Assignment of the Three Methionyl Carbonyl Carbon Resonances in *Streptomyces* Subtilisin Inhibitor by a Carbon-13 and Nitrogen-15 Double-Labeling Technique. A New Strategy for Structural Studies of Proteins in Solution†

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ABSTRACT: The ^{13}C nuclear magnetic resonance (NMR) spectrum of *Streptomyces* subtilisin inhibitor (SSI), whose three methionyl residues were replaced biosynthetically with 90% [1-^{13}C]Met, showed three enhanced signals at 173.8, 174.0, and 175.5 ppm compared with the natural abundance ^{13}C NMR spectrum. In order to assign the three methionyl carbonyl carbon peaks to the specific residues in the amino acid sequence, we have used a new approach that utilizes the fact that the carbonyl carbons are covalently bonded in an amide linkage to the α nitrogen of the next residue on the peptide sequence. In the case of SSI, the three Met-X dipeptides are all different: namely, the carbonyl carbons of Met-70, -73, and -103 are bonded to the α nitrogens of Cys-71, Val-74, and Asn-104, respectively. The ^{13}C NMR spectrum of SSI in which Met and Val residues were labeled simultaneously with [1-^{13}C]Met and [^{15}N]Val showed a doublet signal (J = 15.4 Hz) centered at 173.8 ppm together with a singlet signal at 173.8 ppm due to the carbonyl carbon bonded to [^{14}N]Val. The other two methionyl resonances were unsplit. Since the ^{15}N content of the incorporated Val was found to be 44 atom %, the center peak intensity was reduced to about 60% of the original value. Although each of the two identical subunits of SSI contains 13 Val residues, the effect of incorporated [^{15}N]Val could only be seen in this one carbonyl resonance. Thus the signal at 173.8 ppm was assigned to Met-73. The signal at 174.0 ppm was assigned to Met-70 by the ^{13}C NMR spectrum of doubly enriched SSI in which [^{15}N]Cys was incorporated in addition to [1-^{13}C]Met. The signal at 175.5 ppm was assigned similarly by double enrichment with [1-^{13}C]Met and [^{15}N]Asx. This assignment technique, which requires only the amino acid sequence of a protein and which may be generally applicable at least for any protein of microbial origin, opens up hitherto unexplored possibilities for ^{13}C and ^{15}N spectral studies of the structure and dynamics of the protein backbone. Described in this paper are several such possibilities, including measurements of spin–lattice relaxation rates and line widths of specific resonances, the rates of amide proton exchange at specific residues, and the pattern of thermal unfolding.

Carbon-13 NMR[1] spectroscopy of proteins has several features that distinguish it from its proton NMR counterpart (Allerhand, 1979). The greater chemical shift range is a most frequently claimed advantage of ^{13}C NMR spectroscopy over ^{1}H NMR and is certainly correct as far as the chemical shifts of different amino acid residues are compared (Wüthrich, 1976). It is, however, not necessarily so obvious that the advantage holds as well for the chemical shift dispersion of the same kind of amino acid residue in different microenvironments. The latter criterion should be of far greater importance than the former one, since NMR spectroscopy is the method of choice in solution studies of protein secondary or tertiary structures. In this regard, ^{1}H NMR has been well established and applied as an indispensable spectroscopic method for structural studies of protein in solution (Jardetzky & Roberts, 1981). By contrast, despite its frequently claimed potential usefulness, it seems to be a fair judgement at this moment that there have not been many examples in which ^{13}C NMR has played a key role in solving long-standing biochemical problems. We believe, however, that the situation is not due to an inappropriateness of the method but to the lack of a successful strategy for applying ^{13}C NMR to structural studies of proteins. Now that the sensitivity problem of ^{13}C NMR has been overcome to a great extent by the pioneering work by Allerhand (1979) and others, efforts to develop such a strategy should be explored.

In order to study the molecular details of the inhibitory action of *Streptomyces* subtilisin inhibitor (SSI)[1] to its target proteinase subtilisin, we wished to examine the hybridization state of the carbonyl group in the reactive site peptide bond that is attacked nucleophilically by the active site serine of subtilisin (Laskowski & Sealock, 1971). In principle, this kind of information can be obtained by ^{13}C NMR spectroscopy. The use of carbonyl ^{13}C resonances at natural abundance for structural studies of proteins has been hampered by the congestion of this region (Norton et al., 1977). With use of a high-field spectrometer, however, several single carbon resonances have been observed for the carboxylate groups of hen

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[1] Abbreviations: NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; SSI, *Streptomyces* subtilisin inhibitor; J, coupling constant; T_1 and T_2, spin–lattice and spin–spin relaxation times; ppm, part per million; STI, soybean trypsin inhibitor; BPTI, bovine pancreatic trypsin inhibitor (Kunitz); P_1, reactive site amino acid residue of protein proteinase inhibitors; [M]-SSI, SSI having all methionines labeled with L-[1-^{13}C]-methionine; [M,V]-SSI, doubly labeled SSI containing [1-^{13}C]Met and [^{15}N]Val; [M,C]-SSI, doubly labeled SSI containing [1-^{13}C]Met and [^{15}N]Cys; [M,B]-SSI, doubly labeled SSI containing [1-^{13}C]Met and [^{15}N]Asx; DEAE, diethylaminoethyl; NOE, nuclear Overhauser effect.

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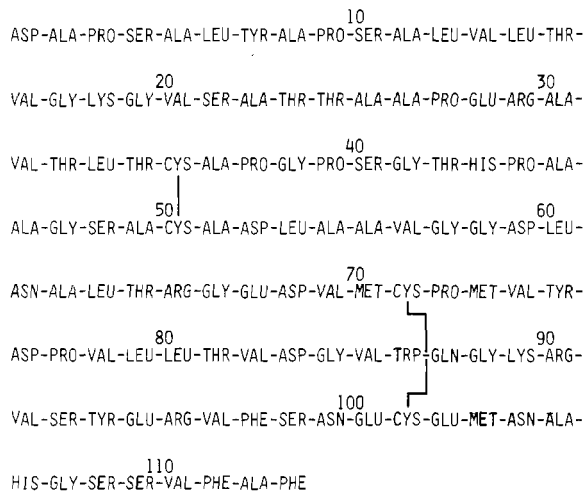

FIGURE 1: Primary structure of SSI (Ikenaka et al., 1974).

egg white lysozyme (Shindo & Cohen, 1976), and a few tentative assignments were given. The lack of a reliable and general assignment technique has limited the use of carbonyl resonances to those from C-terminal (Shindo & Cohen, 1976) and N-terminal residues (Wilbur & Allerhand, 1977).

The reactive site amino acid residue of protein proteinase inhibitors can, in some cases, be replaced enzymatically (Laskowski & Sealock, 1971), and this technique has been used to introduce ^{13}C-enriched amino acids into STI or BPTI. The reactive site carbonyl carbon resonances of STI and BPTI have been studied by ^{13}C NMR (Baillargeon et al., 1980; Hunkapiller et al., 1979; Richarz et al., 1980). The labeled STI was in its native form; but with BPTI, the labeled protein was in an irreversibly modified state, although it exhibited a full inhibitory activity (Richarz et al., 1980).

At present, enzymatic semisynthesis is of rather limited applicability for isotopic labeling of proteins. Even in the favorable case of proteinase inhibitors, it is limited to P_1 or adjacent reactive site residues; and not all proteinase inhibitors can be labeled by this method.[2] In this paper, we describe a general technique for enabling one to observe and assign the carbonyl resonances of virtually any residue in a small microbial protein. The assignment method involves the biosynthetic labeling of the C-1 carbonyl carbon of one amino acid at position n^i throughout the protein with ^{13}C and then making a series of double-labeled derivatives on the basis of the protein sequence in which possible residues at $n^i + 1$ are labeled in addition at the N^α position with ^{15}N. This straightforward method was used to assign the three methionyl residues of SSI, one of which is the P_1 residue (Ikenaka et al., 1974; see Figure 1). Since our strategy, in principle, can be a most potent approach to studies of protein backbone structure and dynamics, a scope of applications is also discussed.

Experimental Procedures

Materials. Streptomyces albogriseolus S-3253 on an agar slope was supplied generously by Professor S. Murao of the University of Osaka Prefecture. Subtilisin BPN′ (Nagase Co. Inc., Osaka, Japan, lot 7370935) was provided by Professor K. Hiromi of Kyoto University. L-[^{15}N]Cystine (95% ^{15}N) was purchased from Hikari Kogyo Co. Ltd., Tokyo. DL-[1-^{13}C]Methionine was prepared from 3-(methylthio)propanal (Aldrich) and sodium [^{13}C]cyanide (92.2% ^{13}C), according to the method of Holland & Nayler (1952). L-[^{15}N]Valine was prepared by microbial fermentation with *Brevibacterium lactofermentum* (Tsuchida et al., 1974). [^{15}N]Ammonium sulfate (Hikari Kogyo, 98.4% ^{15}N) was used in lieu of normal [^{14}N]ammonium sulfate as the sole nitrogen source. No appreciable isotopic dilution was detected during the fermentation. L-[^{15}N]Aspartic acid was prepared by the enzymatic amination of fumaric acid with dried *Pseudomonas trifolli* cells and [^{15}N]ammonium sulfate, according to the method of Zintel et al. (1969). Hammerstein casein used for the inhibitory activity measurements was purchased from E. Merck Co., and phenol reagent was from Daiichi Chemical Co. Inc., Tokyo. The amino acids used for fermentations were obtained from Ajinomoto Co., Inc. Yeast extract and polypeptone were from Difco Laboratories, MI. Sephadex and DEAE-cellulose were purchased from Pharmacia and Brown, respectively.

Table I: Amino Acid Medium Used to Produce Labeled SSI Molecules[a]

Trp	400	Gly	340
His	540	Ala	560
Lys·HCl	1840	Cys·HCl	520
Arg	660	Val	1240
Asp	1200	Met	540
Asn	140	Ile	1000
Thr	740	Leu	1780
Ser	1000	Tyr	380
Glu	4000	Phe	900
Gln	240	Pro	2060

[a] The composition was close to that of polypeptone. Ammonium sulfate 1560 mg/L.

Aerobic Cultivation of S. albogriseolus S-3253 on a Medium Containing an Amino Acid Mixture. The freeze-dried culture of *S. albogriseolus* S-3253 originally supplied by Professor Murao was suspended in distilled water and inoculated on agar plates containing meat extract (10 g/L), polypeptone (10 g/L), NaCl (3 g/L), and agar (15 g/L) at the initial pH of 7.0. The inoculated plates were incubated at 27.5 °C for 1 week, and the resulting colonies were transferred onto agar slopes of the same composition. Each of the subcultures was then incubated at 27.5 °C with vigorous shaking in a test tube containing a liquid medium composed of polypeptone (40 g/L), starch (20 g/L), yeast extract (1 g/L), NaCl (1 g/L), $K_2HPO_4 \cdot 3H_2O$ (1 g/L), and $MgSO_4 \cdot 7H_2O$ (500 mg/L), at the initial pH of 7.0. The strain that contained the highest inhibitory activity in the culture fluid against the proteolytic activity of subtilisin BPN′ was used for the subsequent experiments. For preparation of the isotopically labeled SSI, the amino acid mixture resembling the composition of polypeptone, Table I, was successfully used as a substitute for polypeptone. The accumulation of SSI was monitored by its inhibitory activity (Murao & Sato, 1972). When the cells were grown on the amino acid medium, maximum activity was reached after 6–7 days of incubation at 45 °C; approximately 200 mg/L of SSI was accumulated. This fermentation profile was quite different from that with polypeptone medium, with which the maximum activity was obtained after 2 days of incubation (Murao & Sato, 1972).

S. albogriseolus S-3253 used for the experiment produced, on an average, about 500 mg/L of SSI with the 4% polypeptone medium under the culture conditions described by Murao & Sato (1972). Cultivation was carried out at 45 °C in a medium containing amino acids (20 g/L; Table I), starch (20 g/L), NaCl (1 g/L), $K_2HPO_4 \cdot 3H_2O$ (1 g/L), and MgS-

[2] In the case of SSI, the active site methionine residue (Met-73) was shown not to be removable by carboxypeptidases (T. Ikenaka et al., personal communication).

$O_4 \cdot 7H_2O$ (500 mg/L). The initial pH was 7.0. The accumulation of SSI was monitored by the pH change of the culture fluid and, more quantitatively, by the inhibitory activity toward subtilisin BPN′. The maximum accumulation of about 200 mg/L was obtained after prolonged incubation, as long as 7 days at 45 °C. Although production of SSI in the amino acid medium was only 40% of that in polypeptone, the preparation of various isotopically labeled SSI's was found to be feasible by following the improved isolation procedure.

Purification of SSI. The procedures used by Murao & Sato (1972) were modified slightly in order to improve the isolation yield of SSI, since the original procedures afforded only 15% of SSI contained in the cultured fluids. A total of 500 mL of the culture fluid was filtered through paper to remove cells, and 330 g of ammonium sulfate was added in small portions with stirring. The solution was left at room temperature overnight; the resulting precipitate was collected by centrifugation at 10 000 rpm for 20 min. The precipitate was then dissolved in 100 mL of 0.01 M borate buffer, pH 7.2, and was dialyzed against the same buffer at 4 °C for 24 h. After the insoluble material was removed by centrifugation, the dialyzate was applied onto a DEAE-cellulose column (1.6 × 30 cm), which was equilibrated with the same buffer. After being washed successively with the borate buffer, the absorbed proteins were eluted with a continuous salt gradient up to 1 M NaCl. The absorbance of the eluant was monitored at 280 nm, and the fractions that showed SSI by HPLC analysis were collected.

The combined fractions were saturated (80%) with ammonium sulfate and left overnight at room temperature. The resulting precipitate was collected by centrifugation at 15 000 rpm for 10 min, after being kept overnight at room temperature. The collected precipitate was dissolved in 2 mL of 0.05 M Tris-HCl buffer, pH 7.5, and was chromatographed on a Sephadex G-100 column (32 mm × 90 cm), which was equilibrated with the same buffer. Fractions of 6.3 mL were collected at a flow rate of 15 mL/h; and the last peak, fractions 56–66, contained SSI. The combined fractions, after being dialyzed and lyophilized, afforded 60 mg of SSI, which was about 70% of the SSI contained in the culture broth as estimated by total activity. The SSI purified in this manner gave a single sharp peak by HPLC analysis and also gave a single band by polyacrylamide disc gel electrophoresis at pH 9.5. The inhibitory activity toward subtilisin BPN′ was as high as thrice crystallized SSI prepared in polypeptone medium.

High-Pressure Liquid Chromatography of SSI. The quantitative analyses of various labeled SSI's were very conveniently and accurately performed on a gel-permeation column (G-2000 SW, 7.5 mm i.d. × 600 mm; Toyo Soda Co. Inc., Tokyo) with a Hitachi 635A liquid chromatograph system. A 0.05 M phosphate buffer (0.1 M KCl) was used as an eluant. The peak area was found to be proportional to the amount of applied SSI, and the calibration line was thus used for quantitative analyses of SSI.

Mass Spectral Determination of Isotopic Purities of Amino Acid Residues Incorporated into SSI. A total of 0.5–1.0 mg of the SSI isolated from the culture medium containing isotopically labeled amino acid(s) was hydrolyzed for 24 h at 110 °C in 0.5 mL of constant-boiling hydrochloric acid. The resultant amino acid mixture was converted to the *N*(*O*)-(heptafluorobutyryl)-*O*-isoamyl derivatives according to the procedures by Felker & Bandurski (1975). The amino acid derivatives were then analyzed by a Hitachi RMU-6M mass spectrometer combined with a Hitachi 063 gas chromatograph equipped with a Pyrex column (2 mm i.d. × 3 m) of 3% SE-30 Gaschrom Q, 80–100 mesh. The assignment of each peak was readily made by the mass spectrogram of the respective peak.

Measurements of ^{13}C NMR Spectra. Unless otherwise specified, NMR spectra were recorded on a Varian XL-100-15 FT NMR spectrometer operating at 25.2 MHz. About 40–60 mg of each of the labeled SSI's was dissolved in 1.5–2 mL of deuterium oxide (Merck Sharp & Dohme); and the pD of the solution was adjusted to 8.2 with $N^2H_4O^2H$ (Merck). A Hitachi-Horiba F7-SS-2 pH meter with a Toa CE-103C combination micro glass electrode was used, and the measurements were made in 12 mm i.d. sample tubes. The pD values were the direct meter readings without any correction for the isotope effects. ^{13}C NMR chemical shifts were measured from the internal dioxane peak and then converted to a tetramethylsilane (Me_4Si) reference by the conversion factor of 67.8 ppm. Typical spectral parameters were the following: spectral width 1000 Hz; pulse width 43 μs (90°); data points 4K supplemented with 4K zero points; ambient probe temperature 38 °C (unless specified); proton noise decoupling.

Results and Discussion

Production of Isotopically Labeled SSI's. In order to prepare [1-^{13}C]Met-labeled SSI, we replaced the Met in the amino acid mixture listed in Table I with DL-[1-^{13}C]Met (540 mg/L). Two liters of the culture fluid afforded 280 mg of purified SSI in which the methionine residues were specifically labeled with [1-^{13}C]Met. We, in the following discussion, denote this isotopically labeled SSI as [M]-SSI. The isotopic purity of the Met residues was found to be about 82% ^{13}C as determined by mass spectrometric analysis of the acid hydrolysate of [M]-SSI. Doubly enriched SSI's with [1-^{13}C]Met and [^{15}N]Val ([M,V]-SSI), [1-^{13}C]Met and [^{15}N]Cys ([M,C]-SSI), or [1-^{13}C]Met and [^{15}N]Asx ([M,B]-SSI) were prepared similarly. The enrichment of ^{15}N was, however, not as high as that for ^{13}C, obviously because of enzymatic transamination that took place during fermentation. It was possible to improve the ^{15}N enrichment to some extent by modifying the composition of the amino acid mixture. The conditions used for preparing each of the three doubly enriched SSI's are described below.

[M,V]-SSI was prepared initially in the amino acid medium listed in Table I, except that Val and Met were replaced with L-[^{15}N]valine (1.86 g/L) and DL-[1-^{13}C]methionine (540 mg/L), respectively. A total of 70 mg of the labeled SSI was isolated from 500 mL of the culture fluid, but the ^{15}N enrichment of the incorporated Val was only 29 atom %. Since the initial ^{15}N enrichment of Val was over 90%, the isotopic dilution, possibly due to the transamination reaction catalyzed by branched-chain amino acid transaminase, was quite serious. Since the main source of nitrogen being scrambled with the amino group of [^{15}N]Val was probably glutamic acid, we reduced the amount of Glu and Gln to 50% or 25% of those listed in Table I. The production of labeled SSI with the Glx-reduced media decreased to 98 or 66 mg/L, respectively, but the ^{15}N content of the valine in fact increased to 33% and 44%, respectively. A high ^{15}N enrichment was not required for assignment of the carbonyl resonances, but an enrichment of 50% or more was necessary to obtain a precise value of the coupling constant, $^1J(^{13}C-^{15}N)$, for the scissile peptide bond in the SSI–subtilisin BPN′ complex (Kainosho & Tsuji, 1980). The yield must therefore be compromised to obtain labeled SSI with the isotopic purity needed, in cases where amine scrambling reactions occur. The situation was most crucial in the case of ^{15}N amino acids belonging to the main metabolic pathways, such as Asp or Ala. For these amino acids, the amine scrambling reactions catalyzed by aminotransferases

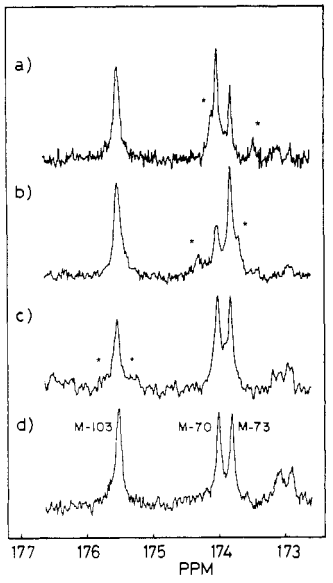

FIGURE 2: Proton-decoupled carbonyl carbon resonances of isotopically labeled SSI's at 25.2 MHz: (a) [M,V]-SSI; (b) [M,C]-SSI; (c) [M,B]-SSI; (d) [M]-SSI. About 40–60 mg of each labeled SSI was dissolved in 1.5–2.0 mL of ^2H_2O, pD 8.2. 20 000–30 000 transients were accumulated. Other conditions: spectral width 1000 Hz; data points 8K; 90° pulse with repetition period of 2 s; 38 °C (ambient temperature). Satellite peaks are indicated by an asterisk (*). These spectra permit the assignment of the three peaks as follows: 173.8 ppm, Met-70; 174.0 ppm, Met-73; 175.5 ppm, Met-103.

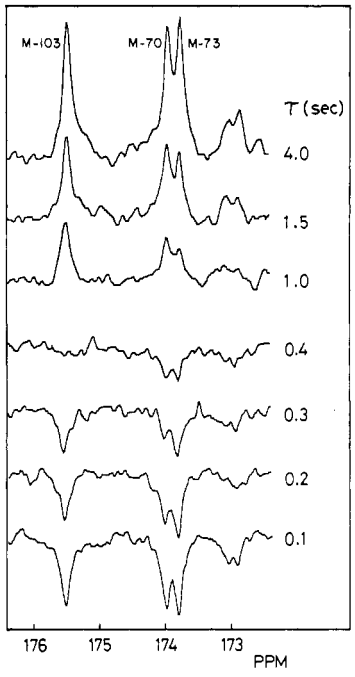

FIGURE 3: Spin–lattice relaxation measurement at 25.2 MHz of the methionyl carbonyl carbons of [M]-SSI (40 mg/1.5 mL of ^2H_2O, pD 8.2). The standard two pulse sequence, $[PD - (\pi - \tau - \pi/2) - AT]_n$, was used. The value indicated on the right-hand side of each spectrum is the delay time between the π and $\pi/2$ pulses. The recycling time (AT + PD) was set to be 7 s, and $n = 4000$. Specral width was 1000 Hz.

should be far more efficient than, for instance, with Val or Cys. We had to conduct several trials in order to optimize the composition of the amino acid mixture used to prepare each of the three doubly labeled SSI's. In fact, our initial attempt to prepare [M,B]-SSI with 400 mg/L of L-[^{15}N]Asp (99% ^{15}N) in place of Asp and Asn (Table I), resulted in no detectable enrichment. Increasing [^{15}N]Asp up to 640 mg/L and at the same time reducing Glx to 50% of the initial amounts listed in Table I resulted in only 6% ^{15}N enrichment. The enrichment was improved eventually to 24% by increasing [^{15}N]Asp to 1200 mg/L. An isotopic enrichment to about 50% ^{15}N was achieved for [M,C]-SSI by using 400 mg/L [^{15}N]Cys and 50% of the normal amount of Glx.

Assignment of Carbonyl Resonances of Methionyl Residues in SSI. Shown in Figure 2 are the carbonyl carbon regions of the 25.2-MHz ^{13}C NMR spectra of (a) [M,V]-SSI, (b) [M,C]-SSI, (c) [M,B]-SSI, and (d) [M]-SSI, at pD 8.2. In the spectrum of [M]-SSI, there appear to be three enhanced carbonyl resonances at 173.8, 174.0, and 175.5 ppm, respectively. These three peaks can be assigned to the three labeled methionyl residues contained in each of the two identical subunits of SSI, namely, Met-70, Met-73, and Met-103, respectively. In Figure 2a for [M,V]-SSI, the signal at 173.8 ppm has decreased in intensity at the expense of the satellite peaks due to spin coupling with the amide ^{15}N of Val-74. We therefore could assign this resonance unambiguously to Met-73. The spin–spin coupling constant of the doublet, ^1J (^{13}C–^{15}N), which is 15.4 Hz, is in the usual range for peptide bonds (Llinas et al., 1977). A small but real isotope shift of 0.03 ppm (high-field shift for the ^{13}C–^{15}N species) was noted. The relative intensity of the central and satellite peaks was close to the value expected from the known ^{15}N enrichment, which was 43% as determined by mass spectrometry. A similar result was obtained for the spectrum of [M,C]-SSI (Figure 3b) in which the cysteinyl residues were partially (50%) replaced with [^{15}N]Cys. In this case, the peak at 174.0 ppm showed satellite peaks around a reduced central peak and therefore could be assigned to Met-70. The spin–spin coupling constant was about 15 Hz. Since two peaks out of the total of three peaks were assigned, the remaining peak at 175.5 ppm can be assigned automatically to Met-103. We, however, obtained the ^{13}C NMR spectrum of [M,B]-SSI, in which the Asx residues were replaced with [^{15}N]Asx to the level of about 24%. (Figure 3c). Although the satellite peaks could not be seen clearly, since their intensities were only about 10% of that of the center peak, a marked decrease in intensity of the center peak was noted. This order of enrichment (24%) seems to be the minimum required to assign a carbonyl resonance decisively.

Table II: Spin–Lattice (T_1) and Spin–Spin Relaxation Times (T_2*) of the Three Methionyl Carbonyl Carbons in [M]-SSI at 25.2 MHz[a]

	chemical shift (ppm)	T_1 (s)	T_2* (s)	w (Hz)
Met-70	174.0	1.10	0.17	1.9
Met-73	173.8	1.10	0.17	1.9
Met-103	175.5	1.00	0.14	2.3

[a] Spin–spin relaxation times were estimated from the line widths of each resonance by the relation $T_2^* = 1/(\pi w)$, where w denotes the line width at half peak height. Chemical shifts are from Me_4Si (primary standard was dioxane, see text).

^{13}C Relaxation Parameters of Methionyl Carbonyl Carbons. Figure 3 shows a sequential plot of the ^{13}C NMR inversion–recovery experiment for the Met carbonyls of [M]-SSI. The T_1 of each resonance is listed in Table II together with the T_2 estimated from the line widths of the respective peaks. The relaxation parameters for Met-103 were shorter than those for Met-70 and Met-73, although the absolute values of these parameters were not very accurate. In the solid state, the carbonyl oxygen of Met-103 is hydrogen bonded to the amide proton of Gly-107, forming part of a short α helix ("α_2 helix") as evidenced by the single crystal X-ray analysis of SSI (Mitsui

et al., 1979a). The other two methionyl residues, namely, Met-70 and Met-73, are in the random coil region of SSI, which contacts subtilisin BPN′ in the SSI–subtilisin BPN′ complex (Mitsui et al., 1979b). Met-73 is especially important because the carbonyl carbon of this residue has been shown to be the site of attack by the active site serine hydroxyl group of subtilisin BPN′ (Mitsui et al., 1979b; Ikenaka et al., 1974; Kainosho & Tsuji, 1980). In the crystalline state, the electron density map of the random coil region around Met-73 (P_1 site) was very sharp, which has been taken as an indication of the rigidity of this stem (Mitsui et al., 1979c). On the contrary, the electron density of the other side of the random coil stem separated by the disulfide bridge between Cys-71 and Cys-101 was so diffuse that only the main chain could be traced from the map (Mitsui et al., 1979c). A plausible interpretation of this stark difference in the electron density map around these two adjacent regions is that the stem including Met-73 must be rigid so as to maintain the precise geometry that satisfies the ideal stereochemical requirement for complex formation with subtilisin BPN′, whereas the stem including Met-70 might be flexible enough to allow the subtle induced fit type geometrical change associated with a contact with the proteinase. In fact, the latter stem was found to become more rigid in the SSI–subtilisin complex as evidenced by X-ray analysis (Mitsui et al., 1979b). In solution, however, there seems to be no such indication of a mobility difference between those two regions, since the line widths and T_1's of the carbonyl carbons of Met-70 and Met-73 are almost identical within experimental error (about 10%). Since the time scales of these two methods are different, there might be a flexibility difference that does not contribute to T_1 and T_2. Relaxation of nonprotonated carbons, however, usually involves relaxation mechanisms other than dipole–dipole relaxation, which dominates in the case of protonated carbons (Allerhand, 1978). For carbonyl carbons, chemical shift anisotropy and dipole–dipole interaction with directly bonded ^{14}N might be vital contributors to the relaxation mechanism. The chemical shift anisotropy can be estimated by measuring the relaxation parameters at several different magnetic fields.

We are now studying various carbonyl carbons other than Met at different magnetic field strengths, and the results will be discussed in a separate paper.

Isotope Shifts of Methionyl Carbonyl Carbon Resonances Induced by Deuterium Exchange of Adjacent Amide Protons. The amide protons of small peptides in deuterium oxide solution exchange rapidly with solvent deuteron. In the case of proteins, however, the exchange rates vary drastically (half-times of 1 s to over 1 year) depending to a large extent on the secondary structure in which the amide group in question is found. Richarz et al. (1979) studied extensively the amide proton exchange rates for BPTI dissolved in deuterium oxide at various deuterium ion concentrations. The exchange rates could be followed by the decrease in intensities of the amide proton peaks, some of which were assigned by elaborate decoupling and NOE experiments. The results clearly indicated that amide protons involved in hydrogen-bond networks such as α helix and β sheets exhibited much slower exchange rates than those of the amide protons in random-coil regions (Wagner & Wüthrich, 1979a,b). Akasaka et al. (1975) have observed numerous slowly exchangeable amide protons in SSI using ^{1}H NMR spectroscopy (220 MHz). Although detailed assignments of those peaks were not possible, because of the rather broad line widths of the amide proton peaks that are typical for a protein of this size (M_r 23 000), Akasaka et al. estimated that about 25 ± 2 protons exchange slowly at pD 6.4. In the crystalline state, SSI shows about 37 hydrogen-bonded protons (Mitsui et al., 1979a), and therefore, about two-thirds of the hydrogen-bonded amide protons should exchange slowly if the hydrogen bonds in solid state are maintained in solution. Nakanishi & Tsuboi (1976) have estimated the rate of amide proton exchange for SSI by infrared spectroscopy and concluded that the protons in the α-helix regions exchange very slowly compared to the other protons. They estimated the number of protons hydrogen bonded in the two α-helix regions in SSI to be 18, which seems to be slightly too many as judged from the result by Mitsui et al. (1979a). A reliable method of evaluating the exchange rates at specific sites in SSI is required.

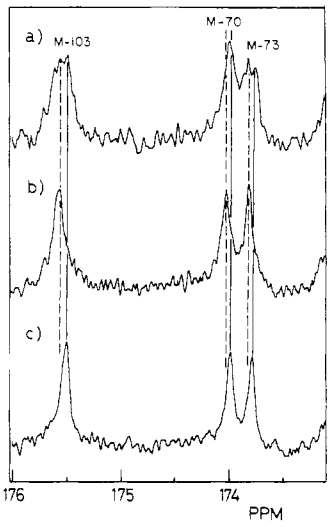

FIGURE 4: Proton-decoupled carbonyl carbon resonances at 25.2 MHz of [M]-SSI dissolved in various ^1H_2O:^2H_2O mixtures: (a) ^1H_2O:^2H_2O 1:1; (b) ^2H_2O:^2H_2O 9:1; (c) pure ^2H_2O. A chemical shift difference was induced by isotopic substitution at the amide hydrogen (proton/deuteron) adjacent to the Met carbonyl carbon. The concentration of [M]-SSI was 30 mg/1.6 mL. Other conditions were as follows: pD 8.2; spectral width 1000 Hz; ambient probe temperature (38 °C); number of transients accumulated 22 000–30 000. The chemical shifts of Met carbonyls in the three spectra were measured by reference to the internal dioxane peak by using wider spectral widths. Prior to running spectra a and c, the sample solutions were incubated at room temperature at least overnight, in order to eliminate slow ^{1}H–^{2}H exchange effects on the resonances (see Text).

We incidentally found that the ^{13}C NMR peaks from the labeled carbonyls of [M]-SSI in spectra obtained immediately after the sample was dissolved in deuterium oxide, at pD 8.2, at room temperature were rather broad, especially those of Met-103 and Met-73. These resonances appeared as sharp singlets in spectra from samples dissolved for several hours at room temperature, as shown in Figure 4c. In the present case, the backbone amide protons of Cys-71, Val-74, and Asn-104 may affect the chemical shifts of the carbonyl carbons of Met-70, Met-73, and Met-103, respectively. In fact, [M]-SSI dissolved in 10% deuterium oxide (deuterium was necessary for the internal lock signal) immediately showed sharp resonances for all of the three signals (Figure 4b). These two sets of spectra, however, exhibited a difference in chemical shift, which is attributed to an isotope shift induced by deuteration of the adjacent amide nitrogen. The spectra indicate that NH exchange at these positions is complete within several hours. The isotope shifts of the Met resonances were more readily seen in the ^{13}C spectrum of [M]-SSI dissolved in 50% deuterium oxide (Figure 4a). Very interestingly, the carbonyl resonances due to Met-103 and Met-73 showed 1:1 "double" peaks, each of which corresponded to either of the chemical shifts in deuterium oxide (99.8%) or in 10% deuterium oxide. On the other hand, the signal due to Met-70 showed a "single"

peak at the chemical shift that is the average of that due to Met-70 in ^2H_2O and ^1H_2O. The results clearly indicate that the amide protons of Val-74 and Asn-104 exchange slowly compared to the inverse of the isotope shift in hertz and that the amide proton of Cys-71 exchanges rapidly on this time scale. Note that what we define here as "slow" and "fast" exchange does not correspond to the much slower time scale used by others (Wüthrich & Wagner 1979; Nakanishi & Tsuboi, 1976). Thus one can monitor two very different time scales by following the chemical shift of the carbonyl carbons in response to a change in the ($^1H/^2H$) composition of the solvent water. A slow change in chemical shift upon transfer from pure ^1H_2O to ^2H_2O (or vice versa) indicates hydrogen exchange that occurs over a period longer than the time required to obtain the NMR spectra. These are the rates generally followed by ^1H NMR. ^{13}C NMR spectroscopy allows one to monitor, in addition, much more rapid hydrogen exchange processes that occur on a time scale more rapid than the inverse of the isotope shift measured in hertz (300 ms at 25 MHz or 70 ms at 125 MHz).

Since the N^{α} H of Asn-104 is hydrogen bonded to the carbonyl oxygen of Glu-100 (Mitsui et al. 1979a), slow exchange monitored by the carbonyl resonance of Met-103 appears reasonable. However, the relatively short half-time for exchange of the amide proton of Asn-104 at room temperature (several hours at most) indicates that the α helix containing Met-103 and Asn-104 fluctuates considerably. Therefore, the "very slowly" exchangeable amide protons observed by infrared spectroscopy (Nakanishi & Tsuboi, 1976) cannot be in this region. The difference in exchange rate of the amide protons of Cys-71 and Val-74 cannot be attributed to differences in hydrogen bonding since both of these belong to the proximate random-coil region and are not hydrogen bonded in the crystalline state (Mitsui et al., 1979a). The results show that the amide proton exchange rate monitored by the isotope shifts of the directly bonded carbonyl ^{13}C resonances affords a new criterion for describing protein microenvironments. We are currently extending this new approach to the other amino acid residues in SSI including Phe, Leu, and Val.

Thermal Denaturation of SSI as Manifested by Methionyl Carbonyl Resonances. SSI has been shown to be very stable toward heat and extremes of pH (Sato & Murao, 1973). SSI exhibits its full inhibitory activity after being kept at room temperature at any pH between 3 and 10 for 25 h; and more than 80% of the activity was preserved even after incubation at 100 °C for 10 min between pH 3 and 9 (Sato & Murao, 1973). The thermal denaturation temperature of SSI was estimated to be 78 °C in water and 83.5 °C in deuterium oxide at pH 6.4 by monitoring the UV absorption change (Nakanishi & Tsuboi, 1976). A similar value was reported by Takahashi & Sturtevant (1981), on the basis of a rigorous thermal analysis of SSI. These studies, however, could only detect a gross structural change, and thus some subtle but important conformational changes may possibly have been overlooked. With the assigned carbonyl resonances in hand as sensitive conformational probes, we should be able to investigate directly such structural changes through ^{13}C spectroscopy.

Figure 5 contains a series of ^{13}C NMR spectra of the carbonyl region of [M]-SSI at various temperatures. The experiment was performed from low to high temperatures. The Met-103 peak started to broaden at about 80 °C; and at 85 °C only a broad hump with reduced intensity was observed. The Met-73 peak also decreased in intensity at 80 °C, but the line width of the remaining "native" peak stayed almost unchanged. The Met-70 peak did not change its apparent

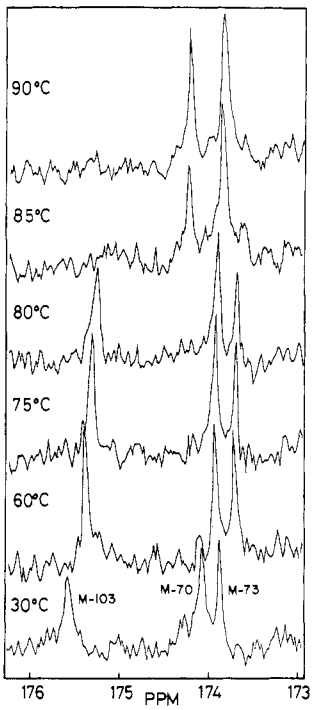

FIGURE 5: Thermal denaturation of SSI as monitored by the methionyl carbonyl resonances at 67.89 MHz of [M]-SSI. Concentration of [M]-SSI was 40 mg/2.0 mL of deuterium oxide, pD 8.2. Temperatures were calibrated with a thermocouple positioned just below the NMR sample tube.

position, but the resonance at this position increased in intensity with the concomitant loss of the intensity of the other two native peaks. At 85 °C, a new peak appeared at about 174.4 ppm, which is obviously due to the thermally "denatured" SSI. A spectrum obtained between 80 and 85 °C showed two sets of resonances of almost equal intensity (spectrum not shown). Apparently, structural changes of SSI monitored at the three different sites, namely, Met-70, Met-73 (closely spaced in the random-coil region), and Met-103 (in the α_2 helix), occur almost simultaneously. Interconversion between the native and denatured forms is slow on the NMR time scale. The thermal denaturation temperature observed by ^{13}C NMR, namely, between 80 and 85 °C at pD 8.2, was in good agreement with the previously reported value in ^2H_2O (Nakanishi & Tsuboi, 1976). At 90 °C, the native peaks had disappeared, and only two peaks appeared at 174.4 and 173.9 ppm. The higher field peak has about twice the intensity of the lower field one.

Since the carbonyl carbons of Met-70 and Met-73 are located in the random-coil region, the chemical shift differences between native and denatured forms should not be large. Moreover, these two carbons are in the same vicinity, and they may be expected to have similar chemical shifts in the denatured form. With these seemingly reasonable assumptions we tentatively assign the resonance at 174.4 ppm to Met-103 (denatured) and the overlapped peaks at 173.9 ppm to Met-70 and Met-73 (denatured). These assignments can be checked by denaturation experiments with [M,V]-SSI and [M,C]-SSI. Note that the above chemical shifts were measured from internal dioxane at an elevated temperature where the native and denatured forms of SSI coexist. With a conversion factor of 67.8 ppm, the chemical shifts from dioxane were converted to the values from Me_4Si. If the above assignments are correct, the chemical shift differences between the native and denatured forms for Met-70, Met-73, and Met-103 are 0, 0.1 (downfield shift), and −1.0 (upfield shift) ppm, respectively. The large

upfield shift observed for Met-103 upon thermal denaturation appears to result from breakage of the hydrogen bond between the labeled carbonyl carbon and the amide proton of Gly-107.

Conclusions. We have proposed a new strategy for assigning carbonyl carbon resonances in proteins and have applied the method to the assign the three methionyl residues of SSI. ^{13}C NMR studies of the assigned, ^{13}C-enriched, carbonyl resonances yielded information concerning the protein backbone that could not have been obtained by other spectroscopic methods including ^{1}H NMR. The assignments are based strictly on the amino acid sequence of the protein (actually the patterns of nearest neighbors) and did not require knowledge of the crystal structure. The independence of our ^{13}C NMR strategy should be regarded as its most important feature. We are now applying this method to the other residues in SSI, including Phe, Leu, and Val. The results obtained so far seem to be quite encouraging and will be discussed in detail elsewhere. Finally, we should point out that the same labeling strategy can be used to observe and assign the ^{15}N resonances of protein amide nitrogens. Recent improvements in the sensitivity of ^{15}N NMR have increased the feasibility of this approach. The carbonyl carbon assignments could be extended to adjacent carbons by using multiply ^{13}C-labeled amino acids. The assignments can then be extended to the resonances of protons directly bonded to these ^{13}C and ^{15}N atoms through heteronuclear double-resonance experiments. Our new strategy and its various variations should be a useful technique for structural studies of proteins in solution.

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