL-[1-^{13}C]Phe was prepared by the Strecker reaction of phenylacetaldehyde (Tokyo Kasei) by using sodium (^{13}C]cyanide (90% ^{13}C; MSD Isotopes) (Gaundry, 1948). DL-[^{15}N]Ala and [^{15}N]Gly were prepared from 2-bromopropionic acid and bromoacetic acid, respectively, by using potassium [^{15}N]phthalimide (Shokotsusho; 99.6% ^{15}N) (Sheehan & Bolhofer, 1950). Purities of these amino acids were analyzed by ^{1}H NMR spectroscopy.

Preparation of the Isotopically Labeled SSIs. Labeld SSIs were prepared by culturing *Streptomyces albogriseolus* S-3253 (kindly supplied by Prof. Murao of Osaka Prefectural University) in amino acid mixture media containing the respective labeled amino acids in place of normal ones (Kainosho & Tsuji, 1982). In the previously described medium (Kainosho & Tsuji, 1982), L-Phe was replaced with DL-[1-^{13}C]Phe to prepare [F]SSI, and both L-Phe and L-Ala were replaced with DL-[1-^{13}C]Phe and DL-[^{15}N]Ala, respectively, to prepare [F,A]SSI. In the case of [F,GS]SSI, Gly was replaced with [^{15}N]Gly and L-Ser was not used in the medium. To facilitate the de novo conversion of Gly to Ser, the concentration of [^{15}N]Gly was increased to 1400 mg/L, which was 3 times the original composition (Kainosho & Tsuji, 1982). The concentration of labeled DL-Phe (900 mg/L), which was the same as in the original medium with L-Phe, was found to be sufficient to achieve high enrichment of the Phe carbonyl carbons in SSI. About 20 mg/dL of the labeled SSI was routinely obtained from the culture filtrate after the procedures described previously (Kainosho & Tsuji, 1982).

NMR Sample Preparation. A typical procedure for the preparation of the sample for ^{13}C NMR measurements was as follows: 20 mg of the lyophilized powder of the labeled SSI was dissolved in 0.5 mL of 0.05 M deuteriated phosphate buffer (pH 7.3) in a vial and lyophilized. The lyophilizate was redissolved in 0.5 mL of D_2O (E. Merck; 99.75% D), and the pH was adjusted to 7.3 by using a small amount of 0.1 M NaOD (MSD Isotopes; 99% D) or 0.1 M DCl (MSD Isotopes; 99% D), if necessary. All pH values were direct meter readings without any corrections. After being transferred into a 5-mm (o.d.) NMR sample tube (Wilmad Co., 507-PP), the solution was incubated at 60 °C for at least several hours to facilitate the hydrogen–deuterium exchange of slowly exchanging amide hydrogens. Sometimes, a small amount of cloudy material appeared after the incubation period; this was removed by centrifugation to obtain a clear solution. A few microliters of dioxane was added to the solution as the internal reference for ^{13}C NMR spectra. In order to obtain the ^{13}C NMR data in H_2O, 90% H_2O–10% D_2O (served as the lock signal) was used in lieu of 99.7% D_2O.

For the DEALS experiments of [F]SSI, a mixture of 50% H_2O–50% D_2O was used in the above procedures and pH was adjusted by using 1 M sodium hydroxide or hydrochloric acid, which were also prepared to be 50% D. It was critical to accurately adjust the sample solutions for the DEALS experiments to be 50% D, because otherwise the two peaks (due to protiated and deuteriated amide carbonyl carbons) show different peak intensities.

NMR Spectral Measurements. All ^{13}C NMR spectra were obtained at 75.4 MHz by use of a Varian XL-300, under a full proton decoupling condition using an MLEV-16 modulation (Levit et al., 1982). With the MLEV-16 modulation, a decoupling power as low as 600 mW was sufficient to obtain completely decoupled resonances for the dioxane (internal reference) and the carbonyl carbons; it was possible to minimize the heat generation (less than a degree) for a sample contained in a 5-mm tube.

For NMR experiments other than DEALS, free induction decay signals after a 34-deg pulse were sampled for 0.94 s by

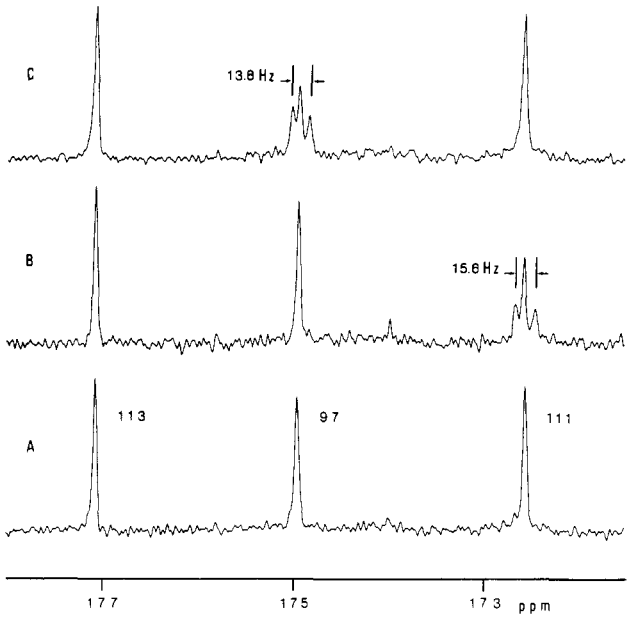

FIGURE 1: Proton-decoupled carbonyl carbon resonances of isotopically labeled SSIs at 75.4 MHz: (A) [F]SSI; (B) [F,A]SSI; (C) [F,GS]SSI. Each labeled SSI (18–20 mg) was dissolved in 0.5 mL of 0.05 M deuteriated phosphate buffer, pH 7.3. A total of 40 000 transients at 50 °C were accumulated and Fourier transformed after being multiplied by a line broadening of 0.5 Hz. The "trios" observed in (B) and (C) represent the superimposed signals due to the ^{13}C–^{15}N and ^{13}C–^{14}N species. J_{CN} values were measured by the separation of the outer two lines in each trio pattern. These spectra allow us to assign the three peaks as shown in (A) (see Table I).

using a 16 000-Hz spectral range (30K data points) and accumulated as indicated in the captions of the figures. For DEALS experiments, a 1000-Hz spectral width and 2-s data acquisition time (4K data points) were used. All data were Fourier transformed after the appropriate digital filtering as the 64K data (supplemented with 34K zero points). The digital resolution of the spectra was 0.5 (usual experiments) and 0.02 Hz (DEALS). The chemical shifts of carbonyl carbon signals were measured from the internal dioxane peak and then converted to the values from tetramethylsilane (Me_4Si) by using a conversion factor of 67.80 ppm.

Results and Discussion

Observation and Assignment of Phe Carbonyl Carbon NMR Signals. As Figure 1A shows, three enhanced resonances are observed in the carbonyl region of the ^{13}C NMR spectrum of [F]SSI, where Phe residues are selectively replaced with [1-^{13}C]Phe. Presumably, these enhanced carbonyl signals originate from the three Phe residues that exist in SSI, i.e., Phe-97,. Phe-111, and Phe-113. Identification of the Phe-113 resonance is straightforward, since this residue is at the C-terminus and therefore has the carboxylate carbon rather than the amide carbon. Carboxyl resonances can easily be differentiated from those of the amide carbons simply by comparing the ^{13}C NMR spectra measurements in H_2O and D_2O; backbone amide carbonyl carbon resonances usually show a high-field shift of about 0.1 ppm in D_2O, due to the secondary isotope shifts caused by the deuteriation of the amide nitrogen (see Table III). The lowest field peak showed a very small isotope shift (less than 2 Hz), whereas the other two signals are shifted to high field by more than 6 Hz. Thus we assign the lowest field peak as Phe-113.

Unambiguous assignments of the carbonyl signals were accomplished by the ^{15}N,^{13}C double-labeling method, which we had used for the assignments of the three methionyl carbonyl carbon signals of SSI (Kainosho & Tsuji, 1982). As

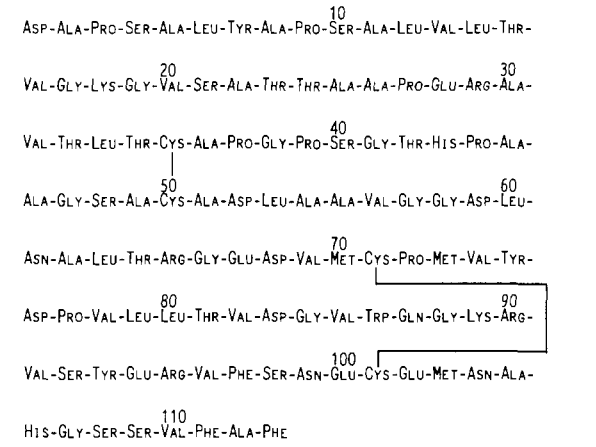

FIGURE 2: Amino acid sequence of the subunit of SSI (Ikenaka et al., 1974).

can be seen in the amino acid sequence of SSI (Figure 2), the residues at the C-terminal side of Phe-97 and Phe-111 are Ser-98 and Ala-112, respectively (Ikenaka et al., 1974). For the assignment of the Phe-111 signal, we prepared [F,A]SSI, in which all of the 18 Ala residues and all of the 3 Phe residues per subunit are uniformly labeled with [^{15}N]Ala and [1-^{13}C]Phe, respectively. The ^{13}C NMR spectrum of [F,A]SSI should show the effect of ^{15}N spins upon the [^{13}C]Phe resonances only for Phe-111, which forms a Phe–Ala linkage. The ^{15}N enrichment of the Ala residues in [F,A]SSI was determined by an integrated peak intensity ratio to be about 45%. The low isotopic content, compared with a high ^{15}N initial enrichment of 99.6%, is due to the transamination reaction that took place during the microbial fermentation (Kainosho & Tsuji, 1982). Figure 1B clearly shows that the highest field resonance is due to Phe-111.

The remaining unassigned signal can automatically be assigned to Phe-97. This was further confirmed by a double-labeling experiment using [1-^{13}C]Phe and [^{15}N]Gly. This double-labeling experiment gave [F,GS]SSI, in which both Gly and Ser residues in SSI were partially labeled with ^{15}N. The de *novo* conversion of Gly to Ser was caused by the action of serine hydroxymethyltransferase and will be discussed in detail elsewhere. A comparison of the spectra of [F]SSI (Figure 1A) and [F,GS]SSI (Figure 1C) clearly indicates that the middle peak originates from Phe-97. This result also shows that the enrichment of [^{15}N]Ser was about 53%. It was thus possible to unambiguously assign all the Phe carbonyl signals.

The ^{15}N–^{13}C spin coupling constant between Phe-111 carbonyl carbon and Ala-112 amide nitrogen was 15.6 ± 0.1 Hz at 50 °C and was nearly temperature independent up to 70 °C (Table I). This value is approximately the same as those observed for Met-70 (14.7 ± 0.4 Hz with Cys-71) and Met-73 (15.4 ± 0.4 Hz with Val-74) and is larger than that for Met-103 (less than 14 Hz with Asn-104) (Kainosho & Tsuji, 1982). Since Met-70 and Met-73 are in an irregular structural region that forms the active site of SSI, a ^{13}C–^{15}N coupling constant of around 15–16 Hz seems to represent a typical value for the coplanar peptide bond without hydrogen bonds. By contrast, Met-103, which is in an α-helix region, gives a smaller J value. Although the precise value could not be determined due to a rather poor ^{15}N enrichment for Asn (about 24%), it appears that the smaller J value is related to the secondary structure.

The ^{15}N–^{13}C coupling constant between the Phe-97 carbonyl carbon and the Ser-98 nitrogen is unusually small (13.8 ± 0.1 Hz) and changes very little at an elevated temperature (Table I). It is of great interest that the coupling constant increases

Table I: Comparisons between NMR Parameters of [F]SSI, [F,A]SSI, and [F,GS]SSI Dissolved in 0.05 M Deuteriated Phosphate Buffer, pH 7.3[a]

		chemical shifts (ppm)				
	temp (°C)	Phe-113	Phe-97 [J_{CN} (Hz)]		Phe-111 [J_{CN} (Hz)]	
[F,A]SSI	50	177.07	174.95		172.57	172.55 (15.6)
	70	177.01	174.90		172.48	172.46 (15.7)
[F,GS]SSI	50	177.06	174.94	174.92 (13.8)	172.56	
	70	177.01	174.90	174.88 (13.6)	172.47	
[F]SSI	50	177.05	174.93		172.55	
	70	177.01	174.90		172.48	

[a] Concentrations of the samples were about 20 mg/0.5 mL. Chemical shifts together with ^{13}C–^{15}N coupling constants represent the carbonyl carbons bonded with ^{15}N.

on thermal denaturation of SSI; the value observed at 95 °C was 15.8 ± 0.1 Hz (Table III), which is typical for the coplanar peptide bond (see above). Formation of hydrogen bonding on the peptide carbonyl carbon has been shown to *increase* the ^{15}N–^{13}C coupling constant (Walter & Wright, 1979). Therefore, the most plausible explanation at this moment for the unusually small coupling constant is that it is a result of the nonplanarity of peptide bonds in β-sheets and α-helices (Schulman & Venanzi, 1976). We are currently trying to confirm this hypothesis in more detail using many other residues in SSI.

The isotope shifts observed for the carbonyl carbon on substituting ^{14}N with ^{15}N were always small high-field shifts. Both of the values for Phe-97 and Phe-111 were 0.02 ppm and were independent of temperature (Table I). The isotope shifts observed for Phe-97 in the native and thermally denatured states were also the same (Table III). Therefore, the isotope shift, unlike the spin coupling constant, may not be used as a criterion for the nonplanarity of the peptide bond.

Solvent Accessibilities of the Backbone Polypeptide Chain and Deuterium–Hydrogen Exchange Rates of Amide Hydrogens. The amide hydrogens of the backbone polypeptide chain of a protein in aqueous solution can exchange with hydrogen atoms of the surrounding water. The exchange rates are determined by many factors including hydrogen ion concentration, temperature, local structural dynamics, and solvent accessibility of each of the amide hydrogens. In the case of small globular proteins, the exchange rate of each of the amide hydrogens can be studied by following the rate of decrease in intensity of the particular amide proton resonances of a protein dissolved in D_2O (Woodward & Hilton, 1980; Wagner & Wüthrich, 1982; Roder et al., 1985). Although this kind of approach has been found to be extremely useful for small proteins, it obviously has limitations in the study of larger proteins such as SSI, where most of the amide proton resonances are extensively overlapped with each other and cannot be unambiguously assigned (Akasaka et al., 1985).

In this paper we propose an alternative and completely different approach to the investigation of the amide proton exchange rates of proteins. This method utilizes the effect of deuterium–hydrogen exchange of amides on the line shapes, which will be denoted by the acronym "DEALS" hereafter, of the carbonyl carbon resonances of a protein dissolved in 50% D_2O (Kainosho & Tsuji, 1982; Kainosho et al., 1985a,b). The method is essentially an extension of the method developed by Feeney et al. (1974) and Hawkes et al. (1978), used for studying the hydrogen–deuterium exchange rates of small peptides. Since one could dramatically reduce by selective ^{13}C enrichment the complexity of the carbonyl carbon region of the ^{13}C NMR spectrum, proteins with much larger sizes can be studied by the DEALS method as if they were small peptides. It should be pointed out that the deuterium-induced secondary isotope shifts observed for the amide carbonyl carbon of the *i*th residue are caused by two different factors. One is the deuteriation of the amide nitrogen of the $(i + 1)$th residue: this value (designated as the β-shift) is usually 4–7 Hz. The other is the deuteriation of the amide nitrogen of the *i*th residue (the γ-shift), which is usually smaller than 2 Hz (Feeney et al., 1974; Newmark & Hill, 1976; Hawkes et al., 1978). Although the DEALS experiments can be done by using both isotope shifts, the γ-shifts may not always visible because of their smaller values (Hawkes et al., 1978; Yashiro et al., 1986).

In the case of *moderatly slow* and *fast* exchange rates, the line shapes observed by the DEALS experiments can be related to the ratios $K = \tau(\nu_D - \nu_H)$ (Jardetzky & Roberts, 1981). Here, ν_D and ν_H denote the chemical shifts (Hz) of the amide carbonyl carbons with a deuteron and a proton, respectively. By use of a typical β-shift of 5 Hz (at 75 MHz) for $\nu_D - \nu_H$ and K values within the range of 0.2–2, the lifetime τ giving rise to the rate-dependent signal shapes for the amide carbonyl signals can be estimated to be in a range of about 40–400 ms. At the coalescence point, where K is 0.45, τ should be around 90 ms. On the other hand, in the cases of the *slow-* or *fast-exchange limit*, the lifetime should be longer than 4 s or shorter than 4 ms. In these limiting cases, the observed line widths, whether they are double lines or a single line, should be the same in 50% D_2O and 100% D_2O. A similar rationale can be applied for the DEALS experiments using the γ-shift values. The time resolution attained by the DEALS method is not retarded by the time required for the spectral measurements, since the method utilizes steady-state spectra.

Some of the spectra obtained for the DEALS experiment of [F]SSI at pH 7.3 are shown in Figure 3. All of these spectra were resolution enhanced by using the parameters given in the caption of Figure 3. The lowest field peak, Phe-113, appeared as a double-peak pattern with a 1.7-Hz splitting throughout the temperature range. Since Phe-113 is the C-terminal residue, the observed small isotope shift should be attributed to the γ-shift. Two clearly resolved peaks for Phe-113 were also observed at 80 °C, indicating that the amide hydrogen exchange rate for Phe-113 was slower than a few seconds even at this temperature. The Phe-97 signal showed a temperature-dependent line shape in 50% D_2O. At 50 °C, it appeared to be composed of four poorly resolved lines, indicating that both β- and γ-shifts contributed to the complex line shape. At 60 °C, however, the line shape of Phe-97 turned into a slightly broadened single peak (compare these results with those observed at the same temperature in D_2O; Figure 4). These results indicate that the hydrogen exchange rates became rapid at least for the Ser-98 amide at elevated temperatures. On the other hand, the carbonyl signal due to Phe-111 stayed as a double-line pattern with a splitting of 5 Hz at all the temperatures. This separation is undoubtedly due to the 50% deuteriation of the Ala-112 amide protons. This result shows that the lifetime of the Ala-112 amide hy-

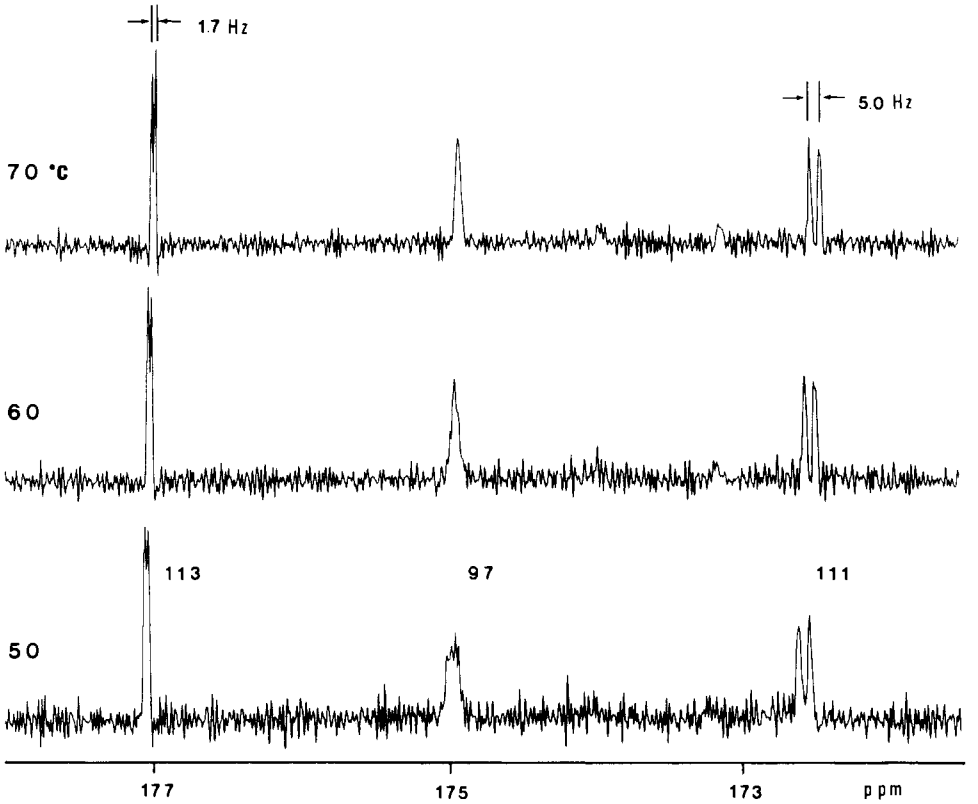

FIGURE 3: DEALS experiment on [F]SSI at various temperatures. [F]SSI (20 mg) was dissolved in 0.5 mL of 50% D_2O, pH 7.3. A total of 20480 transients were accumulated for each temperature. The spectra shown in this figure were processed by using RE = 0.12 s (the inverse exponential filter) and AF = 0.3 s (the Gaussian apodization function). Without digital resolution enhancement filtering, the γ-shift was not clearly observed. The spectrum observed at 70 °C remained almost unchanged at 80 °C (see the text).

Table II: Temperature Variations of Chemical Shifts and Line Widths of Three Phe Carbonyl Signals Observed for [F]SSI Dissolved in 0.05 M Deuteriated Phosphate Buffer, pH 7.3[a]

	chemical shifts (ppm) [line widths (Hz)] at temp (°C)						
	RT[b]	30	40	50	60	70	80
Phe-113	117.15 (10)	177.13 (6)	177.08 (5.6)	177.05 (3.1)	177.04 (3.0)	177.01 (2.6)	176.97 (2.3)
Phe-97	174.93 (20)	174.95 (10)	174.93 (7.9)	174.93 (4.6)	174.93 (3.5)	174.90 (3.1)	174.86 (2.6)
Phe-111	172.69 (20)	172.66 (9)	172.60 (7.0)	172.55 (4.8)	172.52 (3.6)	172.48 (3.3)	172.42 (2.8)

[a] Concentration was 23 mg/0.5 mL. [b] 17–20 °C.

drogen is longer than 200 ms even at 70 °C, pH 7.3. Apparent failure to observe the γ-shift for Phe-111 does not necessarily mean that the lifetime of the Ala-112 amide hydrogen is too short in the temperature range. More probably, since the line widths for Phe-97 and Phe-111 are considerably larger in 50% D_2O than in 100% D_2O, the isotope shift (γ-shift) is too small to be observed (Hawkes et al., 1978; Yashiro et al., 1986).

A marked difference among the amide hydrogen lifetimes of Ser-98, Ala-112, and Phe-113 may be interpreted as originating from the difference of the accessibilitites of the amide hydrogens to the surrounding medium. Although Ser-98 exists in the β-5 strand, its amide hydrogen faces to the outside and does not seem to be involved in the hydrogen-bonding network (Mitsui et al., 1979). The relative accessibility R_a (Lee & Richards, 1971), calculated by Mitsui and co-workers [pp 433–442 in Hiromi et al. (1985)] for the Ser-98 amide nitrogen, was 50%. On the other hand, both of the R_a values for the amide nitrogens of Ala-112 and Phe-113 were estimated to be 0%. We suggest that this is why the amide hydrogen exchange is very slow for Ala-112 and Phe-113 in spite of the fact that the C-terminal segment forms an irregular polypeptide chain and thus the amide hydrogens are free of hydrogen bondings. The results obtained by the DEALS experiments indicate that the C-terminal region is also hydrophobic in solution and the Ala-112 and Phe-113 amides have very little solvent accessibility even at elevated temperatures.

Temperature Dependence of Chemical Shifts of the Three Phe Carbonyl Carbons and Local Structural Flexibilities around the C-Terminal Segment. The Phe carbonyl resonances of [F]SSI dissolved in D_2O, pH 7.3, were measured at various temperatures (Figure 4). The chemical shift of Phe-97, which resides in the fringe of the 5-fold β-pleated sheet ("β-5 strand"; Mitsui et al., 1979), showed almost no temperature dependence up to 50 °C. However, a high-field shift of 0.07 ppm was observed on increasing the temperature from 60 to 80 °C. This value is very close to those observed for Phe-113 (0.07 ppm) and Phe-111 (0.10 ppm) for the same temperature range (Table II). This suggests that the local structure around the β-5 strand is rigid at temperatures below 50 °C but it becomes flexible above 50 °C. Such a local structural fluctuation of the β-5 strand is possible without changing the overall structure of SSI drastically, since this strand forms the outermost one of the 5-fold β-pleated sheets.

The carbonyl signals of Phe-111 and Phe-113, respectively, showed high-field shifts of 0.23 and 0.15 ppm between 30 and 80 °C. These resonances, unlike that of Phe-97, showed gradual changes of chemical shifts even at lower temperatures. This means that conformational changes, which are responsible for the temperature-induced chemical shifts, must have oc-

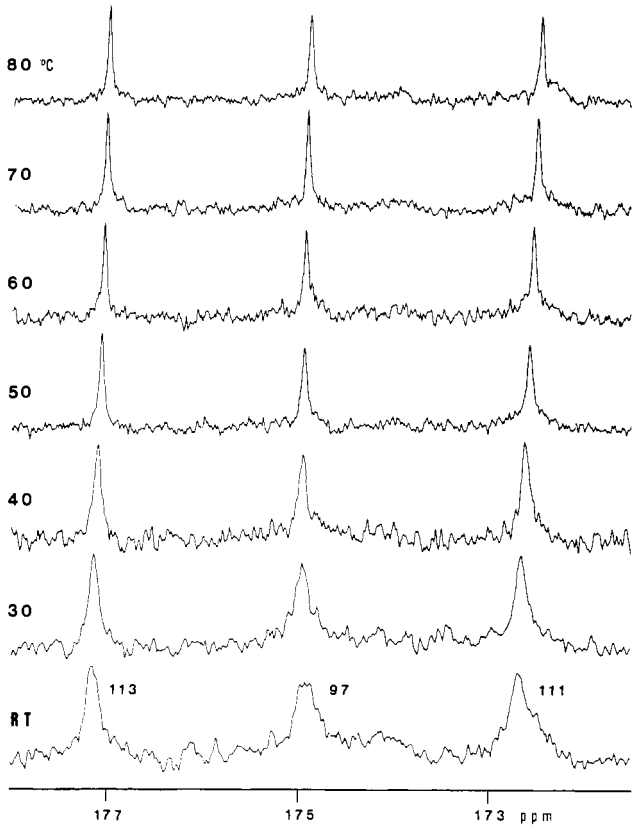

FIGURE 4: Temperature dependency of the carbonyl carbon NMR spectra for [F]SSI at pH 7.3. The sample (23 mg) was dissolved in 0.05 M deuteriated phosphate buffer, and about 2000–5000 transients were accumulated for each run. All spectra were processed by using 0.5-Hz line broadening. Line widths were measured by using the command "DRES" for each signal, and the line-broadening factor (0.5 Hz) was subtracted from the values obtained by the command (see Table II).

curred even at lower temperatures. As we have discussed above, the amide hydrogens of Ala-112 and Phe-113 are separated from contact with the surrounding medium. Therefore, we conclude that the local environment around Phe-111 and Phe-113 is not only hydrophobic but also fairly mobile. The hydrophobic environment around Phe-111 was further confirmed by a 0.65 ppm low-field shift of the Phe-111 carbonyl resonance on thermal denaturation (see below).

Thermal Denaturation of [F]SSI. The variation of the Phe carbonyl carbon resonances of [F]SSI at extremely high temperatures, shown in Figure 5, indicates that the thermal denaturation of SSI takes place primarily in a single step as far as the C-terminal segment is concerned. At 85 °C, there appeared a small amount (ca. 10%) of the denatured form (D), and the ratio between native (N) and denatured forms at 90 °C, N/D, was 0.80. This equilibrium constant corresponds to a free energy difference of 0.67 kJ/mol at 90 °C between the two states. At 95 °C, only a small amount of the N form (less than 10%) survived. The transition temperature for thermal denaturation of SSI obtained by this particular experiment was about 91 °C. The coexistence of the N and D forms for each of the three Phe carbonyl resonances indicates that the exchange rate between the two states was slow enough to give two sets of discrete signals. Saturation-transfer experiments (Fox et al., 1986) involving any of the Phe resonance sets could be used to obtain in more detail information concerning the denaturation processes. It should be noted that the transition temperature for thermal denaturation of [F]SSI dissolved in D_2O is about 10 °C higher than those obtained either by thermal analyses (Takahashi & Sturtvant, 1981) or

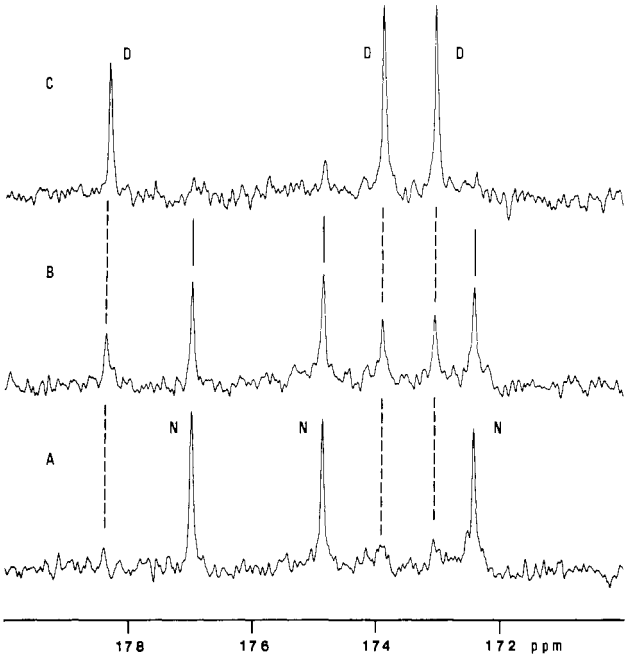

FIGURE 5: Thermal denaturation of [F]SSI dissolved in 0.05 M deuteriated phosphate buffer, pH 7.3. The same sample used for the temperature dependency (Figure 4) was used. The temperature was (A) 85, (B) 90, or (C) 95 °C. A total of 2048 transients were accumulated for each temperature, and 1-Hz line broadening was used. D and N denote thermally denatured and native [F]SSI, respectively.

by variable-temperature UV measurements (Nakanishi & Tsuboi, 1976). This is probably due to the high concentration of SSI used for the ^{13}C NMR experiments (23 mg/0.5 mL). There have been discussions on the concentration dependence of the thermal denaturation temperature of SSI (Hiromi et al., 1985). Use of D_2O for NMR measurements was also responsible for a high denaturation temperature (Nakanishi & Tsuboi, 1976). The results obtained by ^{1}H NMR spectroscopy using a concentrated SSI solution (18 mg/0.5 mL) gave the transition temperature, which is similar to that obtained in the present work (Akasaka, 1984).

Unambiguous assignments of the signals due to the thermally denatured SSI were carried out by using [F,GS]SSI and also by using isotope shifts observed in D_2O and H_2O. In Figure 6, the carbonyl carbon regions of [F]SSI in H_2O (A) and D_2O (B) are compared at the temperatures where the D form of SSI dominates. Since the transition temperature for the thermal denaturation of SSI in H_2O is considerably lower than that in D_2O, the NMR spectra of SSI in D_2O and H_2O were measured at different temperatures, in order to maintain similar N/D ratios: i.e., 95 °C in D_2O (B) and 90 °C in H_2O (A). The transition temperature difference of about 5 °C observed in the present study is consistent with the result of Nakanishi and Tsuboi (1976). Since chemical shifts showed only a little temperature dependence for a temperature difference of 5 °C, we compared the chemical shifts of the denatured SSI in D_2O and H_2O at 90 °C (Table III). Among six resonances observed from both the D and N forms, only a newly appeared lowest field signal due to the D form and the Phe-113 signal of the N form do not show any large isotope shifts. Therefore, we assign the lowest field signal to Phe-113 in the D form. The other four signals from Phe-97 and Phe-111 showed deuteriation shifts of 0.04–0.07 ppm (Table III). Further assignments for the D form were made by using a double-labeled SSI.

By comparing the spectra of denatured [F,GS]SSI (Figure 6C) and [F]SSI (Figure 6B) at 95 °C in D_2O, we were able

Table III: Comparisons between NMR Spectral Parameters of Native (N) and Thermally Denatured (D) [F]SSI and [F,GS]SSI[a]

		Phe-113		Phe-97		Phe-111	
	temp (°C)	N	D	N	D	N	D
[F]SSI (D_2O)	85	176.98		174.86		172.42	
	90	176.97	178.34	174.84	173.89	172.40	173.05
	95		178.31		173.87		173.03
[F]SSI (H_2O)	80	177.00	178.41	174.94	173.96	172.50	173.13
	90	176.98	178.34	174.90	173.93	172.47	173.10
[F,GS]SSI (D_2O)	95		178.31		173.89 (178.87; 15.8)[b]		173.03

[a] As the denaturation temperatures in D_2O and H_2O (10% D_2O) are different, chemical shifts (ppm) should be compared at a constant temperature. [b] Values in parentheses are the chemical shifts of carbonyl carbons bonded to ^{15}N and the spin coupling constants (Hz) between ^{15}N and ^{13}C.

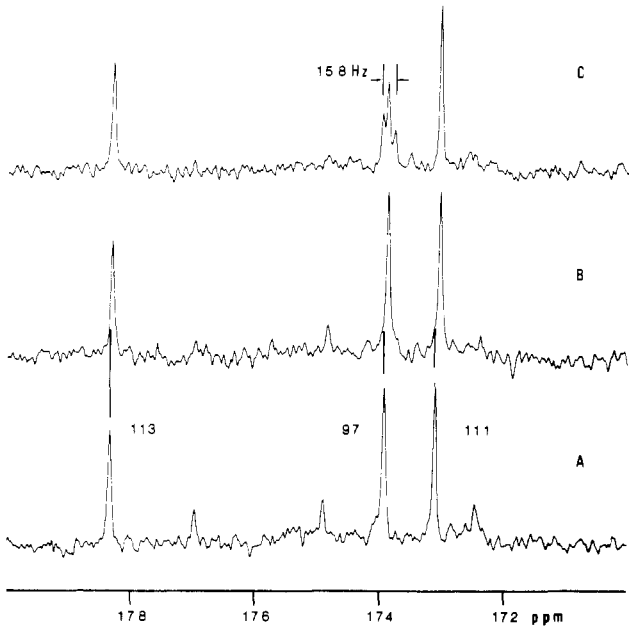

FIGURE 6: Comparisons of ^{13}C spectra of thermally denatured SSIs: (A) [F]SSI in deuteriated phosphate buffer at 95 °C (the same spectrum as in Figure 5C); (B) [F]SSI in 0.05 M phosphate buffer, in 90% H_2O–10% D_2O, 23 mg/0.5 mL, at 90 °C; (C) [F,GS]SSI in 0.05 M deuteriated phosphate (pH 7.3), 20 mg/0.5 mL, at 95 °C. These spectra can be used to assign three Phe signals of the denatured [F]SSI, and the result is shown in (A). A trio pattern in (C) shows a J_{CN} of 15.6 Hz for the Phe-97 carbonyl carbon (see the text).

to assign unambiguously the other two peaks of the denatured [F]SSI as shown in Figure 6. The differences of the chemical shifts in the two states are shown in Table III for various temperatures. The carbonyl signal of Phe-111 in D_2O was shifted on denaturation to low field by 0.65 ppm (at 90 °C). The observed low-field shift may further indicate that in the native state Phe-111 resides in a hydrophobic environment. The observed low-field shift may thus be explained by the hydration of the carbonyl oxygen of Phe-111 in the denatured state. On the other hand, the carbonyl signal of Phe-97 showed a rather large high-field shift of 0.95 ppm. It is tempting to explain this high-field shift by the destruction of the hydrogen bonding between the Phe-97 carbonyl and Val-78 amide (Mitsui et al., 1979), which might cause a low-field shift of the Phe-97 signal in the native state. However, the unusually small coupling constant (J_{CN}) of 13.7 Hz between Phe-97 and Ser-98 in the native state (Table I) returned to normal (15.8 Hz) on thermal denaturation. As discussed before, if this unusually small coupling constant is in fact due to the nonplanar peptide bond in the native state, the observed high-field shift would, at least, partly be ascribed to the relaxation of such a strained conformation. Obviously, further studies for the relations among the carbonyl chemical shifts, coupling constants, and the secondary or tertiary structure are needed in order to understand this sort of phenomenon in more detail.

It should be noted that a large chemical shift differences (0.8 ppm) was observed for the Phe-97 and Phe-111 even in the thermally denatured state. A smaller (0.4 ppm) but significant difference in chemical shift was also observed for the Met resonances of the thermally denatured SSI (Kainosho & Tsuji, 1982). Judging from the ^1H NMR spectra observed at high temperatures, the spectral characteristics of the native SSI were almost lost above 90 °C (Akasaka, 1984). It might be expected to observe nearly identical chemical shifts for the same amino acid residues existing in different sequential positions, if the protein exists in a truly random coil structure. Our results using the carbonyl resonances should therefore be, at least partially, accounted for by the residual conformational effect on the chemical shifts of the denatured SSI. Although these chemical shift differences observed for the denatured SSI may also be due to the sequence effects of these two Phe residues, the observed values seem to be too large to be accounted for only by such effects (Wüthrich, 1976). If such residual nonrandom conformations in fact exist for SSI in the thermally denatured state, two disulfide bridges between Cys-35 and Cys-50 and between Cys-71 and Cys-101 must play key roles to stabilize them. There have recently been interesting discussions about the nature of the residual conformation of the "open form", which was believed to exist in the processes of the hydrogen–deuterium exchange phenomena of small globular proteins (Dempsey, 1986; Wagner & Wüthrich, 1986). Further studies using other carbonyl resonances of SSI might clarify the structural features of the thermally denatured state of globular proteins.

A large low-field shift of the carboxylate carbon resonance of Phe-113 on thermal denaturation, 1.37 ppm, was somewhat unexpected, since this carboxylate group forms a salt bridge with the guanidinium cation of Arg-29 in the crystalline state. By formation of a salt bridge, the electron of the carboxylate anion would be withdrawn by the guanidinium cation of Arg-29, causing a low-field shift of the Phe-113 carboxyl resonance. Therefore, if the salt bridge also exists in solution, cleavage of the salt bridge on thermal denaturation would have resulted in a *high-field shift* of the Phe-113 carbonyl signal, rather than the observed *low-field shift*. The contradiction may be explained by taking the conformational feature of the side chain of Phe-113 into consideration. In the crystalline state, the aromatic ring of Phe-113 is folded over to the carboxyl group and shields the carbonyl carbon (Mitsui et al., 1979). If the structure of the side chain of Phe-113 is similarly maintained in solution, the diamagnetic shielding of the phenyl ring would contribute to the *high-field shift* of the carboxylate carbon in the native state. Such a ring current shift could certainly be large enough to cancel the low-field shift effect of salt bridge formation between Phe-113 and Arg-29. On thermal denaturation, all of these specific conformational effects would be lost and the Phe-113 signal eventually shifts to low field. As we have discussed already, a rather large

temperature dependency of the Phe-113 carboxyl carbon chemical shift may also indicate a crucial role of the side-chain conformation in its chemical shift in the native SSI.

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