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Reductive cleavage and regeneration of the disulfide bonds in *Streptomyces* subtilisin inhibitor (SSI) as studied by the carbonyl ^{13}C NMR resonances of cysteinyl residues

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SUMMARY

Four enhanced carbonyl carbon resonances were observed when *Streptomyces* subtilisin inhibitor (SSI) was labeled by incorporating specifically labeled [1-^{13}C]Cys. The ^{13}C signals were assigned by the ^{15}N, ^{13}C double-labeling method along with site-specific mutagenesis. Changes in the spectrum of the labeled protein ([C]SSI) were induced by reducing the disulfide bonds with various amounts of dithiothreitol (DTT). The results indicate that, in the absence of denaturant, the Cys^{71}-Cys^{101} disulfide bond of each SSI subunit can be reduced selectively. This disulfide bond, which is in the vicinity of the reactive site scissile bond Met^{73}-Val^{74}, is more accessible to solvent than the other disulfide bond, Cys^{35}-Cys^{50}, which is embedded in the interior of SSI. This half-reduced SSI had 65% of the inhibitory activity of native SSI and maintained a conformation similar to that of the fully oxidized SSI. Reoxidation of the half reduced-folded SSI by air regenerates fully active SSI which is indistinguishable with intact SSI by NMR. In the presence of 3 M guanidine hydrochloride (GuHCl), however, both disulfide bonds of each SSI subunit were readily reduced by DTT. The fully reduced-unfolded SSI spontaneously refolded into a native-like structure (fully reduced-folded state), as evidenced by the Cys carbonyl carbon chemical shifts, upon removing GuHCl and DTT from the reaction mixture. The time course of disulfide bond regeneration from this state by air oxidation was monitored by following the NMR spectral changes and the results indicated that the disulfide bond between Cys^{71} and Cys^{101} regenerates at a much faster rate than that between Cys^{35} and Cys^{50}.

Nomenclature of the various states of SSI that are observed in the present study: Fully oxidized-folded = native or intact (without GuHCl or DTT); *half reduced-folded* (Cys^{71}-Cys^{101} reduced; DTT without GuHCl); *inversely half reduced-folded* ($Cys^{35}-Cys^{50}$ reduced; a reoxidation intermediate from fully reduced-folded state); *fully reduced-unfolded* (reduced by DTT in the presence of GuHCl); *fully reduced-folded* (an intermediate state obtained by removing DTT and GuHCl from the fully reduced-unfolded SSI reaction mixture).

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INTRODUCTION

Disulfide bonds in proteins stabilize their native conformations (Anfinsen and Scheraga, 1975; Thornton, 1981). They also play key roles in folding pathways (Ghelis and Yon, 1982; Kim and Baldwin, 1982). Recent developments in protein engineering have shown the practical importance of disulfide bond formation. Many proteins produced in *E. Coli* by recombinant DNA techniques tend to form insoluble inclusion bodies in the periplasmic space (Morehead, 1984; Tsuji et al., 1987). In most cases, such inclusion bodies contain extensive intermolecular disulfide bonds, and fully active protein can only be obtained by reducing and regenerating the disulfide bonds to the correct pairs that exist in the native protein (Morehead, 1984; Tsuji et al., 1987). The molecular mechanisms that convert incorrectly folded proteins into correctly folded ones have thus been important subjects of investigation (Creighton and Goldenberg, 1984; Pace and Creighton, 1986).

In many native proteins, one can reduce some, if not all, of the disulfide bonds without significantly losing biological activity or native-like conformation. Partially reduced proteins, however, are in general less stable toward heat, pH extremes and proteolysis. These effects are believed to be caused by increased dynamic fluctuations associated with the reductive cleavage of disulfide bonds (Wagner et al., 1979). More precise knowledge of the roles of disulfide bonds in conformational fluctuations may assist in the design of proteins with higher stability.

Despite the strong interest in disulfide chemistry in proteins described above, the experimental methodologies available for studying the detailed behavior of cysteinyl residues in folding pathways are still very limited (Ghelis and Yon, 1982; Kim and Baldwin, 1982). A chemical trapping method has been successfully applied in some cases to identify kinetically populated intermediates (Creighton, 1977; Creighton and Goldenberg, 1984; Pace and Creighton, 1986), but possible artifacts due to the protecting groups used to trap free thiols in the intermediates cannot be ignored. It is known that thiols embedded in the interior of a protein do not react with trapping reagents at a sufficiently rapid rate (Goto and Hamaguchi, 1981). An alternative method that does not use a trapping reagent is therefore called for.

We show here that disulfide bond formation can be monitored selectively at individual cysteines by ^{13}C NMR spectroscopy provided that the carbonyl carbons are enriched with ^{13}C. The method described here should be useful for studies of the structural roles of disulfide bonds, especially in larger proteins. *Streptomyces* subtilisin inhibitor (SSI) is a microbial protein which strongly inhibits bacterial alkaline serine proteinases such as subtilisin BPN' (Hiromi et al., 1985). SSI is a dimer of two identical subunits each containing 113 amino acid residues. The molecular weight is 23 kDa as a dimer. We have been using SSI as a model system to develop various new stable isotope-assisted NMR methodologies (Kainosho and Tsuji, 1982; Kainosho et al., 1985a,b; Kainosho et al., 1987). This paper describes detailed studies on the reductive cleavage and regeneration of two disulfide bonds in SSI subunit.

MATERIALS AND METHODS

Selective reduction of Cys^{71}-Cys^{101} bond in SSI

6 Mg of native SSI, dissolved in 1 mL of 0.1 M borate buffer containing 0.1 M KCl and 1 mM EDTA, pH 8.0, was reacted under nitrogen with 5 mL of freshly prepared 20 mM DTT solution

in the same buffer (190-fold excess). The solution was kept at room temperature, and aliquots of the reaction mixture were withdrawn for analysis. The reaction was quenched by passing the solution through a 1.1 × 5 cm Bio Gel P-4 column (Bio Rad) which was equilibrated with 50 mM borate buffer containing 50 mM KCl, pH 8.0. The fractions containing protein were collected under nitrogen. The amount of free thiol and the inhibitory activity toward subtilisin BPN' were immediately analyzed by the methods described below. Half reduced-folded SSI, in which only the Cys^{71}-Cys^{101} bond was completely reduced, was obtained after 1.5 h.

Reduction of both disulfide bonds in SSI

Both disulfide bonds in SSI were reduced by modifying the conditions described above: the buffer in which SSI was dissolved contained 6 M GuHCl; the same volume of 20 mM DTT solution without GuHCl (190-fold excess) was added. The GuHCl concentration of the resultant reaction mixture was 3 M. The reaction was completed within 30 min at room temperature.

Regeneration of the Cys^{71}-Cys^{101} bond from half reduced-folded SSI

The Cys^{71}-Cys^{101} reduced SSI used in this experiment was prepared under slightly modified conditions from those described above. Namely, 20 mg of native SSI, dissolved in 1 mL of 0.1 M borate buffer containing 0.1 M KCl and 1 mM EDTA, pH 8.0, was reacted under nitrogen for 1.5 h at room temperature with 2 mL of 100 mM DTT dissolved in the same buffer. The reaction mixture was applied to a 1.0 × 22 cm Bio Gel P-4 (Bio Rad) column which was equilibrated with 50 mM borate buffer containing 50 mM KCl, pH 8.0 and the fractions containing protein were collected. The combined fractions were incubated in an unstopped test tube at 37°C (note that the test tube must be open to allow air oxidation to occur). The time course of the reaction was followed by measuring the amount of free thiol and the inhibitory activity.

Regeneration of two disulfide bonds from fully reduced-unfolded SSI

Fully reduced-unfolded SSI was prepared by DTT reduction of SSI in the presence of 3 M GuHCl as described above. The time course of the regeneration of the two disulfide bonds, Cys^{35}-Cys^{50} and Cys^{71}-Cys^{101} was followed by the same method described for the case of half reduced-folded SSI.

Quantitative analysis of free thiols in reduced SSI

The quantity of free thiol in reduced SSI was determined by the Ellman method (Ellman, 1959): 0.5 mL of 0.13% 5,5'-dithiobis(2-nitrobenzoic acid), DTNB (Wako Pure Chemicals) dissolved in 0.1 M borate buffer, pH 8.0, containing 0.1 M KCl and 1 mM EDTA, was added to 2.5 mL protein solution in the same buffer. All buffer solutions were degassed under reduced pressure and then saturated with nitrogen. The concentration of reduced DTNB was calculated using a molar coefficient of $1.36 \times 10^4\ M^{-1}cm^{-1}$ at 412 nm.

Measurement of the inhibitory activity of native and reduced SSI

The inhibitory activity of SSI against subtilisin BPN' was determined by the method of Sato and Murao (Sato and Murao, 1973). In the case of reduced SSI, the preincubation time was shortened to 3 minutes from the originally 10 minutes. In order to avoid disulfide bond regeneration, the buffer was degassed and saturated with nitrogen.

Preparation of isotope-labeled amino acids

DL-[1-^{13}C]-S-benzylcysteine was synthesized from 2-bromoacetaldehyde diethylacetal (Aldrich) and sodium [^{13}C]cyanide (90% ^{13}C, MSD Isotopes) by the Strecker method. The L-form was resolved by using a microbial acylase (Amano Seiyaku). L-[1-^{13}C]-S-benzylcysteine was debenzylated with sodium metal in liquid ammonia and was oxidized by air to afford L-[1,1'-$^{13}C_2$]cystine (Uchida and Kainosho, to be published). DL-[^{15}N]alanine was prepared from ethyl 2-bromopropionate and potassium [^{15}N]phthalimide (Shokotsusho, 99.6% ^{15}N) (Sheehan and Bolhofer, 1950). The other ^{15}N-labeled amino acids were produced by microbial fermentation methods using ^{15}N-labeled ammonium sulfate as the sole nitrogen source (Central Research Laboratories, Ajinomoto Co.).

Preparation of isotopically labeled SSI and mutant SSI

Wild-type SSI and its isotopomers were prepared by culturing *Streptomyces albogriseolus S-3253* in the amino acid mixture media containing the respective labeled amino acids in lieu of normal ones (Kainosho and Tsuji, 1982). For example, [C]SSI was prepared by replacing L-Cys HCl with L-[1,1'-$^{13}C_2$]cystine. In the case of doubly labeled SSI analogues such as [C,P]SSI, both Cys and Pro were replaced with the appropriate amino acids: i.e., L-[1,1'-$^{13}C_2$]cystine and L-[^{15}N]Pro. In the case of [C,A]SSI, we attempted to increase the incorporation rate of [^{15}N]Ala with minimal scrambling with glutamate nitrogen. In order to retard the transaminase activity which leads to amino nitrogen scrambling, a potent suicide substrate 3-chloro-L-alanine (Showadenko) was added at a concentration of 1 mM through a Millex-GS (Millipore, 0.22 μm) membrane filter (see below).

[C,G] A36G SSI, which was used to confirm the assignment of the Cys^{35} carbonyl carbon signal, was produced using *Streptomyces lividans* which was transformed by a multicopy plasmid pIJ702 containing a recombinant gene constructed by oligonucleotide directed site-specific mutagenesis (Nirasawa et al., unpublished). Full account of the assignment technique using site-directed mutagenesis will be given elsewhere (Abe et al., to be published).

NMR experiments that use the assigned carbonyl carbon resonances to study cleavage and regeneration of disulfide bonds in SSI

Conformational changes, associated with the reductive cleavage or oxidative regeneration processes of the disulfide bonds in SSI, affect the Cys carbonyl signals as well as those of many other carbonyl carbons in the protein (Uchida and Kainosho, to be published). In this study we concentrated on the cysteine signals that provide the most direct indication of the chemical state of the thiol groups.

(1) Selective reduction of the Cys^{71}-Cys^{101} disulfide bond in native [C]SSI: To the [C]SSI solution in a 5 mm o.d. NMR tube, which was prepared from 20 mg protein and 0.5 mL 0.1 M borate buffer, pH 8.3, containing 10% D_2O for lock, 10 μL of 354 mM DTT solution was added. This quantity of DTT represented two moles per mole of SSI subunit; therefore it was sufficient to react both disulfide bonds in each SSI subunit if the reaction proceeded to completion. After filling up the NMR tube with nitrogen, the reaction mixture in an NMR tube was mixed well. It was allowed to react for 1.5 h at room temperature prior to initiation of the ^{13}C NMR measurement (Fig. 5b). After 1 h accumulation of the NMR spectrum, another aliquot of the DTT solution was added in order to reduce the second disulfide. By this procedure, the two disulfide bonds in SSI could be reduced at quite different rates (see below).

(2) Renaturation of [C]SSI which was fully reduced in the presence of denaturant: 20 mg [C]SSI was dissolved in 1 mL 0.1 M borate buffer, pH 8.3, containing 6 M GuHCl. 1 mL of a 100 mM DTT solution in 0.1 M borate buffer was added under nitrogen and allowed to react for 1.5 h at room temperature. The GuHCl concentration of this solution was 3 M and SSI remained denatured in this solution. Therefore, both disulfide bonds were completely reduced. The reaction mixture was applied to a 1.2 × 20 cm Bio Gel P-4 (Bio Rad) column equilibrated with the 0.1 M borate buffer. The fractions were monitored by a flow cell UV detector at 280 nm and fractions containing reduced SSI were collected and then concentrated by ultrafiltration with a YM-5 membrane filter (Amicon, cut-off molecular weight of 5 kDa). Next, the buffer was substituted with 0.1 M borate buffer containing 10% D_2O, pH 8.3 and the final volume was adjusted to 0.5 mL. This solution was transferred into a 5 mm NMR sample tube for the ^{13}C NMR measurements (Fig. 8).

NMR measurements

^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer at 75.4 MHz under conditions of full proton decoupling. The chemical shifts of the carbonyl signals were measured from the internal reference peak of 1,4-dioxane and then converted to the value from tetramethylsilane (TMS) by adding 67.8 ppm. The DEALS experiment on [C]SSI used procedures described previously (Kainosho et al., 1987).

RESULTS

Assignment of the Cys carbonyl carbon resonances of [C]SSI

The enhanced resonances observed in the ^{13}C NMR spectrum of [C]SSI (Fig. 1a), in which Cys residues were selectively labeled with [1-^{13}C]Cys, have been assigned to each of the four Cys residues in SSI subunit (Cys^{35}, Cys^{50}, Cys^{71} and Cys^{101}). The most reliable assignment method of the carbonyl carbon resonances for very large proteins is the ^{15}N,^{13}C double-labeling method which is based on sequence differences of dipeptide units (Kainosho and Tsuji, 1982). The sequences of the four Cys-X dipeptides in SSI are Cys^{35}-Ala^{36}; Cys^{50}-Ala^{51}, Cys^{71}-Pro^{72} and Cys^{101}-Glu^{102}. The assignments of Cys^{71} and Cys^{101}, which exist in unique dipeptide sequences, should be straightforward, if one can prepare the appropriate doubly labeled SSI isotopomers: [C,P]SSI and [C,E]SSI*. As expected, the spectrum of [C,P]SSI, in which a labeled [^{15}N]Pro is present together with [1-^{13}C]Cys, showed a double-peak (Fig. 1b). The signal at 171.05 ppm was thus unambiguously assigned to Cys^{71}.

We could not get, unfortunately, highly and selectively ^{15}N-enriched SSI for either Glu or Ala residues. Numerous trials using different culture conditions were unsuccessful, owing to α-amino nitrogen scrambling reactions catalyzed by amino transferases. Glutamate nitrogens are transferred to the other amino acids so efficiently that the [C,E]SSI spectrum was nearly identical with that of [C]SSI. The situation for Ala was not that bad, but we could not get as an high enrichment as we had observed before for [F,A]SSI (Kainosho et al., 1987). Since the strain used in the present study was different from that used before, we tested its transaminase activity by preparing [F,A]SSI. The results were similar to those observed for [C,A]SSI. Obviously, for some reason,

* Note that in our designation for the ^{15}N, ^{13}C doubly labeled SSI, the first one-letter code denotes [1-^{13}C]amino acid and the second [^{15}N]amino acid.

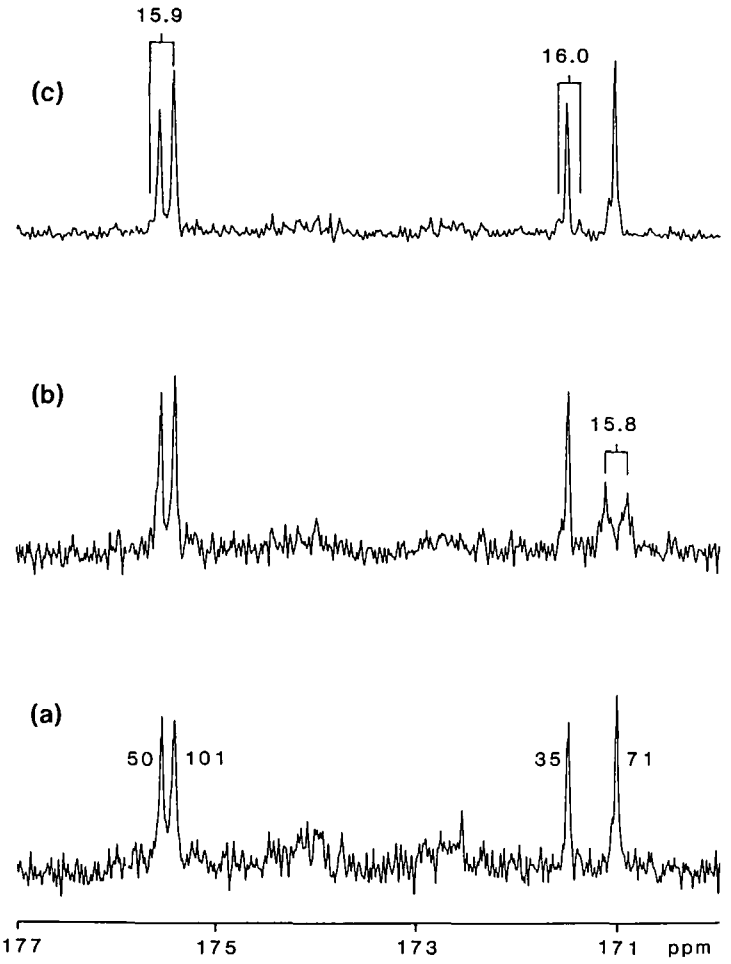

Fig. 1. Carbonyl carbon ^{13}C NMR spectra of *Streptomyces* subtilisin inhibitor (SSI) labeled with: (a) [1-^{13}C]Cys, [C]SSI; (b) [1-^{13}C]Cys and [^{15}N]Pro, [C,P]SSI; (c) [1-^{13}C]Cys and [^{15}N]Ala, [C,A]SSI. 20 Mg of each SSI isotopomer was dissolved in 0.5 mL 50 mM deuterated phosphate buffer, pH 7.3. 75.4 MHz ^{13}C NMR spectra were measured at 50°C (*see text for assignments*).

this newer strain has more efficient transaminase activity than the previous one. We succeeded, to some extent, in retarding the nitrogen scrambling reaction by adding a small amount (1 mM) of the suicide substrate 3-chloro-L-alanine into the culture media. [C,A]SSI, which was prepared this way, showed an appreciable decrease in intensity for two out of the three unassigned peaks (Fig. 1c). Judging from the satellite signal intensities of these signals, only about 20% ^{15}N enrichment was achieved, while there was virtually no ^{15}N scrambling into glutamate. Therefore, the two signals at 171.51 and 175.55 ppm should be from Cys^{35} and Cys^{50}, whose C-terminal neighbors are Ala. The remaining peak at 175.42 ppm was thus assigned by difference to Cys^{101}.

We might speculate, at this point, that the chemical shift of the Cys^{50} signal should be similar to that of Cys^{101}, since both these residues lie in α-helices. If so, the signal at 175.55 ppm must be from Cys^{50}. This tentative assignment was confirmed by the line shapes of Cys carbonyl signals

observed for [C]SSI dissolved in 50% D_2O. This experiment, which we call the DEALS experiment (acronym from *the effect of deuterium-hydrogen exchange at the amide on the line shapes*) (Kainosho et al., 1987), was found to be useful in estimating hydrogen exchange rates of amides in larger proteins by observing the steady-state line shapes of amide carbonyl carbons, which are affected by the secondary isotope shifts associated with deuterium substitution of two hydrogen atoms: namely, the amide hydrogen belonging to the same residue gives rise to a γ-shift, which is usually small or invisible, and the amide hydrogen belonging to the C-terminal neighbor gives rise to a β-shift, which is usually large enough to be observed (Kainosho and Tsuji, 1982; Kainosho et al., 1987).

^{13}C NMR spectra of [C]SSI dissolved in 50% deuterated 50 mM phosphate buffer, pH 7.3, measured at 50°C and 70°C are shown in Fig. 2. The signal due to Cys^{71}, which has no hydrogen responsible for the β-shift, appeared as a singlet even at 50°C, which confirmed the Cys-Pro sequence for this residue. This effect, which we denote the *Proline Effect*, was described earlier as a versatile method for assigning the carbonyl carbon signals of X residues in X-Pro sequences (Kainosho et al., 1985a,b). A marked temperature dependence was noticed for the line shape of one of the two Cys-Ala carbonyl signals (the signal with asterisk in Fig. 2). A broad double peak found at 50°C coalesced into a broad single resonance at 70°C. The results strongly indicate that this peak is from Cys^{35} which exists in an irregular region on the SSI surface (Mitsui et al., 1979). On the other hand, the two low-field peaks remained double at 70°C, showing that the amide hydrogens of the C-terminal neighbors of those residues exchange at much slower rates. Since Cys^{50} and

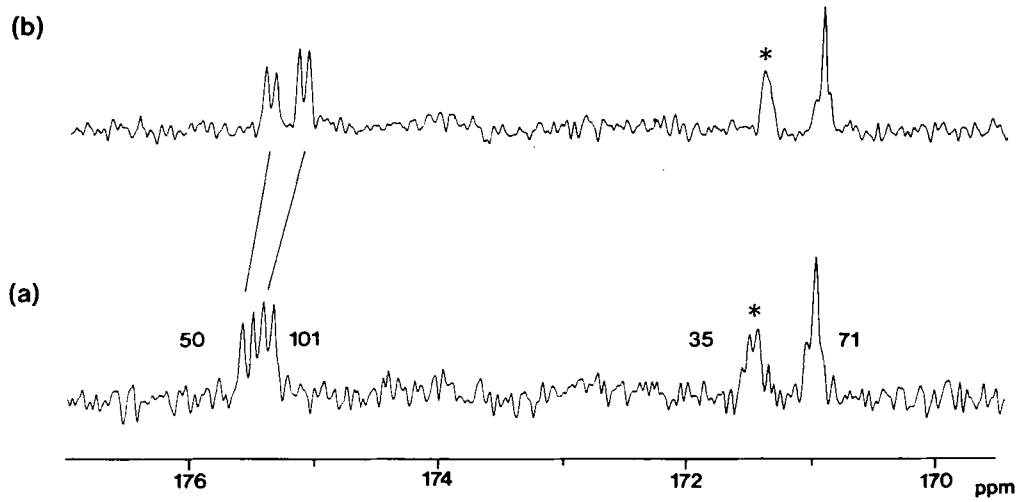

Fig. 2. DEALS experiments (effect of deuterium-hydrogen exchange at the amide on the line shapes of the carbonyl carbon resonances; Kainosho et al., 1982, 1987) of the Cys carbonyl carbon resonances at 75.4 MHz: (a) 50°C; (b) 70°C. 20 Mg of [C]SSI was dissolved in 50 mM phosphate buffer made of 50% H_2O-50%D_2O, pH 7.3. The double peak patterns of Cys^{50} and Cys^{101} are due to slow exchange rates at the Ala^{51} and Glu^{102} amide hydrogen (deuterium), respectively (β-shift, Kainosho et al., 1987). The results indicate that these amide protons are hydrogen bonded (actually they are in α-helix regions) even at 70°C. The Cys^{35} signal (*) appeared as a broad double peak at 50°C but became a broad single peak at 70°C. Therefore, the amide hydrogen of Ala^{36} exchanges at an intermediate rate (Kainosho et al., 1987). The carbonyl carbon of Cys^{71} always appeared as a sharp single peak; since the C-terminal neighbor of Cys^{71} is Pro^{72} which has no amide hydrogen (*the Proline Effect*, Kainosho et al., 1985a,b).

Cys^{101} are in α-helices, the amide hydrogens of their C-terminal neighbors are hydrogen bonded (Mitsui et al., 1979) and thus exchange slower even at high temperature.

The most decisive assignment of Cys^{35} and Cys^{50} was obtained by site-directed mutagenesis on a cloned gene of SSI (Obata et al., 1989a,b). We mutated Ala^{36} to Gly. The recombinant gene on a *Streptomyces* multicopy plasmid pIJ702 was expressed using *Streptomyces lividans* (Nirasawa et al., unpublished). The ^{13}C NMR spectrum of [C,G] A36G SSI is shown in Fig. 3. As expected, the higher-field signal which has been assigned to Cys^{35} showed a doublet peak due to ^{13}C-^{15}N spin coupling (J_{CN} 15.8 Hz), although the chemical shift value of Cys^{35} was about 1.1 ppm lower than that of wild-type SSI. The assignments of the Cys carbonyl carbons of SSI are summarized in Table I, together with those for the reduced forms of SSI and the A36G mutant.

Selective reduction of the disulfide bonds and its effect on the structure and function of SSI

The time course of the generation of free thiols by the reduction of native SSI with a large excess of DTT (about 190 molar excess) at room temperature is shown in Fig. 4, together with the accompanying changes in the inhibitory activity against subtilisin BPN'. The number of detected free thiols was gradually increased to reach two per subunit after 60 min. At this point, one of the two disulfide bonds was presumed to be selectively reduced.

We might have determined the cleaved disulfide bond by modifying the free thiol groups by protecting groups and analyze the digested peptides to establish the site of cleavage. Instead, we used the ^{13}C NMR signals of [C]SSI to determine the cleavage site. By the NMR method, we could determine not only the site of reduced disulfide bond, but also the structural features of this partially reduced state of SSI.

The ^{13}C NMR spectrum of [C]SSI at 23 C gave three, instead of four, signals (Fig. 5a), since the signals from Cys^{50} and Cys^{101} are completely overlapped at this temperature and give a large signal at the lowest field. By adding a twofold molar excess of DTT to the NMR sample, the intensities of Cys^{71} and the lower-field overlapped peak decreased significantly with the concom-

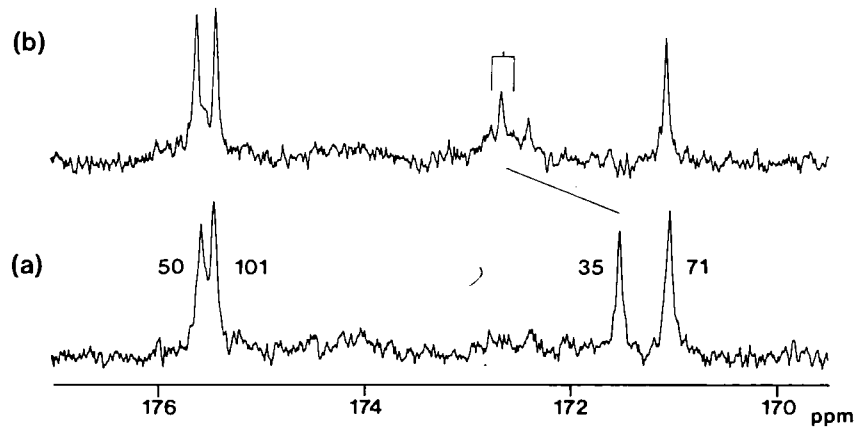

Fig. 3. Comparison of the 75.4 MHz carbonyl carbon ^{13}C NMR spectra of WT SSI and A36G SSI labeled with: (a) [C]SSI, 20 mg/0.5 mL; (b) [C,G] A36G SSI, 5 mg/0.5 mL. Spectra were measured at 50 C, pH 7.3. Among four signals of [C]SSI, only that of Cys^{35} was affected by replacing Ala^{36} with Gly by site-directed mutagenesis (Nirasawa et al., to be published). The Cys^{35} signal, which shifted to low field by 1.1 ppm, showed satellite signals due to the spin coupling with ^{15}N of Gly^{36}. The size of the satellite signals showed that the level of ^{15}N enrichment of Gly^{36} was about 50%.

TABLE I
CYS CARBONYL ^{13}C NMR CHEMICAL SHIFTS (PPM) OF [C]SSI IN VARIOUS REDUCTION STATES

		^{35}Cys		^{50}Cys		^{71}Cys		^{101}Cys	
	temp (C)	intact[a]	reduced[b]	intact	reduced	intact	reduced	intact	reduced
native [C]SSI	50	171.51	–	175.56	–	171.03	–	175.43	–
	23	171.75	–	175.83	–	171.05	–	175.83	–
^{71}Cys-^{101}Cys red. [C]SSI	23	171.66	–	175.78	–	–	171.12	–	176.20
^{35}Cys-^{50}Cys red. [C]SSI	23	–	172.03	–	175.82	171.06	–	175.82	–
fully red. [C]SSI	23	–	172.03	–	175.83	–	171.23	–	176.19
native [C,G] A36G SSI	50	172.66 (J = 15.6 Hz)	–	175.56	–	171.07	–	175.42	–

[a] Denotes that the respective disulfide bond is preserved.
[b] Denotes that the respective disulfide bond is cleaved.

itant appearance of new small peaks with asterisks at the lower-field side of those signals (Fig. 5b). These new signals are obviously from the Cys^{71} and Cys^{101} in the reduced disulfide bond. These peaks are getting larger at the sacrifice of the intensity of the native signals and finally, by the addition of a 10 molar excess of DTT, the Cys^{71}-Cys^{101} bond was completely reduced (half reduced-folded state: Fig. 5d).

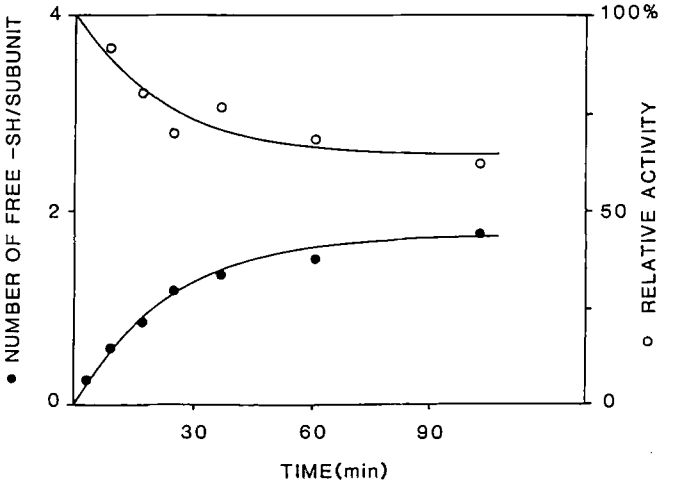

Fig. 4. Time course of the reduction of native SSI with dithiothreitol, DTT. 6 Mg of SSI was dissolved in 1 mL 0.1 M borate buffer containing 0.1 M KCl and 1 mM EDTA, pH 8.0. 5 mL of 20 mM of DTT in the same buffer was added to the SSI solution and allowed to react at room temperature. The time course for the amount of free thiols and the residual inhibitory activity were measured using aliquots of the reaction mixture (see *Materials and Methods* for details). The open circles are the inhibitory activity relative to native SSI and the closed circles are the number of free thiols generated per subunit.

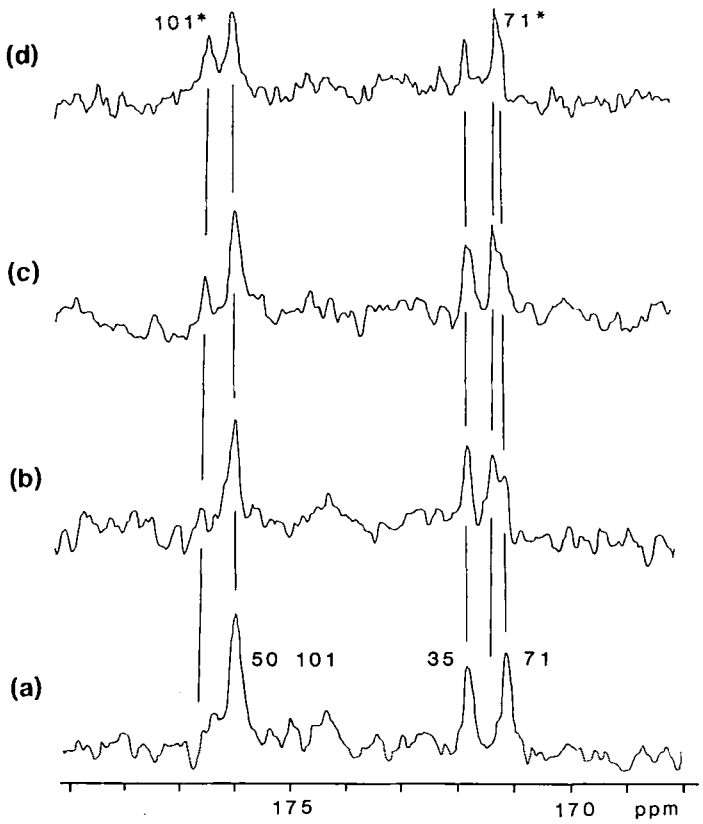

Fig. 5. Effect of disulfide bond reduction on the carbonyl carbon ^{13}C NMR signals of [C]SSI. Samples contained various molar ratios of dithiothreitol (DTT) to SSI subunit: (a) without DTT; (b) 2 molar (the theoretically equivalent amount to reduce the two disulfides in each SSI subunit); (c) 4 molar ratio; (d) 10 molar ratio. 75.4 MHz ^{13}C NMR spectra were measured at room temperature (23° C) using 20 mg of [C]SSI dissolved in 0.5 mL 0.1 M borate buffer (10% D_2O) containing 0.1 M KCl at pH 8.3. Each spectrum with DTT was measured over approximately 1 hour (2,000 transients using a recycling time of 2 s) immediately following addition of the DTT solution to the sample solution in an NMR tube (see *Materials and Methods* for details).

Regeneration of the disulfide bond between Cys^{71} and Cys^{101} in half reduced-folded SSI

The time course of the regeneration reaction of the reduced disulfide bond by air oxidation is shown in Fig. 6. The amount of free thiols in the half reduced-folded SSI was found to decrease almost linearly, while the inhibitory activity against subtilisin BPN' also increased linearly. Nearly full activity was restored at the end of the reaction when free thiol could not be detected. The results indicate that air oxidation of selectively reduced SSI restores its native structure. The ^1H NMR spectrum of the regenerated SSI was identical to that of the fully oxidized-folded (intact) SSI.

Renaturation of fully reduced-unfolded SSI to the native form

Fully reduced-unfolded SSI, in which two disulfide bonds were completely cleaved, was prepared by the method described above (see *Materials and Methods*). We found that the disulfide

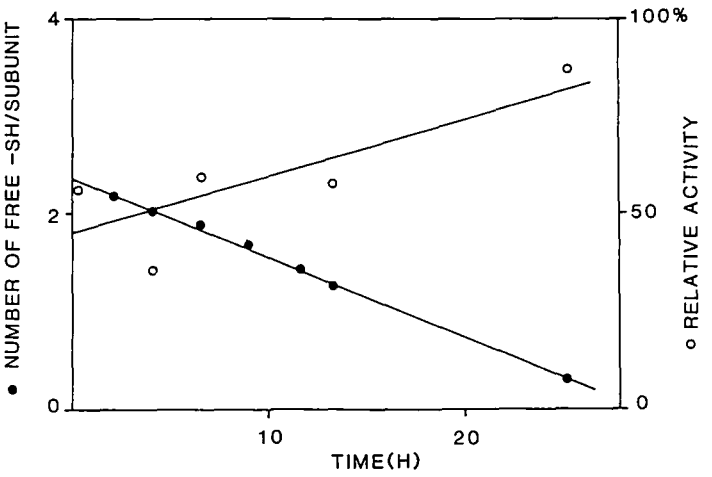

Fig. 6. Time course of the regeneration of reduced disulfide bond in the selectively reduced SSI. A solution containing 20 mg of the selectively reduced SSI in 2 mL of 0.1 M borate buffer with 0.1 M KCl, pH 8.0, was incubated at 37 C, exposed to air. The amount of free thiols and the restored inhibitory activity were measured for aliquots of the reaction mixture at various incubation times (see *Materials and Methods* for details). The open circles are the inhibitory activity relative to the native SSI and the closed circles are the number of remaining free thiols per subunit.

bond between Cys^{35}-Cys^{50}, which exists in the interior of SSI, could also be reduced by DTT in the presence of 3 M GuHCl, within 30 minutes. In this reaction mixture, SSI is in fully reduced-unfolded state. The native-like structure, fully reduced-folded state, is immediately restored by removing GuHCl and DTT from the mixture. By standing the solution of the fully reduced SSI to allow the air oxidation, the amount of free thiols gradually decreased and could not be detected after 72 h (Fig. 7). It should be noted that two out of four free thiols were oxidized within a few hours, while the other two thiols remained for a much longer time. This is in qualitative agreement with the NMR results described below. The inhibitory activity against subtilisin BPN' also was restored in a similar way and the original activity was fully recovered. These results suggest that the two cleaved disulfide bonds were regenerated in a correct manner as they were in the native SSI. The detailed molecular events in the process, however, could not be obtained by this type of experiments. ^{13}C NMR spectroscopy using the assigned Cys carbonyl carbon resonances were found to be useful to elucidate the detailed scheme. Note that all our regeneration experiments on the reduced disulfide bonds used air oxygen as the oxidant, the actual reaction rates were quite different for each experiment. The difficulties in controlling the oxygen concentration did not, however, affect any conclusions of our experiments.

In Fig. 8, shown are the time-dependent spectral changes observed for the fully reduced-folded state of [C]SSI in solution contained in a 5 mm (o.d.) NMR sample tube (see *Materials and Methods*). In order to facilitate the air oxidation, the NMR tube was kept open during the entire experiment. Because of the smaller surface area, the reaction rate was slower in the NMR tube than during the experiment carried out in a test tube (Fig. 7). The first spectrum (Fig. 8a) was obtained 3 h after removing the denaturant (GuHCl) and reducing reagent (DTT) from the reaction mixture. Since all the sample preparation procedures were carried out under nitrogen, the carbonyl carbon signals due to SSI with two cleaved disulfide bonds were seen in this spectrum. Apparently, the se-

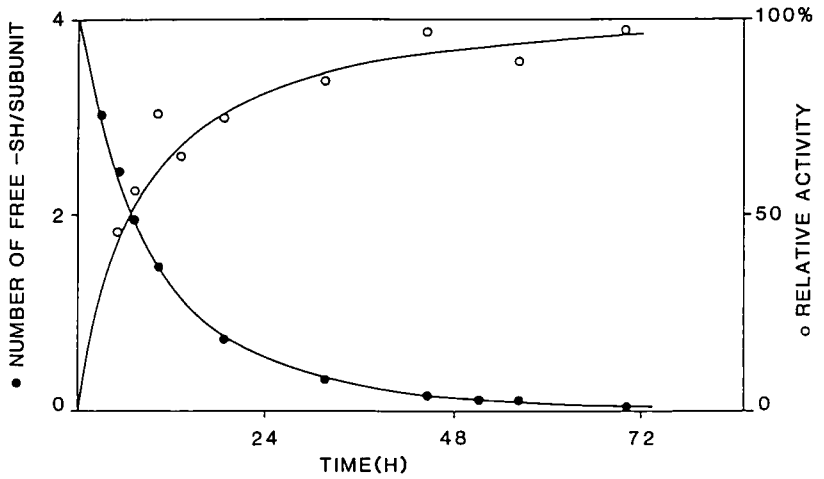

Fig. 7. Time course of the regeneration of disulfide bonds from fully reduced SSI by air oxidation. A solution of fully reduced-folded SSI in 0.1 M borate buffer containing 0.1 M KCl, pH 8.0, was incubated at 37 C exposed to air. The open circles are the inhibitory activity relative to native SSI and the closed circles are the number of remaining free thiols per subunit at various incubation times (see *Materials and Methods*).

condary structure as well as the overall conformation around these four cysteines are almost identical to those of native SSI (fully reduced-folded state). This result is not in agreement with previous results on the effects of DTT on SSI structure as studied by CD (Komiyama and Miwa, 1985). We suspect that their results are due to DTT in their sample, since they did not remove it before the CD measurement.

The ^{13}C NMR signals of fully reduced-folded SSI change with the incubation time in the presence of atmospheric oxygen (Fig. 8). Within several hours, signals due to *Cys^{71} and *Cys^{101} (*reduced*) were strongly diminished and new signals due to Cys^{71} and Cys^{101} (*oxidized*) were observed. The chemical shifts of the latter are almost identical to those for native SSI (Fig. 8b). At this point, *Cys^{35} still has almost full intensity, showing that the Cys^{35}-Cys^{50} bond is not formed. Therefore, a predominant species in this period should be Cys^{35}-Cys^{50} reduced SSI which is the inversely half reduced-folded SSI, a different species from the one we observed for the reduction of native SSI without GuHCl. After a day, approximately half of the protein molecules are already in the fully oxidized-folded state (Fig. 8c) and finally, after 216 h, all molecules are completely in the native form (Fig. 8e).

DISCUSSION

We should first point out that each of the disulfide bonds in SSI joins an α-helix with an irregular peptide chain (Fig. 9). Topologically, the SSI subunit can be regarded as being composed of two structurally similar subdomains, *domain 1* (around residues 1-60) and *domain 2* (around residues 60-113), each of which is composed of two long antiparallel β-sheets and one short α-helix connected by a disulfide bond. The idea behind this has come from our extensive studies on SSI and SSI-proteinase complexes (Kainosho and Miyake, to be published). We concluded that *domain 2* is responsible for proteinase binding and thus can be regarded as *the functional domain,*

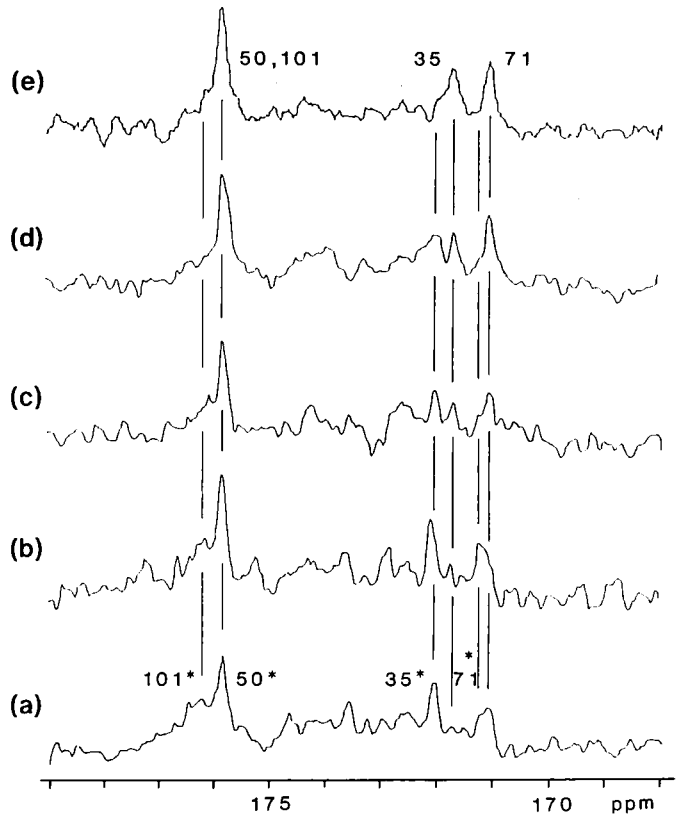

Fig. 8. Time course of the regeneration of the disulfide bonds in fully reduced [C]SSI as followed by ^{13}C NMR. A solution of 20 mg of fully reduced [C]SSI dissolved in 0.5 mL of 0.1 M borate buffer (10% D_2O) containing 0.1 M KCl at pH 8.3 was transferred into a 5 mm NMR tube without a cap to facilitate air oxidation and was incubated at room temperature (23 C). Each spectrum was accumulated over approximately 1 h (2,000 transients with 2 s recycling time) after a specific incubation period: (a) 0 h; (b) 4 h; (c) 22 h; (d) 69 h; (e) 213 h. Assignments are shown in the figure: numbers without asterisks (top) refer to reformed disulfide; numbers with asterisks (bottom) refer to reduced disulfides.

while the *domain 1* seems to be essential to maintain the overall dimeric structure and thus can be regarded as *the structural domain* (Kainosho and Miyake, to be published). The disulfide bond in *domain 2*, Cys^{71}-Cys^{101}, appears to stabilize the conformation of the flexible irregular polypeptide chain containing the reactive site scissile bond Met^{73}-Val^{74}. Therefore, it was interesting to see the effect of reducing this disulfide bond on the inhibitory activity and overall conformation of *domain 2*. For this purpose, we had to reduce this bond selectively. The relative solvent accessibilities of each of the four sulfur atoms calculated using the atomic coordinates determined by X-ray analysis are indicated in Fig. 9 (Hiromi et al., 1985). It is thus expected that the disulfide bond in *domain 2*; Cys^{71}-Cys^{101}, is more exposed to the solvent and is more susceptible to reducing reagents. This expectation was confirmed by the experimental results in which conditions were found to reduce this bond selectively.

The results showed several important features of this reaction. First of all, the disulfide bond having larger relative solvent accessibility was in fact selectively reduced. Secondly, as the chemical shifts of newly formed half-cystines are very close to those for the native SSI, the secondary

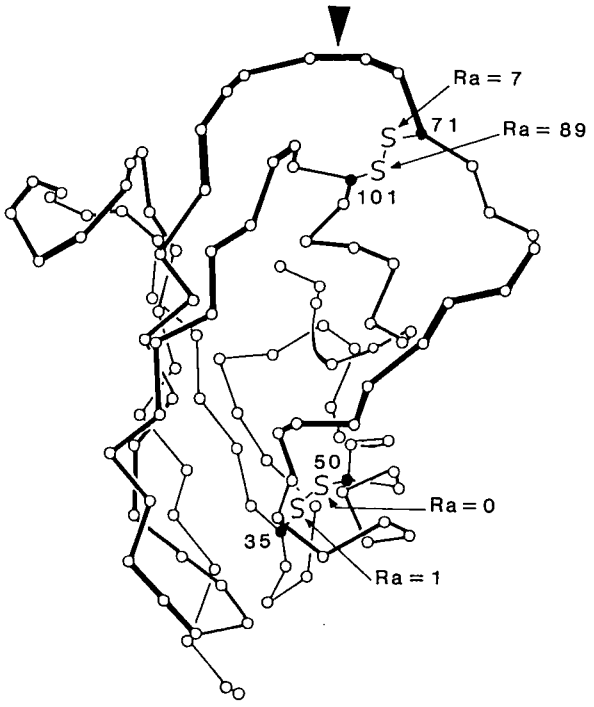

Fig. 9. Schematic chain trace of an SSI subunit showing positions of α-carbons (Mitsui et al., 1979). The Cys α-carbons are shown by the closed circles together with the residue numbers. The disulfide bonds at Cys^{71} and Cys^{101}, Cys^{35} and Cys^{50} are shown by '-S-S-'. The relative solvent accessibilities (Ra%) for the Cys sulfur atoms were calculated from the atomic coordinates obtained by X-ray analysis (Hiromi et al., 1985).

structures and local conformation around these residues have been maintained even without the disulfide bond. A similar result was observed for the C_L fragment of immunoglobulin which has only one disulfide bond (Goto and Hamaguchi, 1979). Thirdly, the signals due to Cys^{50} and Cys^{35} were not at all affected by reductive cleavage of the Cys^{71}-Cys^{101} bond (Table I). Therefore, structural perturbations occurring in *domain 2* are not transmitted to *domain 1*. This is in fact evidence in support of our subdomain hypothesis for the SSI subunit. Overall, the structure of half reduced-folded SSI is similar to that of native SSI. This conclusion is supported by an analysis of the other carbonyl signals in SSI, which will be described elsewhere (Uchida and Kainosho, manuscript in preparation).

Interestingly, even with one disulfide bond completely reduced, the remaining activity of partially reduced SSI was as high as 65% (Fig. 4). The results also indicate that the biological activity of the native SSI is maintained to a large extent even at the half reduced-folded state. As the buried thiol group might be much less reactive with protecting groups such as iodoacetamide (Creighton, 1986), the quantitative analysis of the free thiol group was also performed in the presence of 3 M GuHCl. Both values agreed well and we thus concluded that one disulfide bond is in fact much more sensitive toward DTT reduction than the other. This agrees with the NMR results.

The renaturation process of SSI with its disulfide bonds being fully reduced by DTT in the pres-

ence of GuHCl actually involves two fundamental processes, namely refolding of overall conformation and regeneration of disulfide bonds. In the following discussion, we show the evidence that these two processes are in fact quite different in their time scale.

Perhaps the most important result is that disulfide bond formation is not essential for SSI to refold from the denatured state. Since SSI cannot be folded as a monomer (Hiromi et al., 1985), the fully reduced-folded state must be dimeric. The signals labeled with asterisks (Fig. 8) are those from half-cystine groups. Their chemical shifts are very similar to those of the oxidized form (Table I).

Unlike the case of BPTI (Creighton, 1986), we did not see any signals due to incorrect disulfide pairing. In contrast to BPTI, where an intermediate with incorrectly paired disulfides is on the refolding pathway, in SSI folding and disulfide formation are not related. We suspect that a very flexible monomeric SSI without disulfide bonds is stabilized by the dimeric association through the hydrophobic interface composed of antiparallel β-sheet. If this assumption is correct, the nucleation site of the folding pathway in SSI is this β-sheet region.

CONCLUSION

We have used the assigned carbonyl carbon NMR signals of Cys residues to study the molecular details of the reduction-oxidation reactions of two disulfide bonds in *Streptomyces* subtilisin inhibitor (SSI). Fully reduced-unfolded SSI was found to refold to a native-like conformation without the formation of any disulfide bond. We have shown that the disulfide bond close to the surface regenerates (or cleaves) faster than the interior disulfide bond. The method described here should prove valuable for investigation of the structural and functional roles of disulfides in larger proteins.

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