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Solution X-ray Scattering Analysis of Cold- Heat-, and Urea-denatured States in a Protein, *Streptomyces* Subtilisin Inhibitor

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Streptomyces subtilisin inhibitor (SSI), a homo-dimeric protein with a subunit of 113 residues with two disulfide bonds, is known to exist at low pH in at least three distinct thermodynamic states namely, the native (N), cold-denatured (D') and heat-denatured (D). Small-angle X-ray scattering was used to analyze and to compare overall chain conformations of SSI in typical, N, D', D and urea-denatured states (D_{urea}). Molecular masses were determined from scattering intensities extrapolated to a scattering angle of zero, which showed that SSI exists as a homo-dimer in the N state, but as dissociated monomers in the D', D and D_{urea} states. From Guinier plots of the scattering intensities, radii of gyration (R_g) were determined to be 20.1($\pm$1.8) Å for N, and 20.7($\pm$1.3), 25.8($\pm$1.5) and 32 to 35 Å for D', D and D_{urea}, respectively. Kratky plots for both N and D' exhibited a bell-shape indicating that the polypeptide chain has a globular part not only in N but also in D', while Kratky plots for D and D_{urea} showed that the polypeptide chain has no globular part either in D_{urea} or D. Combined with the results from circular dichroism and ^{1}H NMR spectra, a picture emerges for the polypeptide chain conformation of SSI such that in N it is a globular dimer close to that in the crystal, in D_{urea} it is totally disordered and expanded nearly to a fully random chain with restrictions only from the disulfide bridges, in D the entire chain is disordered and expanded but with considerable local intra-chain interactions, and in D' the chain consists of a part with a unique tertiary structure and a part disordered and expanded to a degree comparable to D.

Keywords: small-angle X-ray scattering; *Streptomyces* subtilisin inhibitor; cold denaturation; radius of gyration; globularity

Introduction

The structural characterization of the unfolded polypeptide chain and its folding intermediates has a fundamental importance in the understanding of the mechanisms of protein folding and stability (Dill & Shortle, 1991). Much effort has recently been focused on the structural and dynamic characterization of non-native species. One of the most commonly observed non-native species is the molten globule species found in equilibrium in high salt conditions and as a kinetic intermediate of folding (Ohgushi & Wada, 1983; Kuwajima, 1989; Goto *et al*., 1990; Dobson, 1992; Ptitsyn, 1992).

Another non-native species of interest is the unfolded species at low temperature, which has been observed in various globular proteins (Privalov *et al*., 1986; Griko *et al*., 1988, 1989, 1992; Tamura *et al*., 1991a,b). Cold denaturation is a general phenomenon expected for a globular protein, which is considered to be driven by the negative free energy of hydration of non-polar groups at lower temperatures (Privalov, 1979). This contrasts with heat denaturation, which is driven by an increase

Abbreviations used: SSI, *Streptomyces* subtilisin inhibitor; N, native state of SSI; D', cold-denatured state of SSI; D, heat-denatured state of SSI; D_{urea}, urea-denatured state of SSI-; SAXS, small-angle X-ray scattering; R_g, radius of gyration; CD, circular dichroism; NMR, nuclear magnetic resonance; PGK, yeast 3-phosphogylcerate kinase; MW, molecular mass; ppm, parts per million.

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in chain entropy upon the unfolding of a protein chain. Although the two denatured states are usually treated as belonging to the same thermodynamic state (Privalov, 1979), because the major driving forces are different in the two processes, we expect that chain conformations may generally be different in the two denatured states. Furthermore, because the cooperativity due to hydrophobic interactions within the polypeptide chain decreases at lower temperatures, we may expect a more subtle variety of conformation in the cold-denatured species than in those in the heat-denatured state. In fact, this theory seems to have been proved for yeast 3-phosphoglycerate kinase (PGK) (Griko *et al*., 1989; Freire *et al*., 1992). More studies are clearly needed to characterize the cold-induced unfolding of globular proteins, and such studies are expected to increase our understanding of the subtle structural organization of a globular protein.

In the present study, we used small-angle X-ray scattering (SAXS) to characterize and compare the overall chain conformations of the heat-, cold- and urea-denatured conformers of *Streptomyces* subtilisin inhibitor (SSI), with particular emphasis on the cold-denatured conformer. Recent studies have shown that SAXS analysis is a unique and powerful tool for providing information on the compactness and the chain shape of various conformers of proteins (Damaschun *et al*., 1992, 1993; Flanagan *et al.*, 1992, 1993; Kataoka *et al*., 1993, 1995; Sosnick & Trewhella, 1992). However, to the author's knowledge, this technique has not been applied for characterization of the conformation of a cold-denatured state, except in the case of PGK (Damaschun *et al*., 1993). In addition to the information from the SAXS data, high-angle X-ray scattering profiles (Stuhrmann, 1973; Fedorov, 1975) and nuclear magnetic resonance (NMR) and circular dichroism (CD) spectra were used to supplement the SAXS experiments.

SSI is a dimeric protein consisting of two identical subunits, each having 113 amino acids and two disulfide bridges, the two subunits being associated strongly through a hydrophobic interface consisting of a five-stranded β-sheet (Figure 9 of Mitsui *et al*., 1979; Hiromi *et al*., 1985). Tamura *et al*. (1991a,b) found, based on CD, NMR, and differential scanning calorimetry measurements, that the native, folded conformation is altered both by heating and by cooling under acidic conditions. It was notable that a cooperative structural transition was accompanied by heat absorption between the cold-denatured (D') and heat-denatured (D) states, which means that the D' and D states are thermodynamically distinct in SSI. These authors presented spectroscopic evidence that a limited tertiary structure is present in the D' conformer (Tamura *et al*., 1991a,b). The equilibrium among the three states (N, D' and D) of SSI was represented by a phase diagram as functions of pH and temperature (Figure 6 of Tamura *et al*., 1991a), which forms the basis of the present study.

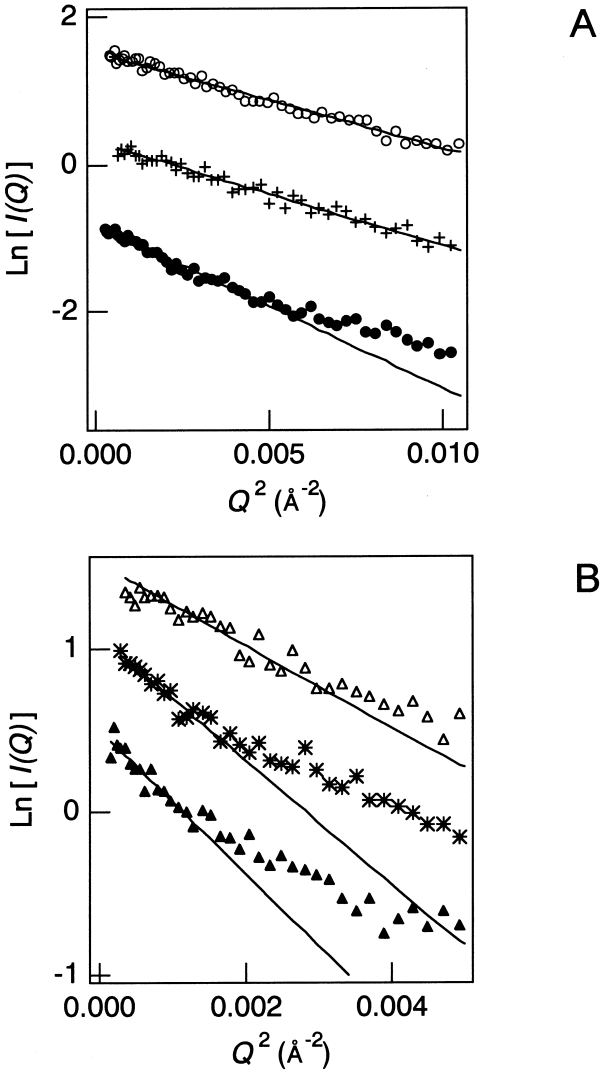

Figure 1. A, Guinier plots of SSI in the N (○), D' (+) and D (●) states. Each plot was obtained by the extrapolation to zero protein concentration of the data for four different protein concentrations (Kataoka *et al*., 1989). Experimental conditions were as follows: pH 7.0 and 20°C for N; pH 1.8 and 3°C for D'; and pH 1.8 and 35°C for D. B, Guinier plots of SSI in D_{urea}, pH 3.0, 20°C, 5 M urea (△); pH 1.8, 3°C, 5 M urea (*); pH 1.8, 35°C, 5 M urea (▲). [SSI] = 8 mg / ml. For clarity, each plot in A and B is shifted on the ln[I (*Q*)] axis. The regression lines were fitted to the data within $R_g \cdot Q < 1.3$.

Results

Guinier plot

Figure 1A shows Guinier plots of the small angle X-ray scattering (SAXS) curves of SSI at infinite dilution, in the typical solution conditions for the native (N), cold-denatured (D') and heat-denatured (D) states (the conditions listed in the figure legend). These conditions for N, D' and D were selected as typical conditions, producing the respective conformers from the phase diagram determined in the previous work (Figure 6 of Tamura *et al*., 1991a).

Table 1. Estimates of MW and R_g.

	R_g (Å)	MW (kDa)
Without urea		
pH 7.0, 20°C (N)	20.1 ± 1.8	19.7
pH 1.8, 3°C (D')	20.7 ± 1.3	9.8
pH 1.8, 35°C (D)	25.8 ± 1.5	11.1
With 5 M urea (D_{urea})		
pH 3.0, 20°C	32	11
pH 1.8, 3°C	32	12
pH 1.8, 35°C	35	12

Estimates of MW and R_g were made for different solutions of SSI, obtained from the intensity of X-ray scattering extrapolated to zero angle and the slope in the Guinier plot. RNase A was used as a reference protein for MW determination (see the text for details).

Neither significant aggregation nor interparticle interference was observed in any measurements of N, D' or D. The curves in Figure 1A for N, D' and D clearly possess a region where the plot approximates a straight line (the Guinier region; Guinier & Fournet, 1955). Radius of gyration (R_g) values were deduced from the slopes of the regression lines within the Guinier region, defined as $R_g \cdot Q < 1.3$ (where Q is the momentum transfer; see Materials and Methods) in this study (Olah *et al*., 1993), and are listed in Table 1. The above restriction corresponds to $Q < 0.062$, 0.062 and 0.050 for N, D' and D, respectively.

Scattering intensites at zero angle, $I(0)$, were determined from the linear fitting of scattering profiles for different concentrations of SSI. The plot $I(0)/[SSI]$ against [SSI], obtained from a series of experiments, did not show any significant increase with increasing [SSI] (Figure 2A), confirming that no significant aggregation occurred for any of the N, D' or D state. Molecular masses (MW) of SSI in the N, D' and D states were estimated from the $I(0)$ values, with the $I(0)$ values of RNase A solutions under exactly the same solution conditions as for SSI as standards. For the estimation, the specific volumes of SSI and RNase A under all the conditions were assumed to be 0.73 mg/ml (Inouye *et al*., 1978) and 0.71 mg/ml (Sober, 1970), respectively, which are the values for the native states of the proteins. That is:

$$I(0)_{X,nor} = \frac{I(0) \text{ for SSI in the X state}}{I(0) \text{ for RNase A under the same condition}} \quad (1)$$

The $I(0)_{X,nor}$ value extrapolated to [SSI] = 0 is proportional to the MW of SSI in the X state. Figure 2B shows the plot of $I(0)_{X,nor}/I(0)_{N,nor}$ against [SSI] for X = D' and D, which indicates that the MW for X = D' and D are both almost half that of the N state in the concentration range of less than 8 mg/ml. Molecular masses estimated in this way for various states of SSI are listed in Table 1. The MW value for the N state, 19.7 kDa, is close to the formula weight (23 kDa) of SSI as the homodimer. On the other hand, the estimated MW values for the D' and D states (9.8 kDa and 11.1 kDa) are almost half that of the N state, in agreement with the earlier conclusion drawn from the sedimentation equilibria, that SSI exists as dissociated monomers in the D and D' states (Tamura *et al*., 1991b).

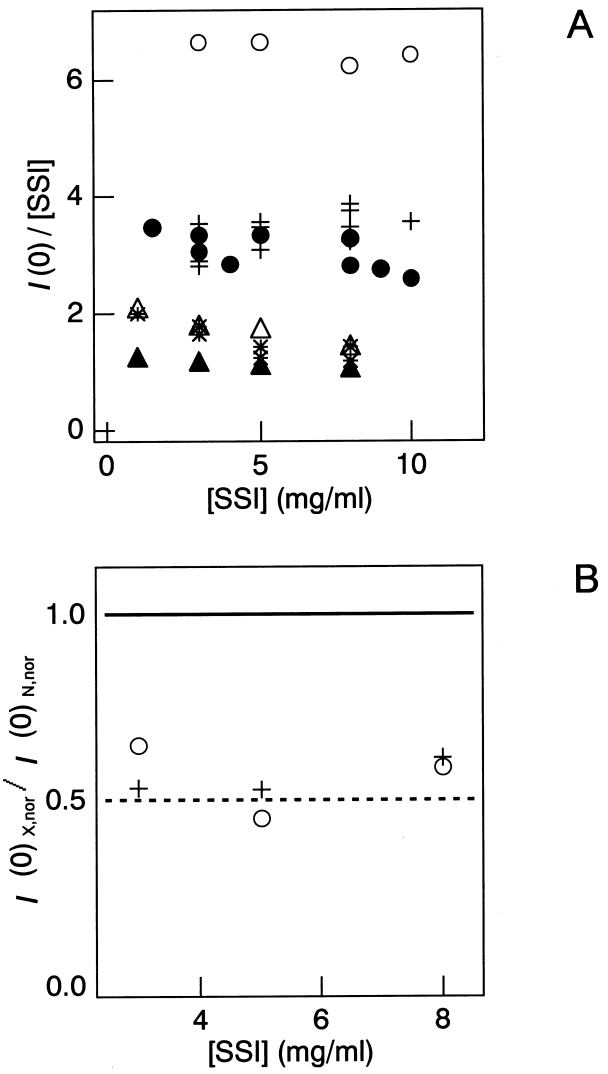

Figure 2. A, The dependence of $I(0)/[SSI]$ on the concentration of SSI. All the data were obtained in a series of experiments. N, pH 7.0, 20°C (○); D', pH 1.8, 3°C (+); D, pH 1.8, 35°C (●); and D_{urea}, pH 3.0, 20°C, 5 M urea (△); pH 1.8, 3°C, 5 M urea (*); pH 1.8, 35°C, 5 M urea (▲). B, The dependence of $I(0)_{X/N}$ on the concentration of SSI for X = D' (+) and D (○). Data were obtained for 3, 5 and 8 mg/ml protein. See the text for the definition of $I(0)_{X/N}$.

As references for the above data, we also obtained X-ray scattering profiles of SSI in the presence of 5 M urea (D_{urea}). The quality of the data was worse than that of N, D' and D, partly because of low relative scattering from the protein molecules in 5 M urea. Moreover, since the scattering profiles have a relatively strong concentration dependence, reliable Guinier plot based on an extrapolation to zero concentration was difficult to obtain by the same procedure as in Figure 1A. Thus, a Guinier plot was made separately for each concentration. The plots at 8 mg/ml for three different conditions are shown in Figure 1B, all of which depict linear regions for $Q < 0.036$, used for determining R_g values. The

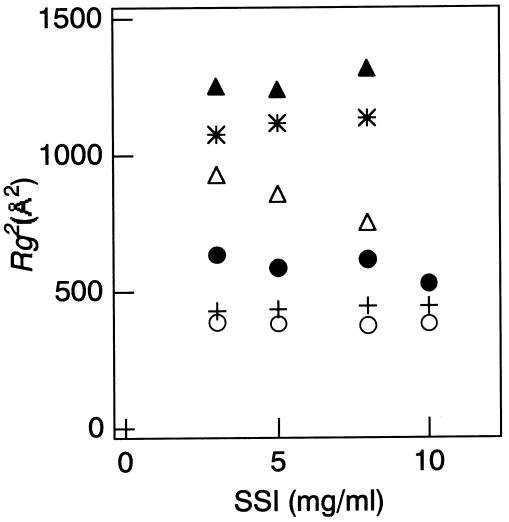

Figure 3. The dependence of R_g^2 on the concentration of SSI. All the data were obtained in a series of experiments. N, pH 7.0, 20°C (○); D', pH 1.8, 3°C (+); D, pH 1.8, 35°C (●); and D_{urea}, pH 3.0, 20°C, 5 M urea (△); pH 1.8, 3°C, 5 M urea (*). pH 1.8, 35°C, 5 M urea (▲).

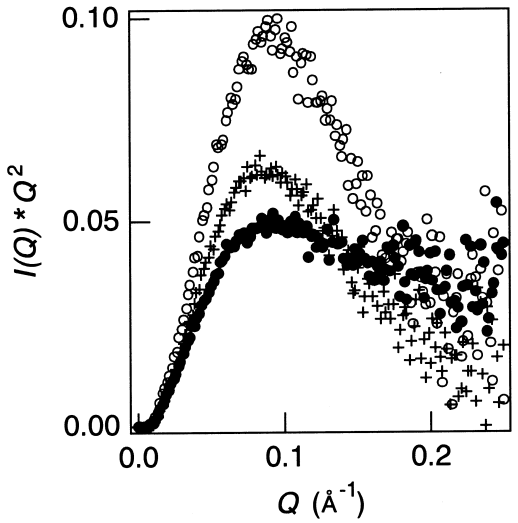

Figure 4. Kratky plots of SSI in the N, pH 7.0, 20°C (○); D', pH 1.8, 3°C (+); and D, pH 1.8, 35°C states (●). The protein concentrations were kept at 8 mg/ml for all the profiles.

squared R_g values so determined at different concentrations of SSI are plotted in Figure 3 for the three solution conditions of 5 M urea, along with those for N, D' and D for comparison. Linear extrapolation to zero SSI concentration gave tentative R_g values for D_{urea} (Table 1) which are significantly larger than those of N, D' and D.

In the presence of urea, the relation between $I(0)$ and molecular mass is not straightforward because of the interaction of the protein with urea, the third component, which might differ from protein to protein (Zaccai & Jacrot, 1983), and because of a contrast variation effect of urea (Luzzatti & Tardieu, 1980). Here, by assuming that the interaction between urea and the protein is the same for SSI and RNase A, we tentatively determined MW values for D_{urea}, using the $I(0)$ value of RNase A as a reference (Table 1). The MW values (11 to 12 kDa) obtained suggest that SSI in D_{urea} is in a monomeric form. The plot of $I(0)/[SSI]$ against [SSI] for D_{urea} did not show any significant increase with increasing [SSI] (Figure 2A), confirming that no significant aggregation occurred for D_{urea} for concentrations below 8 mg/ml.

Kratky plot

The Kratky plot, $I(Q)\cdot Q^2$ *versus* Q, a useful expression to describe the structural characteristics of a chain molecule (Glatter & Kratky, 1982), has been widely used to study the folding intermediates and structures of the non-native states of proteins (Damaschun *et al*., 1991, 1993; Flanagan *et al*., 1992, 1993; Kataoka *et al*., 1993, 1995). The essence of the Kratky plot is as follows. The scattering curve for the globular conformation follows Porod's law, $I(Q) \propto Q^{-4}$, at large Q values, while the scattering from the expanded unfolded conformer is proportional to Q^{-2} at moderate Q and is then proportional to Q^{-1}. Therefore, the Kratky plot shows a clear peak in the former case, while it has a plateau and then increases monotonically in the latter case (Flanagan *et al*., 1992; Kataoka *et al*., 1993, 1995).

Figure 4 shows Kratky plots of SSI (8 mg/ml) in the N, D' and D states, demonstrating clear differences in global conformation among them. The plots for both the N and D' states exhibit distinct peaks, indicating that both of them have globular conformations. The coincidence of the peak positions for the two states at about $Q = 0.08$ indicates that the R_g values of the two states are similar, whereas the fact that the peak height is much higher for N than for D' is simply an indication that the N conformer has a larger MW than the D' conformer. On the other hand, the Kratky plot for the D state has a much less distinct peak than those for N and D', representing a disordered chain conformation with little hint of globularity. This means that the overall shapes of the polypeptide chain are totally different between the D and D' conformers.

Figure 5A and B demonstrates the effects of the addition of 5 M urea on the Kratky patterns in the D' and D states, respectively. The addition of urea dramatically altered the profile of the Kratky plot for D', indicating that its globularity was completely destroyed in 5 M urea (Figure 5A). The further addition of urea did not change the profile. Although less pronounced, a slight change in the Kratky plot in the direction of further disorder was reproducibility observed for D in the presence of 5 M urea (Figure 5B). Comparison of the Kratky patterns (D_{urea}) in Figure 5A and B indicates that the polypeptide chain takes a similar, nearly fully random-coiled conformation, irrespective of the initial denatured states, D or D', in the presence of urea.

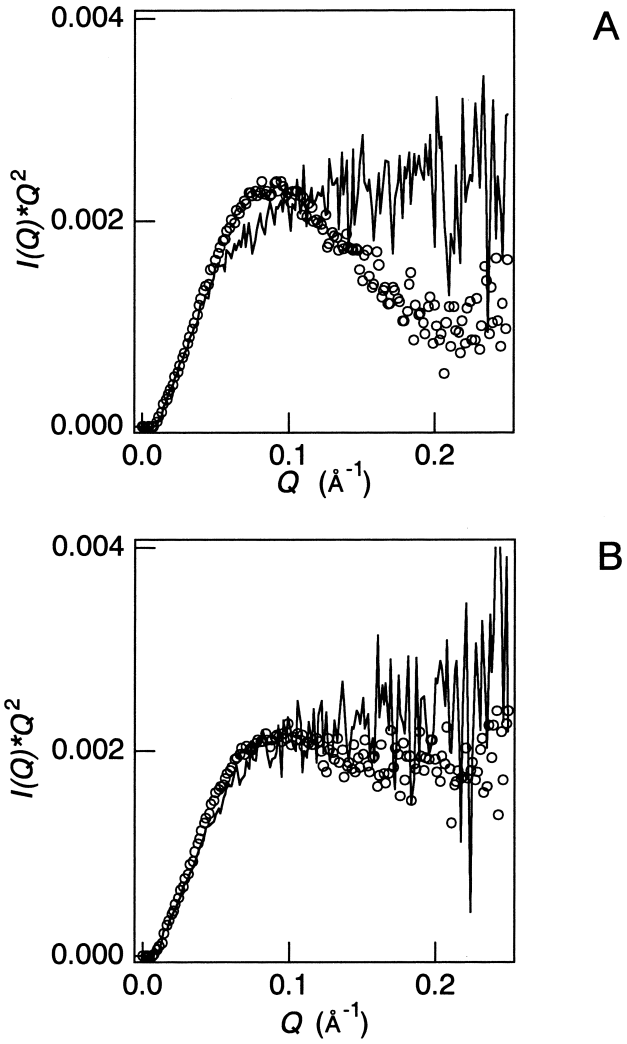

Figure 5. Kratky profiles of the D' and D states in the absence (○), and presence (continuous line) of 5 M urea. A, pH 1.8, 3°C; and B, pH 1.8, 35°C.

Scattering curves in the high *Q* region

Curves 1, 2 and 3 in Figure 6 show the scattering profiles in the high-angle region for the native, cold-denatured and heat-denatured states, respectively. Broad maxima are obvious in the profiles for the N and D' states. The profile of the N state has an evident peak at $Q = \sim 0.54$ Å^{-1}, and probably more peaks at lower values of Q. Also, for the D' state, a broad peak is evident around $Q = \sim 0.60$ Å^{-1}, the profile being distinct from that of the N state.

The X-ray scattering profile at the high-angle region ($0.2 < Q < 1.0$) reflects a fluctuation of electron density distribution within a protein molecule corresponding to distances of from 6 Å to 30 Å, i.e. it represents some internal structures rather than an overall shape (Stuhrmann, 1973; Ueki *et al*., 1985). Internal structures spanning from 6 Å to 30 Å would correspond largely to the spatial packing of

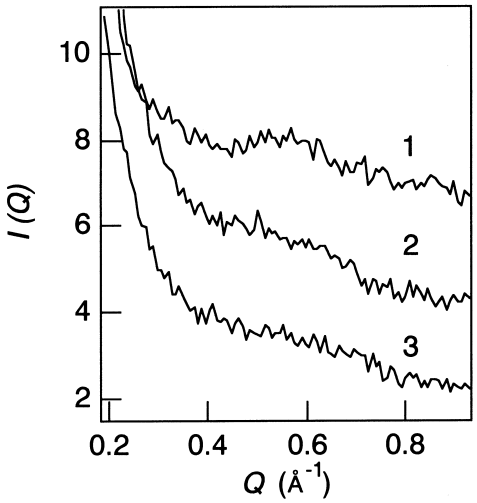

Figure 6. X-ray scattering profiles in the high-angle region for the N, D' and D states. 1, pH 3.0 and 20°C for N; 2, pH 1.8 and 3°C for D'; 3, pH 1.8 and 35°C for D. For clarity, each plot is shifted on the $I(Q)$ axis.

secondary structure elements, e.g. the mutual arrangement of α-helices (Stuhrmann, 1973; Fedorov, 1975; Kataoka *et al*., 1995). Thus it is clear that, in the D' state, some distinct spatial packing of secondary structure elements takes place, which is different from that of the native state. On the other hand, the scattering profile of the D state, showing no distinct peak, suggests that the D conformer contains no distinct mutual arrangement of secondary structure elements.

CD and NMR spectra

A major purpose of measuring CD and NMR spectra in the present work was to supplement and confirm the results in the previous studies (Tamura *et al*., 1991a,b), with additional data on urea-denatured SSI and with higher resolution and sensitivity in the NMR spectrum. In Figure 7, the CD spectra in the N, D', D and D_{urea} states in the far-UV region are compared. The results show that in 5 M urea, the secondary structure is totally absent, as in 8 M guanidine hydrochloride (Komiyama *et al*., 1984), while in heat- and cold-induced denaturation, considerable degrees of secondary structure are present.

The ^{1}H NMR spectra for N, D' D and D_{urea} are compared in Figure 8 with better resolution and sensitivity than previously reported (Tamura *et al*., 1991b). All the proton signals for D_{urea} are quite sharp, without any sequence-specific dispersion in chemical shifts. For D, the chemical shifts are nearly the same as those for D_{urea}, but the linewidths are much broader, suggesting the presence of limited sequence-specific dispersion of chemical shifts. The characteristic features of the spectrum for D' will be examined in detail in the Discussion.

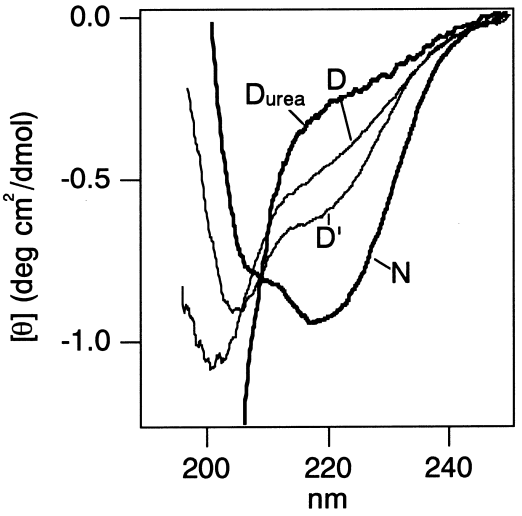

Figure 7. CD spectra of the N, D', D and D_{urea} states of SSI in the far-UV region. Experimental conditions were as follows: N, pH 3.0 and 25°C; D', pH 1.8 and 3°C; D, pH 1.8 and 35°C. D_{urea}, pH 3.0, 25°C and 5 M urea. The protein concentration was kept at 100 μM (as monomer) throughout the experiments.

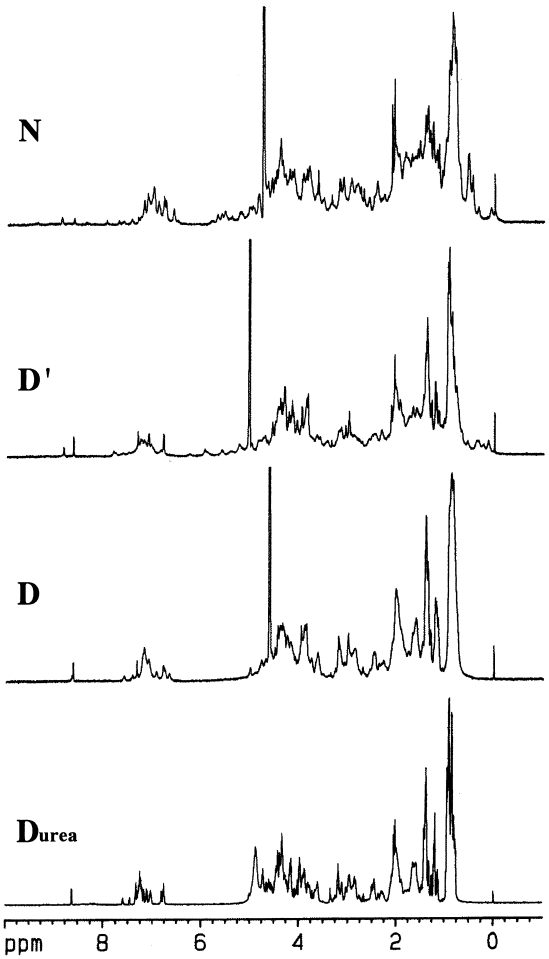

Figure 8. The ^{1}H NMR spectra at 600 MHz for the N, D', D and D_{urea} states of SSI. Experimental conditions were as follows: N, pH 2.6 and 25°C; D', pH 1.4 and 3°C; D, pH 1.4 and 35°C; D_{urea}, pH 2.6, 25°C and 5 M urea.

Discussion

Structural characteristics of the N conformer

The R_g value obtained for the native state of SSI was 20.1 Å, which is similar to a calculated R_g value (18.4 Å) of the native SSI using Debye's formula (Guinier & Fournet, 1955) and the Protein Data Bank coordinates (2SSI). The bell-shaped Kratky profile of the native SSI (Figure 4) and the clear peak in the higher range of the scattering angle (Figure 6) demonstrate that the N conformer is globule-shaped, with substantial packing of secondary structural elements. These features are consistent with the folded dimeric structure determined in the crystal (Mitsui *et al*., 1979).

Structural characteristics of the urea-denatured conformer

The Kratky plots in Figure 5 demonstrate a complete lack of globularity and a highly disordered chain conformation for SSI in 5 M urea. The observed R_g value of 32 to 35 Å comes closer to the value of 41 Å expected for a fully random-coiled polypeptide chain with 113 amino acids without disulfide bridges (Miller & Goebel, 1968). Thus the chain conformation in D_{urea} is close to a fully random one, except for the restriction due to the two disulfide bonds. The CD spectrum shows that the chain contains no secondary structures. The ^{1}H NMR spectrum, with distinctly sharp signals, all at positions of normalized chemical shifts (Wüthrich, 1986), suggests strongly that the entire polypeptide chain is mobile and that all the side-chains are well exposed to the solvent, with no involvement in the formation of a tertiary structure. Thus, the urea-denatured state can serve as a good reference state of disorder for other denatured states.

Structural characteristics of the D conformer

The R_g value of the D form (which is monomeric with an MW of 11.5 kDa and two disulfide bonds) is 25.8 A (Table 1), even larger than the R_g value of 19.8 Å for N (a homo-dimer with an MW of 23 kDa). This result indicates that the polypeptide chain of the D conformer is substantially expanded. The R_g value is comparable to that of acid-denatured cytochrome *c*, 24.2 Å (Kataoka *et al*., 1993; this has an MW of 12.4 kDa and no disulfide bond), and lies in between the R_g value for the heat-denatured RNase A, 19.3 Å (Sosnick & Trewhella, 1992; this has an MW of 13.7 kDa and four disulfide bonds) and the R_g value for reduced RNase A, 28 Å (Sosnick & Trewhella, 1992; this has no disulfide bond). In view of these, the R_g value determined for the D state of SSI seems to be what is normally expected for a denatured polypeptide chain with two disulfide bonds.

The Kratky pattern for the D state of SSI is close to that expected for a random-coiled chain (Figure 5B),

but the R_g value discussed above is significantly smaller than that of D_{urea}. This indicates that the heat-denatured form of SSI still contains significant intrachain interactions, which restrict the spatial distribution of the polypeptide chain. In accordance with this, the far-UV CD spectrum (Figure 7) demonstrates the presence of a significant amount of secondary structure, with the spectrum of D_{urea} as reference. On the other hand, the weak X-ray scattering profile in the high-angle region (Figure 6) suggests that mutual interactions among secondary structure elements are improbable. Altogether, the results present a view of the D state of SSI such that the entire polypeptide chain is considerably disordered, but with a limited amount of local intra-chain interactions, e.g. secondary structures, present.

Structural characteristics of the D' conformer

The R_g value of 20.7 Å for the D' state of SSI (which is monomeric with an MW of 11.5 kDa and two disulfide bonds) is considerably smaller than that for D, which is 25.8 Å (Table 1). This indicates that, on average, the polypeptide chain is more compact in D' than in D. What is striking is that the Kratky analysis in Figure 4 demonstrates a globular pattern, which is at least qualitatively similar to that in the N state. In addition, the clear peak in the high-angle scattering data in Figure 6 suggests the presence of well-defined packing arrangements of secondary structure elements in the D' state, different from those in the N state. A comparison of CD intensities of D', N and D_{urea} at 220 nm (Figure 7) shows that the D' conformer retains nearly half the secondary structure of N.

One of the features of the NMR spectrum for D' (Figure 8) is that several peaks in the aliphatic region (<5 parts per (ppm)) share commonality with the spectrum for D, e.g. intense peaks at ~0.9 ppm (due to the methyl protons of Val and Leu), 1.2 ppm (due to the methyl protons of Thr) and 1.4 ppm (due to the methyl protons of Ala), all at their positions normalized for a random polypeptide chain (Wüthrich, 1986). These observations clearly indicate that part of the polypeptide chain in D' is disordered to a comparable degree to that in D. Comparison of the intensities of the Ala and Thr methyl peaks between the two spectra gives an estimate that about half of the polypeptide chain is disordered in the D' state. On the other hand, the spectrum for D' in Figure 8 confirms the presence of all the characteristically shifted signals at exactly the positions as previously reported (Tamura *et al*., 1991b). These include signals at high-field (<0.7 ppm) previously assigned to the methyl protons of Val2 and Leu1 and in other regions of the spectrum, i.e. the ring protons of His43, His106 and Tyr7, and the methyl protons of Met70 and -73 (Tamura *et al*., 1991b). In particular, the presence of a group of low-field shifted α-proton signals in the 5.5 to 6.5 ppm region may be taken as an indication for the presence of a hydrogen-bonded β-structure (Wishart *et al*., 1992). None of these coincides with any of the signals in N, clearly suggesting a formation of unique secondary and tertiary structures in D', distinct from those in N.

Thus, a picture emerges for a conformation in D' such that about half of the polypeptide chain is involved in the formation of unique secondary and tertiary structures, while the rest of the polypeptide chain is disordered and expanded to a comparable degree to that in D. It is clear that such a chain conformation is quite different from that of a typical molten globule, which retains a large part of the intact secondary structure with nearly complete loss of the tertiary structure (Ohgushi & Wada, 1983; Dobson, 1992). The polypeptide conformation in the D' state is unique for a denatured conformer.

Granted that solubilization of the hydrophobic core is a major driving force in the cold-induced unfolding of a polypeptide chain (Privalov, 1979), some expansion of the polypeptide chain should be expected in the cold-denatured state, as in PGK (Damaschun *et al*., 1993). The formation of a distinct tertiary structure involving hydrophobic residues encountered in the D' state of SSI is in apparent contradiction of such an expectation. However, since low temperatures would reduce cooperativity of the hydrophobic core, probably in a characteristic manner, depending on the particular protein architecture (e.g. dimers, multi-domains, etc.), incomplete collapse of a hydrophobic core could happen, leading to an unfolding intermediate (Freire *et al*., 1992). It would be interesting to investigate, in this context, whether the structured part of the D' conformer of SSI was further destroyed if the temperature of the solution could be lowered much below 0°C without freezing.

Materials and Methods

Materials

Crude solutions of SSI were obtained by cultivating *Streptomyces albogriseolus* S-3253, and were purified on chromatographic columns with DE52 (Whatman, Maidstone, England) and Sephacryl S-200 (Pharmacia, Uppsala, Sweden), as described by Sato & Murao (1973). Ribonuclease A (from bovine pancreas, Sigma type XII) was used for MW reference purposes without further purification. Protein solutions for all the experiments were freshly prepared before experimentation by dissolving lyophilized powders into 25 mM Mops (pH 7.0) or 25 mM glycine buffer (pH 3.0 or 1.8). For NMR experiments, deuterated glycine was used for the buffer. The concentrations of SSI were determined photometrically after diluting the solution in a concentrated phosphate buffer (0.1 M, pH 7.2) using $A_{0.1\%}$ = 0.796 at 280 nm (Inouye *et al*., 1977).

Methods

Solution X-ray scattering measurement

Solution X-ray scattering experiments on the native and denatured states of SSI were carried out at the solution scattering station (SAXS camera) installed at BL-10C, the

Photon Factory, Tsukuba, Japan (Ueki *et al*., 1985; Kataoka *et al*., 1991). The sample-to-detector distance was about 90 cm for the measurement of SAXS, or about 50 cm for the measurement of high-angle scattering, calibrated by meridional diffraction of dried chicken collagen. The wave length of the X-ray was 1.488 Å. The sample cell was 50 µl in volume with 15 µm thick quartz windows, and had a 1 mm X-ray pathlength. The temperature of the sample was controlled by circulating temperature-controlled water. For SAXS measurements, protein concentrations were varied within the range of 3 to 10 mg / ml, and the measurement time was 10 to 15 minutes. For higher-angle scattering measurements, the protein concentration of the specimen was 15 mg / ml, and the measurement time was 20 minutes. In both cases, buffer, solutions with no SSI were measured as background.

Analysis of SAXS data

Data processing was carried out with both Macintosh (Apple) and PC0901 (NEC) personal computers. The scattering curve at infinite dilution was obtained from a series of scattering data with different protein concentrations (Kataoka *et al*., 1989). X-ray scattering intensities at the small-angle region were given as $I(Q) = I(0) \exp(-R_g^2 \cdot Q^2 / 3)$, where Q and $I(0)$ are the momentum transfer and the intensity at zero scattering angle, respectively (Glatter & Kratky, 1982). Q is defined by $Q = 4\pi \sin\theta / \lambda$, where 2θ and λ are the scattering angle and the wavelength of the X-ray, respectively. The radius of gyration (R_g) should then be obtained from the slope of the Guinier plot, a plot of $\ln[I(Q)]$ against Q^2. Extrapolation of $I(Q)$ to $Q = 0$ gives $I(0)$, which is proportional to the molecular mass of the scatter. Thus the $I(0)$ values were used to estimate the molecular mass (MW) of SSI in the N, D', D and D_{urea} states, with scattering profiles of RNase A as standards.

CD measurement

CD spectra were measured in the wavelength range of 190 to 250 nm with a Jasco J-720 spectropolarimeter, using a quartz cell with a light path of 1 or 10 mm. The protein concentration was kept at 100 µM (as the monomer) throughout. The sample solutions contained 25 mM glycine buffer; the pH being adjusted with HCl. The temperature of the solution was maintained with a thermostatically controlled water bath within (±0.1) deg.C.

NMR measurement

The ^{1}H NMR spectra of SSI were obtained on a Bruker AMX-600 spectrophotometer, using a sample tube of 5 mm diameter at a protein concentration of 10 mg / ml. The pH values given are direct pH meter readings adjusted with dilute ^{2}HCl calibrated against standard ^1H_2O buffers. The temperatures of the samples were controlled within (±0.1) deg.C.

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