Urea-induced conformational changes in cold- and heat-denatured states of a protein, *Streptomyces subtilisin inhibitor*

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Abstract

Streptomyces subtilisin inhibitor (SSI) is known to exist in at least two distinct denatured states, cold-denatured (D') and heat-denatured (D) under acidic conditions. In the present work, we investigated the manner how increasing urea concentration from 0 to 8 M changes the polypeptide chain conformation of SSI that exists initially in the D' and D states as well as in the native state (N), in terms of the secondary structure, the tertiary structure, and the chain form, based on the results of the experiments using circular dichroism (CD), small-angle X-ray scattering (SAXS) and ¹H-NMR spectroscopy. Our results indicate that the urea-induced conformational transitions of SSI under typical conditions of D' (pH 1.8, 3 °C) occur at least in two steps. In the urea concentration range of 0–2 M (step 1), a cooperative destruction of the tertiary structure occurs, resulting in a mildly denatured state (D_U), which may still contain a little amount of secondary structures. In the concentration range of 2–4 M urea (step 2), the D_U state gradually loses its residual secondary structure, and increases the radius of gyration nearly to a maximum value. At 4 M urea, the polypeptide chain is highly disordered with highly mobile side chains. Increasing the urea concentration up to 8 M probably results in the more highly denatured or alternatively the stiffer chain conformations. The conformational transition starting from the N state proceeds essentially the same way as in the above scheme in which D' is replaced with N. The conformational transition starting from the D state lacks step 1 because the D state contains no tertiary structures and is similar to the D_U state. The fact that similar conformations are reached at urea concentrations above 2 M from different conformations of D', D, and N indicates that the effect of urea dominates in determining the polypeptide conformation of SSI in the denatured states rather than the pH and temperature.

Keywords: circular dichroism; nuclear magnetic resonance; small-angle X-ray scattering; *Streptomyces subtilisin inhibitor*; urea-denatured state

Much effort has recently been made on structural characterization of protein folding intermediates and unfolded polypeptide chains (Dill & Shortle, 1991; Shortle, 1993). For example, a compact denatured state or the molten globule state is commonly produced in acid-denatured proteins under high ionic strength or in folding steps (Ohgushi & Wada, 1983; Kuwajima, 1989; Goto et al., 1990; Dobson, 1992; Ptitsyn, 1992). The addition of denaturing agents

such as alcohol also produces a compact denatured state as well as a highly extended helical conformation (Kamatari et al., 1996). However, except some special cases, much is still unknown about the detailed structures and their variation of the non-native species of proteins. Here we report the result of our investigation in conformational variations of denatured states of a protein as a function of concentration of a typical denaturant, urea, by a combined use of circular dichroism (CD), proton nuclear magnetic resonance (¹H-NMR) and small-angle X-ray scattering (SAXS). We chose, as a system of study, *Streptomyces subtilisin inhibitor* (SSI), because we can compare the effect of urea in two distinctly different denatured structures of the protein, i.e., in the cold-denatured (D') and heat-denatured (D) states.

SSI is a well-characterized dimeric protein consisting of two identical subunits, each having 113 amino acids and two disulfide bonds, the two subunits being associated strongly through hydro-

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Abbreviations: SSI, *Streptomyces subtilisin inhibitor*; SAXS, small-angle X-ray scattering; R_g, radius of gyration; CD, circular dichroism; NMR, nuclear magnetic resonance; N, the native state; D, the heat-denatured state; D', the cold-denatured state; D_U, the mildly urea-denatured state; U_R, the randomly urea-denatured conformation; U_E, the extended urea-denatured conformation.

phobic interface consisting of a five-stranded β -sheet (Mitsui et al., 1979; Hiromi et al., 1985). Below pH about 3.0, SSI is acid denatured and is dissociated into monomeric units (Tamura et al., 1991b; Konno et al., 1995). At least two forms of the acid-denatured states, one heat-denatured (D) above about 20 °C and the other cold-denatured (D') below about 20 °C are known (Tamura et al., 1991a, 1991b). In the D' state of SSI, part of the polypeptide chain forms a globular structure, while the rest of the chain is disordered and expanded to a comparable degree as in D (Konno et al., 1995). The D and D' states give two independent systems to examine the effect of urea; a typical heat-denatured state, and a representative of a denatured conformer with a limited tertiary structure. Thus, in the present work, we compare the manner how increasing urea concentration from 0 to 8 M changes the polypeptide chain conformation of SSI that exists initially (when no urea is present) in the D' and D states, in comparison with the case of the native (N) state, in terms of the secondary structure, the tertiary structure, and the chain form.

Results

Circular dichroism

Changes in ellipticity at far- and near-UV regions for the D', D, and N conformers of SSI were studied as a function of urea concentration (0–8 M). The spectral changes in the near-UV region are shown in detail for the urea concentration range 0–1.5 M (Fig. 1) and the general trends of ellipticity changes for the whole range of urea concentration (0–8 M) are plotted in Figure 2, typically at 222 and 292 nm.

For the D' state (cold-denatured, pH 1.8 and 3 °C; Tamura et al., 1991a, 1991b; Konno et al., 1995), changes in $[\theta]_{292}$ occur quite sharply, in the urea concentration of 0–1.5 M (open circles in Fig. 2A). By increasing the urea concentration further from 1.5 M to 8 M, no more significant change in $[\theta]_{292}$ was observed. As discussed later (see Discussion), we would suggest that the cooperative change in $[\theta]_{292}$ at low urea concentration represents a cooperative destruction of the tertiary structure in D'. As for $[\theta]_{222}$ of D', however, the transition occurs more gradually in a wider urea concentration range (0–8 M) (open circles in Fig. 2B). At 1.5 M urea, where the change of $[\theta]_{292}$ for D' is almost completed, only half of the total loss of $[\theta]_{222}$ is achieved. This suggests that a little amount of the secondary structure exists even after the complete destruction of the tertiary structures of the D' states and that the nearly complete loss of the secondary structure is achieved at 4–8 M urea.

For the D state (heat-denatured, pH 1.8 and 35 °C), the urea-induced change in $[\theta]_{292}$ is small (Fig. 2). This indicates that a tertiary structure involving specific interactions of aromatic residues in the D state is trivial, if at all. As for $[\theta]_{222}$, only a slight decrease in the value occurred gradually in the urea concentration from 0 M to 8 M (Fig. 2B), indicating that only a small amount of residual secondary structure in the D state may be gradually lost by the addition of urea up to 8 M.

For the N state (pH 3.0 and 20 °C), changes in $[\theta]_{292}$ by the addition of urea are almost the same as that of D' (Fig. 2A). As for $[\theta]_{222}$, however, changes in the value in the urea concentration range in 1.5–4 M are less clear than the case of D', indicating that further destruction of secondary structures of the N condition may be smaller in urea concentration exceeding 1.5 M. We also note that the $[\theta]_{222}$ value in 8 M urea is significantly larger for D' than

Fig. 1. CD spectral changes of SSI (10 mg/mL) in the near-UV region induced by low concentrations of urea in N (pH 3.0, 20 °C, A), D' (pH 1.8, 3 °C, B), and D (pH 1.8, 35 °C, C). Urea concentration was varied in 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.5 M.

for D or N. The physical origin of this difference is unclear at present.

Solution X-ray scattering

SAXS experiments were performed for SSI under typical conditions for D' (pH 1.8, 3 °C), D (pH 1.8, 35 °C), and N (pH 3.0,

Fig. 2. Urea-induced changes of SSI (10 mg/mL) in ellipticities at 222 nm (A) and at 292 nm (B) for N (*: pH 3.0 and 20 °C), D' (open circle; pH 1.8 and 3 °C), and D (closed circle; pH 1.8 and 35 °C) states. The dashed lines indicate positions of 1.5 M urea.

20 °C), with varying concentrations of urea (0–8 M). The data were analyzed according to the protocol used in Konno et al. (1995). Typical Guinier plots in the protein concentration of 10 mg/mL are shown in Figure 3, which indicates the existence of a linear region in each curve. Because scattering curves in urea solutions depended significantly on the protein concentration (Konno et al., 1995), R_g values extrapolated to zero protein concentration were obtained by linearly fitting R_g^2 versus [SSI] plots (Table 1). Under the condition stabilizing D' (pH 1.8, 3 °C), addition of urea up to 2 M sharply increased R_g values from 20.7 to 26.0 Å, close to the R_g value of the D state. In the urea concentration range

Fig. 3. Guinier plots of SSI for D' (open circle), D' + 4 M urea (closed circle), and D' + 8 M urea (*). The concentration of SSI is 10 mg/mL. For clarity, each plot is shifted on the $\ln[I(Q)]$ axis. The regression lines were fitted to the data within $R_g \cdot Q < 1.3$.

above 2 M, the R_g values for both D' and D conditions continued to increase gradually and reached 29–30 Å at 8 M urea. Similar experiments for SSI in the native condition (pH 3.0, 20 °C) in 2–8 M urea gave R_g values that were indistinguishable from those obtained for the D' and D conditions (Table 1).

The Kratky plot, $I(Q) \cdot Q^2$ versus Q , is a useful expression to describe the overall shape of a chain molecule (Glatter & Kratky, 1982), and has been widely used to study folding intermediates and structures of non-native states of proteins (Damaschun et al., 1991, 1993; Flanagan et al., 1993; Kataoka et al., 1993, 1995; Konno et al., 1995). The essence is that the Kratky plot shows a clear peak at $Q \sim 0.1$ for a globular conformation, while it has a plateau and then increases monotonically for an expanded unfolded conformer (Flanagan et al., 1993; Kataoka et al., 1993, 1995; Konno et al., 1995).

At pH 1.8 and 3 °C, the Kratky plot of SSI in the D' state exhibits a clear peak, indicating a globular shape of at least part of

Table 1. Radii of gyration (R_g in Å) of in the D', D, and N states SSI under various concentrations of urea, obtained from R_g^2 versus [SSI] plots

[urea]	D' (pH 1.8, 3 °C)	D (pH 1.8, 35 °C)	N (pH 3.0, 20 °C)
0 M	20.7 ^a	25.8 ^a	22.9 ^{a,b}
2 M	26.0	25.9	25.3
4 M	28.3	28.2	28.8
8 M	29.3	29.8	29.2

^aData from Konno et al. (1995).

^bSSI is homo-dimeric in N but monomeric in D' and D (Konno et al., 1995).

the molecule (Konno et al., 1995; see also Fig. 4). Upon addition of urea, the globular pattern quickly diminishes and the pattern becomes close to the random-chain pattern at 2 M urea. On the other hand, at pH 1.8 and 35 °C, SSI in the D state shows little hint of globularity even without urea (Fig. 4). However, by addition of urea up to 2 M, the Kratky pattern slightly changes in the direction for more highly disordered structure. The difference in the Kratky pattern between the D' and D states is largely lost in 2 M urea. In the range of 2–4 M urea, the plots for both D' and D exhibit typical random-chain patterns. We also found that, in the presence of 2–8 M urea, the Kratky pattern for the N condition (pH 3.0, 20 °C) was almost identical with those for the D' and D conditions (data not shown).

Another interesting point may be the change in the Kratky pattern between 4 M and 8 M urea (Fig. 5). Both at 3 °C (D') and 35 °C (D), curves in the region of $Q > 0.1$ at 8 M urea seem to have positive slopes that were not found at 4 M urea. A similar result was obtained also at pH 3.0 and 20 °C (the N condition; data not shown). One of the interpretations of this result may be that the SSI chain at 8 M urea is more expanded and highly denatured as compared to that at 4 M urea. Alternatively, the polypeptide chain at 8 M urea has a larger persistent length than that at 4 M urea. The

Fig. 4. Urea-induced changes in Kratky plots of SSI (5 mg/mL) in D' (pH 1.8, 3 °C; left column) and D (pH 1.8, 35 °C; right column) in the urea concentration range of 0–4 M.

Fig. 5. Comparison of the Kratky plots of SSI (5 mg/mL) in 4 M (open circles) and (—) 8 M urea under the D' (A: pH 1.8, 3 °C) and D (B: pH 1.8, 35 °C) condition.

chain may become stiff by binding a large number of urea molecules per protein in 8 M urea. However, these structural implications are not conclusive at present because of relatively high noise level of the data in Figure 5.

¹H-NMR spectra

As for D', the signals with specific chemical shifts at 0 to –1.0 ppm (Val, Leu) or at 5.5–6.0 ppm (β -sheet protons) and at 8.8 ppm (His106 C2 proton) indicate the existence of a specific tertiary structure, while the overall feature of the spectrum indicates the existence of highly disordered segments (Tamura et al., 1991b; Konno et al., 1995; see also Fig. 7 and Discussion). All these signals specific for the tertiary structure of the D' state (arrows in Fig. 6) disappeared completely at 1.5 M urea (Fig. 6), and the resulting spectrum (Fig. 6, 1.5 M) is close to that of D (Fig. 7, no urea).

In the ¹H-NMR spectrum for the D state, most of the signals are relatively broad. This is a typical situation for a ¹H-NMR spectrum of a heat-denatured protein, suggesting that a conformational heterogeneity due to limited intra-chain interactions. By addition of urea under the D condition (pH 1.8, 35 °C), line-narrowing of the proton signals occurred in a wide range of urea concentration,

Fig. 6. Changes in the ^1H -NMR spectra of SSI (10 mg/mL $^2\text{H}_2\text{O}$) induced by low concentration of urea under the D' condition (pH* 1.8, 3°C). Origins of the peaks marked with (open circle) are the same as in Figure 7.

0–8 M (Fig. 7). Similar line-narrowing was also observed in the aliphatic region of the spectra (data not shown). The narrowing of the signals indicates that the limited intrachain interactions in D (pH 1.8, 35°C, no urea) and in D' (pH 1.8, 3°C, 1.5 M urea) are removed gradually by urea and that the polypeptide chain assumes a random conformation with highly flexible side chain at 8 M urea.

Fig. 7. Urea-induced changes in the ^1H -NMR spectra of SSI (10 mg/mL $^2\text{H}_2\text{O}$) at 400 MHz under the D' (pH* 1.8, 3°C) and D (pH* 1.8, 35°C) conditions. Peaks marked with (open circle) are assignable to amide proton signal that increased with an increased fraction of protons in the solvent by addition of originated from urea. The peak at ~0.5 ppm marked with (closed circle) is due to an impurity.

Discussion

Cooperative structural units in the D' state

We have previously reported that the D' state contains the specific tertiary structure that is different from that exist in the N state (Tamura et al., 1993a, 1993b; Konno et al., 1995). The existence of the tertiary structure was mainly indicated by ^1H -NMR spectra of D' state. In the NMR spectrum for D', there are the characteristically shifted signals, which include signals at high-field (<0.7 ppm) assigned to the methyl protons of Val and Leu, the ring protons of His43, His106, and Tyr7, the methyl protons of Met70 and Met73, and a group of low-field shifted α -proton signals in the 5.5 to 6.5 ppm (Tamura et al., 1991b). 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. In addition, ^1H -NOESY spectra for the D' state shows some cross-peaks between the specifically shifted ring protons and the high-field shifted aliphatic protons (Konno, unpubl. obs.). Based on these, together with the other observations by far-UV CD, NMR, and SAXS, we have suggested 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 (Konno et al., 1995).

In the present report, we would suggest another sign for the tertiary structure formation in the D' state, that is, near-UV CD spectra. Although the spectral shape for D' without urea is quite different from that for N, the cooperative change of the spectra in the low urea concentration range (Fig. 2A) well coincides with the disappearance of the specific peaks in the ^1H -NMR spectra (Fig. 6), suggesting that the near-UV CD spectra contain structural information on the tertiary structure of D'.

For a comparison of the urea-induced denaturation processes of N and D', we fitted experimental data using a function;

$$-RT \ln K_d = \Delta G_0^0 - \Delta m_{\text{urea}}[\text{urea}], \quad (1)$$

where K_d is the equilibrium constant of denaturation, and ΔG_0^0 is the free energy difference between unfolded and folded states of the protein without urea. Δm_{urea} is a measure of difference in the number of bound urea molecules between the unfolded and folded states. Experimental K_d values were obtained from the transition curve of $[\theta]_{292}$ for N (Fig. 2) and those of $[\theta]_{292}$ (Fig. 2) and the NMR peak intensity of the C2 proton of His106 for D' (Fig. 6). A non-linear least-squares method was applied for fitting the data sets to the Equation 1. At present, we cannot exclude the possibility that some denatured states other than D' coexisted at pH 1.8, 3°C. Actually, the peak area of the His106 ^1H -NMR signal is around 75% of that expected for a single proton peak estimated from the peaks in the other spectral regions (data not shown; more detailed analysis on the phase properties of denatured SSI is under progress and left for future works). The value 70% may be a lower limit for the fraction of D' at pH 1.8, 3°C without urea. In the present analysis, we use this value as the fraction of D' in this condition not to overestimate the cooperativity of the transition curves of D'. The fitting parameters thus determined are listed in Table 2. Comparison of the estimated parameters ΔG_0^0 between N and D' has no absolute meaning because these values depend on pH and temperature. Δm_{urea} has been interpreted as a measure of difference in exposed surface area between the unfolded and folded

Table 2. Parameters obtained by fitting urea-induced unfolding curves with Equation 1

	ΔG_0^0 (kJ/mol)	Δm_{urea} (kJ/mol per M)
D' (pH 1.8, 3 °C), $[\theta]_{292}^a$	2.2	-6.2
D' (pH* 1.8, 3 °C), His106 ^b	2.3	-5.6
N (pH 3.0, 20 °C), $[\theta]_{292}^a$	8.3	-10.3

^aThe theoretical fitting was performed using a data set of $[\theta]_{292}$.

^bThe theoretical fitting was performed using a data set of intensity of His106(C2) proton signal in the NMR spectra of D'.

states, that is, a larger Δm_{urea} corresponds to a larger cooperative structural unit destroyed (Pace, 1986; Schellman, 1987). The determined values of Δm_{urea} are around -6 kJ/mol per M for D' and -10.3 kJ/mol per M for N. This suggests that the size of the cooperative unit of D' at pH 1.8, 3 °C destroyed by urea is about half in size to that for N at pH 3.0, 20 °C. The Δm_{urea} value for D' is even comparable to those for other proteins with similar sizes, for example, $\Delta m_{\text{urea}} = -4.6$ kJ/mol per M for myoglobin (MW = 17,800; Konno & Morishima, 1993) and -5.9 kJ/mol per M for ribonuclease T1 (MW = 11,100; Pace, 1986).

In the above analysis, in addition to the uncertainty for the fraction of D' at no urea, variations in physico-chemical properties of SSI-urea interactions as a function of pH and temperature are not known at the moment. Thus, the quantitative interpretation of Δm_{urea} may be limited. However, the present analysis leads to a conclusion that the cold denatured state D' at pH 1.8, 3 °C contains a cooperative unit that is significant in size. The present result further supports the view that D' actually exists in a rigidly folded form at least in part of the SSI chain. This implies that SSI (or a part of it) has two stable folds—one in the dimeric N form, and the other in the monomeric D' form. According to this view, the phase transition induced by temperature increase from D' to D at low pH (Tamura et al., 1991a) could be regarded as the thermal denaturation of the monomeric folded form of SSI.

Structures of SSI as a function of urea concentration

In the present study, we investigated the conformational transition in two forms of denatured states (D', and D) as well as in N of SSI in a wide range of urea concentration under acidic conditions. We

found that the conformational changes of SSI under the D' condition proceeds according to the following scheme;

The physical properties of the various conformations in the scheme 1 are summarized in Table 3.

[Step 1] In the low urea concentration range (0–2 M), a cooperative destruction of tertiary structures of D' occurs, resulting in a mildly denatured state (D_U), still containing a small amount of secondary structure (Table 3).

[Step 2] In the concentration range of 2–4 M urea, the polypeptide chain gradually loses their residual secondary structure (Fig. 2B), and its radius of gyration reaches nearly the maximum values (Table 1). At 4 M urea, the Kratky pattern reaches nearly that of a random chain (Fig. 4), and the NMR signals become sharper showing that intra-chain interactions are removed and the side chains become mobile (Fig. 7). Therefore, the denatured state at 4 M urea has a typical property of a highly disordered polypeptide chain (U_R). Further increase in urea concentration up to 8 M probably induce a further small conformational change, that is, the more expansion and denaturation or the elongation of the persistent length in the chain conformation (U_E) (Fig. 5). Note that, because the conformational changes in step 2 occur rather gradually, it is our view that D_U, U_R, and U_E are not distinct thermodynamic states from one another but rather conformational variants in the urea denatured state.

Under the condition where N is stable, the addition of urea causes essentially the same type of transitions as those of D'. The only difference is that the change in secondary-structure contents monitored by $[\theta]_{222}$ in step 2 is less evident for N. Under the condition where D is stable, the tertiary structure lost even at zero urea concentration, and hence, step 1 in Scheme 1 is absent. The conformational properties of the polypeptide chain are quite similar in the entire urea concentration range of 2–8 M, irrespective of whether the original structure at 0 M urea is D', D, or N.

It has been noticed that acid- or temperature-induced denatured states of a protein are less disordered than denaturant-induced denatured states (Dobson, 1992). The present study has clearly demonstrated that the degree of disorder, as expressed in the secondary and tertiary structure, the globularity, and the radius of gyration, is highly dependent on urea concentration. A range of non-native conformation can be distinguished and characterized by the combined use of three major techniques for the structural

Table 3. Summary of structural properties of various conformational states of SSI

	Rg ^a (Å)	$[\theta]_{222}$ (deg · cm ² /mol)	$[\theta]_{292}$	Chemical shift dispersion	Chain shape ^b
D' (pH 1.8, 3 °C)	20.7	-1,270	-50	Intermediate	Globular
D _U (pH 1.8, 3 °C, 2 M urea)	26.0	-560	+2	Small	Random chain
U _R (pH 1.8, 3 °C, 4 M urea)	28.3	-310	-4	None	Random chain
U _E (pH 1.8, 3 °C, 8 M urea)	29.3	-130	-4	None	Extended chain
N (pH 3.0, 20 °C)	22.9	-1,740	-39	Large	Globular
D (pH 1.8, 35 °C)	25.8	-1,020	+10	Small	Restricted chain

^aRg values are determined from Guinier plot of SAXS.

^bChain shapes are determined from Kratky plot of SAXS.

analysis of proteins in solution, CD, NMR, and SAXS. The fact that common conformations are reached from different structures of SSI in the D' and D condition at urea concentrations above 2 M indicates that the effect of urea dominates in determining the polypeptide conformation in the denatured states rather than the difference in pH and temperature.

Materials and methods

Materials

Crude solutions of SSI were obtained by cultivating *Streptomyces albobrisesolus* S-3253 and were purified on chromatographic columns with DE52 (Whatman, Maidstone, England) and Sephacryl S-200 (Pharmacia, Uppsala, Sweden), as described by Sato and Murao (1973). Protein solutions for all the experimentation were freshly prepared before experiments by dissolving lyophilized powders into water without using buffer (pH 3.0 or 1.8). 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).

CD measurement

CD spectra were measured in the wavelength range of 190–250 nm with a Jasco J-720 spectropolarimeter using a quartz cell with a light path of 1 or 10 mm. Protein concentration was kept at 870 μ M (as monomer) throughout. The sample solutions contain no buffer, pH being adjusted with HCl. The temperature of the solution was maintained with a thermostatically controlled water bath within $\pm 0.1^\circ\text{C}$.

Solution X-ray scattering measurement

Solution X-ray scattering experiments on the native and non-native 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 measurements of SAXS, calibrated by meridional diffraction of dried chicken collagen. The wave length of the X-ray was 1.488 Å. The sample cell was 50 μ L in volume with 15- μ m-thick quartz windows, and had a 1 mm X-ray pathlength. The temperature of the sample was controlled by circulating the temperature-controlled water. The protein concentrations used for any urea concentrations were 3, 5, 8, and 10 mg/mL, and the measurement time for each scattering experiment was 10 to 15 min. The scattering from the solution containing no SSI was measured for each concentration of urea to subtract scattering from the background.

Analysis of SAXS data

Data processing was carried out with both Macintosh (Apple) personal computers. X-ray scattering intensities at the small-angle region are 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 0 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) was obtained from the slope of the Guinier plot, a plot of $\ln[I(Q)]$ against Q^2 (Guinier & Fournet, 1955).

^1H -NMR measurement

^1H nuclear magnetic resonance (NMR) spectra of SSI at 400 MHz were measured in $^2\text{H}_2\text{O}$ on a JEOL Lambda-400 using a sample tube of 5 mm diameter at a protein concentration of 10 mg/mL. The pH* values were adjusted with dilute ^2HCl and are given as direct pH meter readings calibrated against standard $^1\text{H}_2\text{O}$ buffers. Temperatures of the samples were controlled by a JEOL temperature controller and were calibrated with a thermistor to the accuracy of $\pm 0.1^\circ\text{C}$. Although the ^1H -NMR spectra were measured in $^2\text{H}_2\text{O}$, not in $^1\text{H}_2\text{O}$ as in CD and SAXS, the effect of this difference on the phase diagram of SSI is small (Tamura et al., 1991b) and causes no problem in the discussion of this study.

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