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Cold Denaturation and Heat Denaturation of *Streptomyces* Subtilisin Inhibitor. 2. ^{1}H NMR Studies†

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ABSTRACT: Structural transitions of the protein *Streptomyces* subtilisin inhibitor (SSI) from the native state to the cold-denatured and heat-denatured states were studied by ^{1}H NMR spectroscopy in the temperature range from −10 to 60 °C in the acidic pH range. Assignments of some of the ^{1}H NMR signals of SSI in the cold-denatured and heat-denatured states were performed by a combined use of selective deuteration and site-directed mutagenesis. Throughout the pH range from 2.1 to 3.1, both transitions were cooperative and basically only three distinct spectra corresponding to structures in the cold-denatured, native, and heat-denatured states were detected. In the cold-denatured state, the side-chain signals of Met73, His106, at least one Val, and two Leu were observed at distinctly shifted positions from those for a random-coiled structure, suggesting the formation of a tertiary structure, while those of Met70, His43, and Ala2 were observed at positions for a random-coiled structure. This tertiary structure in the cold-denatured state is entirely different from that in the native state, as some amino acid residues exposed to the solvent in the native state (e.g., Met73, His106) are buried while those sequestered in the native state (e.g., His43) are exposed. In the heat-denatured state, however, most ^{1}H NMR signals were observed at random-coiled positions, indicating that there is much less tertiary structure in the heat-denatured state than in the cold-denatured state. At pH values below 2.09, a structural transition was observed from the cold-denatured state to the heat-denatured state without passing through the native state. A sedimentation equilibrium experiment indicated that the two subunits of SSI were dissociated in both the heat-denatured and cold-denatured states, eliminating the possibility of formation of a tertiary structure in the cold-denatured state by intersubunit interaction, such as by aggregation.

The preceding paper using circular dichroism (Tamura et al., 1991b) indicated that neither the cold-denatured (D′) nor the heat-denatured state (D) of *Streptomyces* subtilisin inhibitor (SSI;[1] see Figure 1 for the tertiary structure) is perfectly random coiled and that the two states differ slightly but distinctly in their secondary structure. Furthermore, DSC experiments suggested that a tertiary structure might be formed from hydrophobic amino acid residues in the D′ form of SSI.

In the present paper, a detailed study of the structural transitions of SSI was undertaken using ^{1}H NMR spectroscopy, which reflects the tertiary structure of a protein more directly than CD and DSC. Our points of interest here are (1) whether SSI takes three distinct states, N, D, and D′, differing in their tertiary structure, (2) whether and how the tertiary structure of SSI in the D′ state is different from that in the D state, and (3) whether the transitions of tertiary structures between the N and D′ forms (cold denaturation),

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[1] Abbreviations: SSI, *Streptomyces* subtilisin inhibitor; NMR, nuclear magnetic resonance; CD, circular dichroism; DSC, differential scanning calorimetry.

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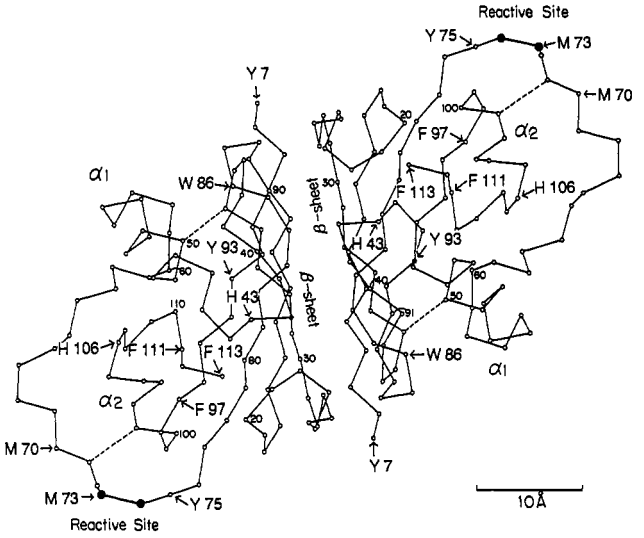

FIGURE 1: Tertiary structure of SSI in the crystal (Mitsui et al., 1979) with location of amino acid residues of Met (70, 73, and 103), Tyr (7, 75, and 93), Phe (97, 111, and 113), His (43 and 106), and Trp (86).

between the N and D forms (heat denaturation), and between the D′ and D forms are all cooperative and proceed as two-state transitions.

One of the difficult points for the ^1H NMR study of the denatured states is the specific assignment of signals because of the overlap of signals from the same kind of amino acid residues (Wüthrich, 1986). Specific signal assignments may be possible for some signals of SSI because of the indications from the CD and DSC studies that SSI in the denatured states does not assume random-coiled structures. We employ selective deuteration and site-directed mutagenesis for signal assignments, since specific isotope labeling of any kind of amino acid residues of SSI has been successfully performed biosynthetically (Kainosho & Tsuji, 1982; Akasaka et al., 1988; Hiromi et al., 1985), and site-directed mutagenesis of specific amino acid residues in SSI has also been established (Kojima et al., 1990, 1991). Finally, the question of whether SSI exists as a dimeric protein or a dissociated monomeric unit in the cold-denatured and heat-denatured states will be answered by the sedimentation equilibrium experiment.

Materials and Methods

^1H NMR Measurements. ^1H NMR spectra were measured at 400 MHz on a JEOL GX-400 spectrometer equipped with a variable-temperature accessory. The temperature values given are those of the sample solutions in NMR tubes, precalibrated with a Cu–Constantan thermocouple prior to the NMR measurements. For most experiments, the SSI samples were dissolved in 25 mM deuterated glycine buffers (in a pH range from 2.0 to 3.1) at a fixed concentration of 5.0 mg/mL. The pH values given are the direct readings of the pH meter calibrated against ^1H_2O buffers. ^1H NMR spectra in ^1H_2O buffers (containing 2.5% ^2H_2O) were measured by using a 1–1 pulse (Clore et al., 1983). 3-(Trimethylsilyl)-3,3,2,2-tetradeuteropropionic acid sodium salt (TSP) was employed as an internal chemical shift reference.

Preparation of Selectively Deuterated SSI. Selective deuterium substitutions of amino acid residues in SSI were performed by cultivating *Streptomyces albogriseolus* S-3253 in synthetic culture media containing the desired deuterium-labeled amino acids (Kainosho & Tsuji, 1982), i.e., Trp (the five ring protons were deuterated), Phe (the five ring protons were deuterated), Val (the six γ-methyl and one β protons were deuterated), Leu (the six δ-methyl, one γ, and two β protons were deuterated), or Met (the three ε-methyl protons were deuterated). The deuterium content of these amino acids was more than 98%. Selective deuterations of the C_2 protons of His43 and of His106 were performed by selective hydrogen–deuterium exchange of either of the C_2 protons with solvent ^2H_2O, by taking advantage of the difference in pK_a of the two His residues (pK_a = 6.40 for His106, His43 is not titratable unless SSI is denatured below pH 3; Fujii et al., 1980).

Preparation of Mutated SSI. Colonies of transformed *Streptomyces lividans* 66 which contained the mutated SSI gene (Met70 replaced by Ile or Met73 replaced by Leu) were prepared by site-directed mutagenesis and were cultivated in tryptone soya broth (OXOID) at 33 °C for 6 days. Secreted SSI was purified by passing it through an anion-exchange (DE52, Whatman) and a gel filtration (Sephacryl S-200, Pharmacia) column.

Sedimentation Equilibrium Experiments. Sedimentation equilibrium experiments were carried out with a Beckman Model E ultracentrifuge equipped with a double-sector cell. The speed of rotation was set at 20 410 or 18 000 rpm. The concentrations of SSI were in a range from 0.98 to 4.47 mg/mL in 25 mM glycine buffers at acidic pH or in 25 mM phosphate buffers at neutral pH.

Results and Discussion

Effects of Solvent Water on the Stability of SSI. In order to correlate the ^1H NMR data which were obtained mainly in ^2H_2O environments to the CD and DSC data obtained in ^1H_2O environments (Tamura et al., 1991b), the structures and the stabilities of SSI in the two environments were examined by ^1H NMR. Figure 2 shows the temperature dependence of the ^1H NMR spectra in the aliphatic region of SSI in ^1H_2O (a, pH 2.50, 2.15) and in ^2H_2O environments (b, pH 2.54, 2.13), respectively. In the ^1H_2O environment (Figure 2a), the spectrum at 15 °C, pH 2.50, is that for the native structure. The spectral changes were observed by both increasing and decreasing the temperature from 15 °C, showing the change in the tertiary structure associated with the heat denaturation and the cold denaturation, respectively. Similar changes were observed in ^2H_2O (Figure 2b), only at somewhat higher temperatures. The transition temperature of heat denaturation (T_d) in ^2H_2O (35 °C at pH 2.54, 37 °C at pH 2.62) was higher than that in ^1H_2O (30 °C at pH 2.50, 31 °C at pH 2.61) by about 5 °C, in agreement with earlier observations (Nakanishi & Tsuboi, 1976; Komiyama et al., 1984; Tamura et al., 1991a). The transition temperature of cold denaturation (T'_d) was almost the same in ^2H_2O (0 °C at pH 2.54, −3 °C at pH 2.62) as in ^1H_2O (1 °C at pH 2.50, −3 °C at pH 2.61). T_d and T'_d were determined here as temperatures at which the signal intensities of Met73 of the native (N) and heat-denatured (D) forms and those of the native and cold-denatured (D′) forms, respectively, were equal (signal assignments will be made in the following section). The ^1H NMR spectra of the N, D, and D′ forms of SSI, however, were not affected by the change of the solvent from ^1H_2O to ^2H_2O, showing that the tertiary structures of the three conformers were not affected by the choice of the solvent. Therefore, in the following, we performed ^1H NMR experiments in ^2H_2O environment, keeping the fact in mind that the heat denaturation occurs at temperatures about 5 degrees higher in ^2H_2O than in ^1H_2O.

Assignments of NMR Signals in the Denatured States. Specific assignments of ^1H NMR signals of SSI in the native state have so far been established for the aromatic protons [Tyr7, -75, and -93, from Fujii et al. (1981); His43 and -106, from Fujii et al. (1980); Trp86, from Akasaka et al. (1988);

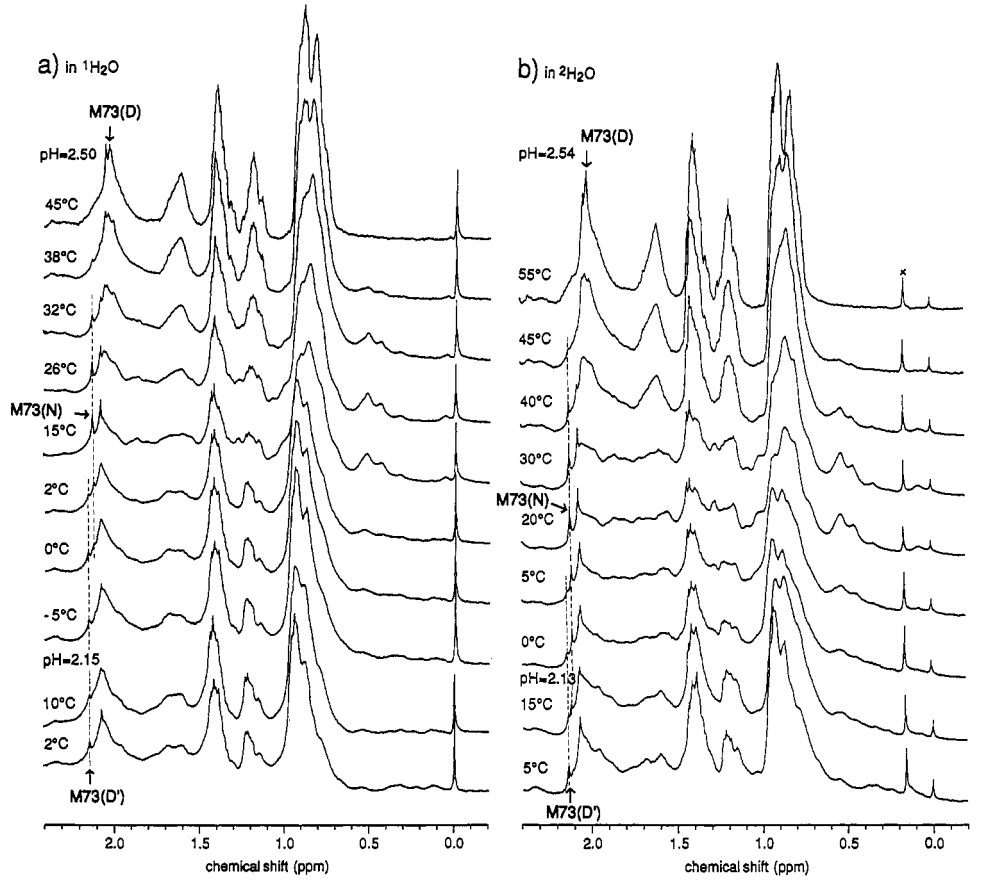

FIGURE 2: Temperature dependence of ^1H NMR spectra of SSI in the aliphatic region: (a) in ^1H_2O (upper, pH 2.50; lower, pH 2.15) and (b) in ^2H_2O (upper, pH 2.54; lower, pH 2.13). M73(N), M73(D'), and M73(D) indicate assigned methyl proton signals of Met73 in the native state (2.124 ppm), in the cold-denatured state (2.155 ppm), and in the heat-denatured state (2.010 ppm), respectively.

Phe97, -111, and -113], Met70, -73, and -103 (Akasaka, 1987), Lys89, and some Ala, Leu, and Val (Hiromi et al., 1985). Structural transitions of SSI accompanying the cold and heat denaturations were studied by following these assigned signals.

Figure 3 shows the temperature dependence of ^1H NMR spectra in the aliphatic region (–0.2–2.4 ppm) of (a) nonlabeled SSI (wild), (b) SSI deuterated for the γ-methyl and β protons of Val residues (13, 16, 20, 31, 56, 69, 74, 78, 82, 85, 91, 96, and 110; abbreviated to wild[Val-^2H]), (c) SSI deuterated for the δ-methyl, γ, and β protons of Leu residues (9, 12, 14, 33, 53, 60, 63, 79, and 80; abbreviated to wild[Leu-^2H]), and (d) SSI deuterated for the ϵ-methyl protons of Met residues (70, 73, and 103; abbreviated to wild[Met-^2H]). No signals were observed at high field (<0.7 ppm) at high temperatures, suggesting the lack of hydrophobic cluster in the heat-denatured state (D). On the other hand, signals shifted at high field (<0.7 ppm; shown by arrows in Figure 3a) were clearly observed at low temperatures (below 0 °C). This indicates the formation of a hydrophobic cluster or a tertiary structure involving some methyl groups and probably some aromatic residues in the cold-denatured state (D'). By comparison of part a with parts b and c of Figure 3 at lower temperatures, we can assign signals at 0.13 and 0.31 ppm to methyl protons of Val and those at 0.55, 0.35, and 0.21 ppm to methyl protons of Leu (the D' form). The result indicates that the tertiary structure of the D' form of SSI is made up of at least one Val and two Leu residues. These signals are unique to the D' form, indicating that the tertiary structure of the D' form is different from that in the N form. The sharp signal at 1.542 ppm in the native SSI has been assigned to the methyl protons of Ala2 whose Asp1 in the N-terminal segment is lost by proteolysis during the culture (Akasaka, 1985). The presence of the same signal in the D' form (Figure 3c) suggests that the N-terminal segment of SSI in the D' form is also exposed to the solvent with high mobility as in the N form.

Specific assignments of the methyl proton signals of Met70, -73, and -103 in both denatured states were performed by utilizing artificial mutants: (a) Met70 replaced by Ile (abbreviated to 70I; Kojima et al., 1990), and (b) Met73 replaced by Leu (abbreviated to 73L; Kojima et al., 1991). It is apparent from Figure 4 that these substitutions scarcely affected the conformation and stability of SSI. By comparison of the spectra of 70I and 73L with the spectra of wild[Met-^2H] (Figure 3d), at low temperatures, two peaks at 2.155 ppm (Figure 4a, pH 2.12 at –4 °C) and 2.070 ppm (Figure 4b, pH 2.10 at –2 °C) were specifically assigned to the methyl protons of Met73 and Met70, respectively, in the D' form of SSI. Similarly, at high temperatures, two peaks at 2.010 ppm (Figure 4a, 60 °C) and 2.028 ppm (Figure 4b, 60 °C) were specifically assigned to the methyl protons of Met73 and Met70, respectively, in the D form of SSI. At 60 °C, the peak at 2.010 ppm remained even in 73L, although the peak disappeared completely in wild[Met-^2H] (50 °C, pH 2.23, Figure 3d). This indicates that there is another Met resonance at 2.010 ppm, assignable to the methyl protons of Met103 in the D form of SSI. The position of the methyl proton signal of Met103 in the D' form could not be recognized in Figure 4, probably because of the broadening. The spectra at 20 °C (Figure 4) confirmed the earlier tentative assignments of the methyl proton signals at 2.078, 2.124, and 1.78 ppm to the methyl protons of Met70, -73, and -103 in the N form of SSI, respectively (Akasaka, 1987).

Figure 5 shows the temperature variation of ^1H NMR spectra in the aromatic region (5.4–10.6 ppm) of (a) nonla-

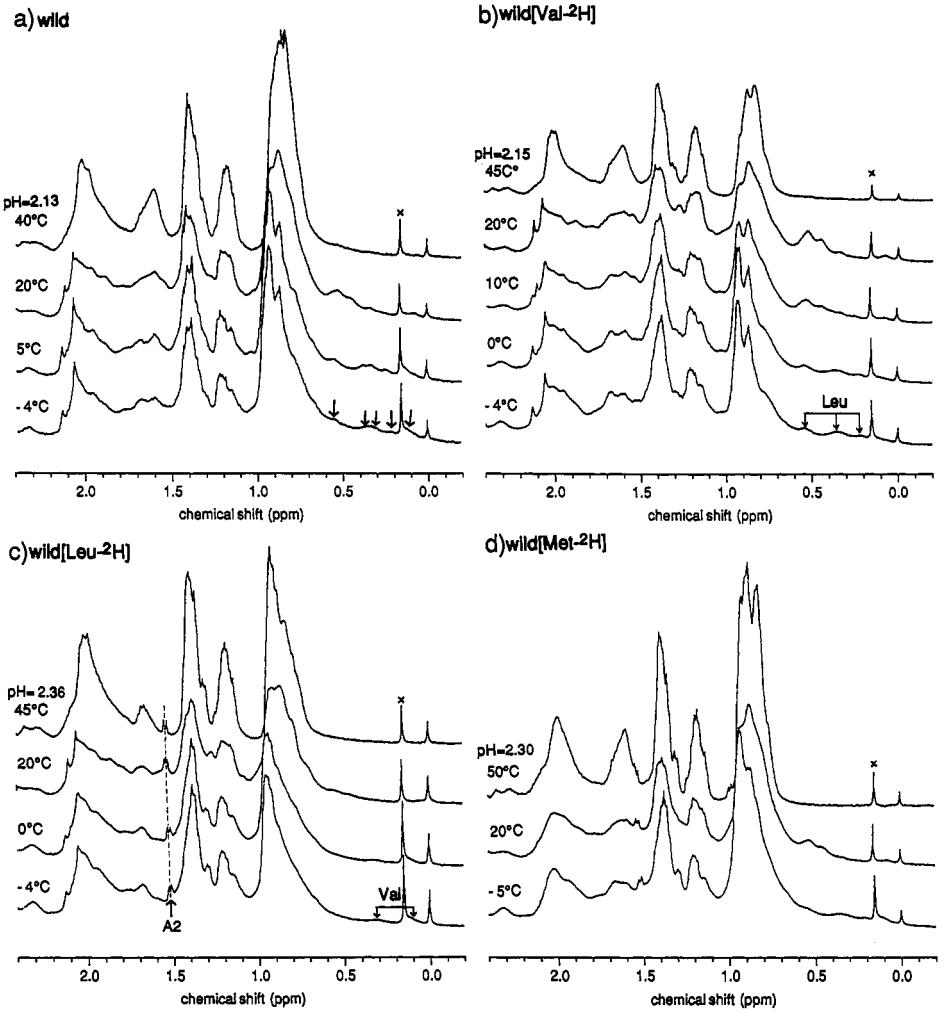

FIGURE 3: Temperature dependence of ^1H NMR spectra of deuterium-labeled SSI in the aliphatic region: (a) nonlabeled SSI, pH 2.13; (b) SSI with deuterated side chains of all Val, pH 2.15; (c) SSI with deuterated side chains of all Leu, pH 2.36; (d) SSI with deuterated ϵ-methyl groups of all Met, pH 2.30. Arrows indicate the methyl proton signals of Leu and Val (a), which are classified into Leu (b) and Val (c).

beled SSI (wild), (b) SSI deuterated for the ring protons of Phe97, -111, and -113 (abbreviated to wild [Phe-^2H]), and (c) SSI deuterated for the ring protons of Trp86 (abbreviated to wild[Trp-^2H]). Comparison of these three spectra gave classification of the aromatic proton signals into those of Phe, Tyr, His, and Trp in both the cold- and heat-denatured states. In Figure 5b, the two sharp doublets at 6.80 and 7.10 ppm at −2 °C, pH 2.10 (D′ form), each with intensities of two protons by integration, are assignable to the 3,5 and 2,6 protons of one well-exposed Tyr residue. Since the N-terminal segment of the D′ form is exposed and mobile as mentioned earlier, it is likely that these signals arise from Tyr7 in the cold-denatured state. Thus, two Tyr residues, probably Tyr75 and Tyr93, are apparently buried in the cold-denatured state in contrast to only one (Tyr93) in the native state. In Figure 5c, the broad signals centering around 7.23 ppm at low temperatures (D′ form) are assignable to the ring protons of the two Tyr and three Phe (97, 111, and 113). The contribution to these signals from Phe can be evaluated by comparing part c with part b of Figure 5. The broadness of the signals of the two Tyr and the three Phe residues suggests that the mobilities are restricted in the D′ state. The signals of Trp86 are also broad at low temperatures (Figure 5b), suggesting that it is immobilized in the cold-denatured state, while the very sharp signals at high temperatures (Figure 5b) indicate that the Trp ring is exposed and mobile in the heat-denatured state. The broad singlet at 7.80 ppm in the D′ form (Figure 5a) is attributable to the C_4 protons of His43 and His106, as it did not disappear by the deuterations of Phe (Figure 5b), Trp (Figure 5c), and His C_2 protons (Figure 6).

Figure 6 shows the temperature dependence of the ^1H NMR spectra in the aromatic region of (a) SSI with the C_2 proton of His106 replaced by deuteron (abbreviated to wild-[His106-^2H]) and (b) SSI with the C_2 proton of His43 replaced by deuterium (abbreviated to [His43-^2H]). The C_2 proton of His43 resonates at a random-coiled position (8.65 ppm) upon increasing or decreasing temperature (Figure 6a), indicating that His43, sequestered in the native state, becomes exposed to the solvent in both the cold-denatured and heat-denatured states. In the course of transition from N to D′ observed at higher pH values (Figure 6b), a signal of the C_2 proton of His43 appeared at 9.45 ppm (Figure 6b), which is abnormally low-field-shifted (Gross & Kalbitzer, 1988). This signal is always accompanied by the C_2 proton signal of His43 of the N form (7.96 ppm). Moreover, the loss of the combined intensities of this and the N form signal of His43 correlates with the increase of the C_2 proton signal of His43 in the D′ form when the temperature is decreased. Thus, we tentatively assign this signal to the C_2 proton of the protonated form of His43 within the native manifold of SSI (abbreviated to the N′ form). Figure 6c shows that the C_2 proton of His106 resonates at a random-coiled position (8.65 ppm) upon increasing temperature, while upon decreasing temperature it resonates at a unique position of 8.85 ppm, shifted to lower

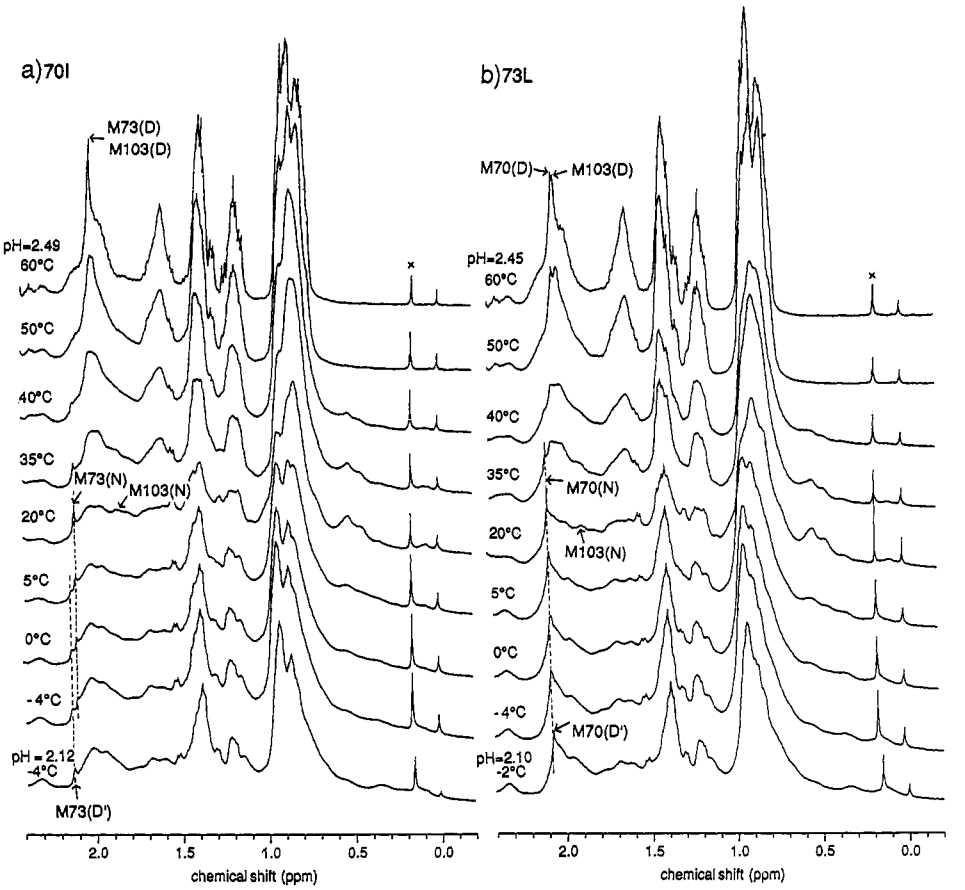

FIGURE 4: Temperature dependence of ^{1}H NMR spectra of mutated SSI in the aliphatic region: (a) SSI with Met70 replaced by Ile (70I); (b) SSI with Met73 replaced by Leu (73L). N, D′, and D represent SSI in the native, cold-denatured, and heat-denatured states, respectively. Determined chemical shift values: M73(N), 2.124 ppm; M73(D′), 2.155 ppm; M73(D), 2.010 ppm; M70(N), 2.078 ppm; M70(D′), 2.070 ppm; M70(D), 2.028 ppm; M103(N), 1.78 ppm; M103(D), 2.010 ppm.

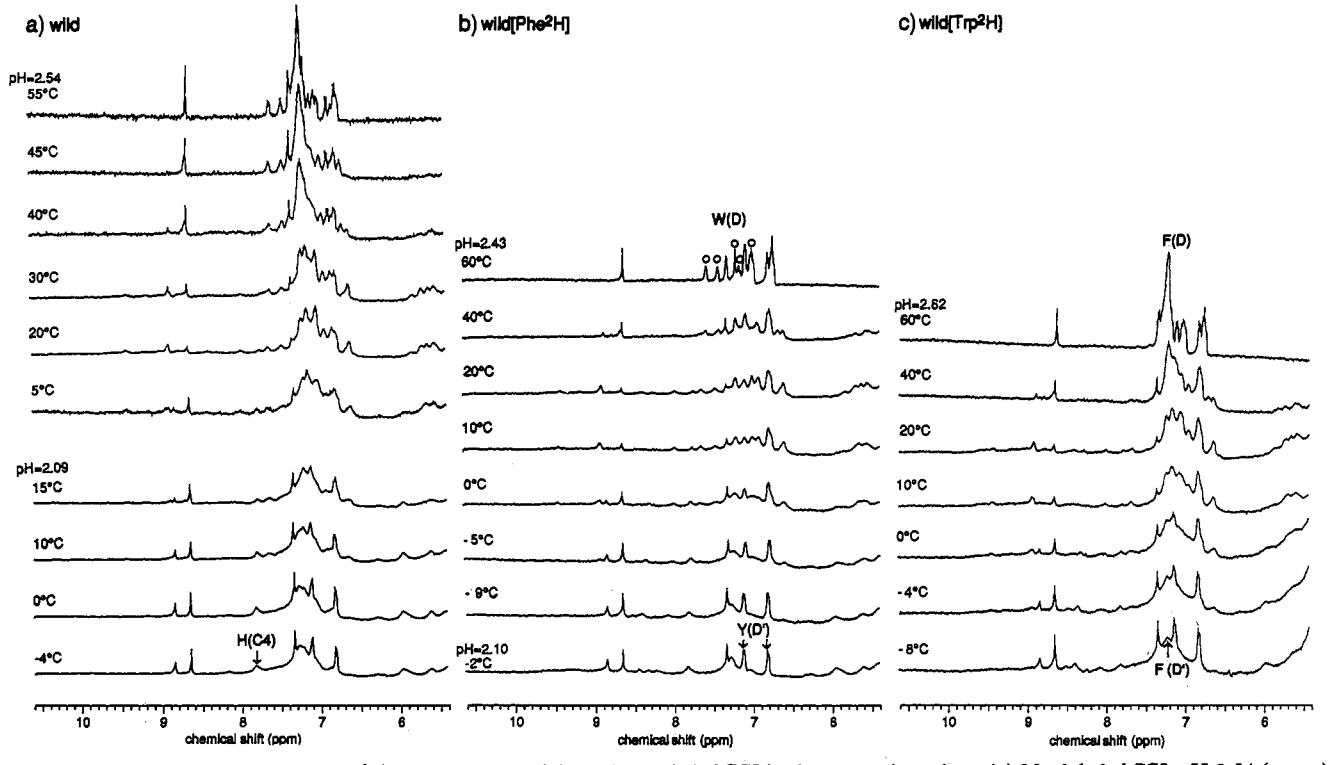

FIGURE 5: Temperature dependence of ^{1}H NMR spectra of deuterium-labeled SSI in the aromatic region. (a) Nonlabeled SSI, pH 2.54 (upper) and 2.09 (lower): H(C4) refers to the C_4 proton of His (−4 °C). (b) SSI with deuterated ring protons of Phe, pH 2.43 (upper) and 2.10 (lower): The open circles indicate the ring proton signals of Trp (60 °C). Y(D′) refers to the 3,5 (6.80 ppm) and 2,6 (7.10 ppm) protons of Tyr in the cold-denatured form (−2 °C), respectively. (c) SSI with deuterated ring protons of Trp, pH 2.62: F(D′) and F(D) refer to the ring proton signals of Phe in the cold-denatured and heat-denatured forms, respectively.

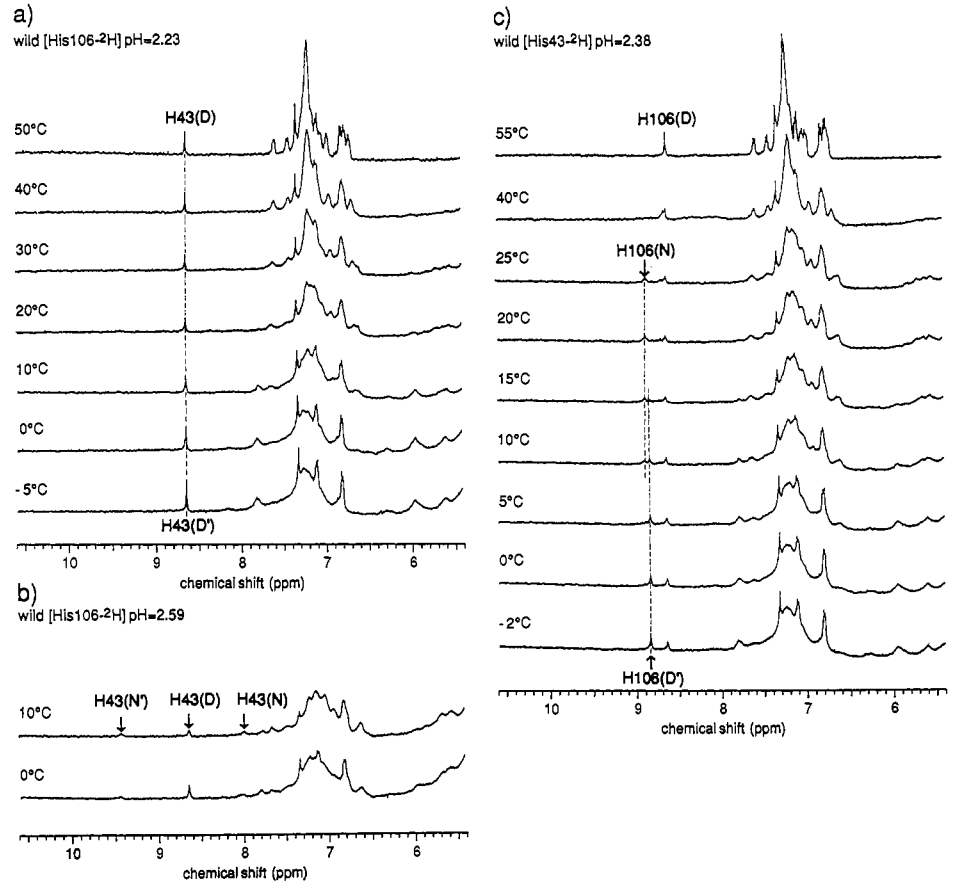

FIGURE 6: Temperature dependence of ^1H NMR spectra of deuterium-labeled SSI in the aromatic region: (a) SSI with deuterated C_2 proton of His106, pH 2.23; (b) same as (a), pH 2.59; (c) SSI with deuterated C_2 proton of His43, pH 2.38.

field by 0.20 ppm from the random-coiled position. This indicates that His106, located in the α_2-helix and exposed to the solvent with a pK_a of 6.40 in the N form (Tamura et al., 1991a), is involved in an interresidue interaction in the D′ form. The small signal at 8.65 ppm (Figure 6c, low temperature) is attributable to the remaining C_2 proton of His43 due to imperfect deuteration.

Transition at a Lower Value of pH. Figure 7 shows the temperature dependence of ^1H NMR spectra of nonlabeled SSI at pH 2.09 (a) in the aliphatic region and (b) in the aromatic region. From the NMR spectra, it is clear that below 0 °C SSI takes almost fully the cold-denatured form and that above 50 °C it takes almost fully the heat-denatured form. Only a low fraction of the native form spectrum is apparent in the entire temperature range studied at pH 2.09. Thus the spectral changes between 0 and 50 °C in Figure 7 represent a direct transition from the cold-denatured structure to the heat-denatured structure in SSI.

Structures in the Cold-Denatured and Heat-Denatured States. The ^1H NMR spectra of SSI in the fully cold-denatured state were identical irrespective of the pH of measurements (Figures 2, 3, 4, 5, 6, and 7). The same was true for the heat-denatured state. These observations indicate that SSI takes distinctive tertiary structures in both the cold-denatured and heat-denatured states. During the transitions from N to D, N to D′, and D′ to D, ^1H NMR spectra consist basically of two kinds of signals attributable to either N, D, or D′ forms. All assigned ^1H NMR signals transformed from N to D, N to D′, or D′ to D at identical temperatures, with an exception of the C_2 proton signal of His43 in the transition from N to D′ (Figure 6b). These observations indicate that all these structural transitions can be considered basically as two-state transitions with respect to the tertiary structure. This cooperative nature of the transitions of the tertiary structure observed by ^1H NMR is in parallel with the cooperative nature of transition of the secondary structure observed by CD at various wavelengths (Tamura et al., 1991b).

Almost all the protons of SSI in the heat-denatured state resonate at positions close to random-coiled peptides, indicating that its tertiary structure is highly disrupted. In contrast, some signals in the cold-denatured state showed distinct deviation from random-coiled positions, although many other signals are at random-coiled positions. This indicates that the cold-denatured state has certain distinctive tertiary structure, in agreement with the suggestion from the DSC experiments (Tamura et al., 1991b). The tertiary structure in the D′ form, however, should be entirely different from that in the N form, as the NMR spectra indicate that some amino acid residues exposed to the solvent in the N form (e.g., Met73, His106) are buried in the D′ form, while those sequestered in the N form (e.g., His43) are exposed in the D′ form. The observed high-field shifts of the methyl protons of at least one Val and two Leu of the D′ form are likely to be due to ring current effect of some aromatic groups. The broad signals of ring protons of three Phe, two Tyr, and one Trp residues, reflecting restricted motions, also suggest involvement of these aromatic groups in the formation of this tertiary structure (Figure 3). On the other hand, the CD spectra indicate that the secondary structure of SSI in the D′ form is lost almost to the same extent as that of the D form (Tamura et al., 1991b). The presence of a distinctive tertiary structure and the low content of a secondary structure in the D′ form of SSI make sharp contrast to the concept of the molten globule state (Ogushi & Wada, 1983), which is characterized by the lack of a tertiary structure and the high content of a secondary structure.

Sedimentation Equilibrium Experiments. In order to ex-

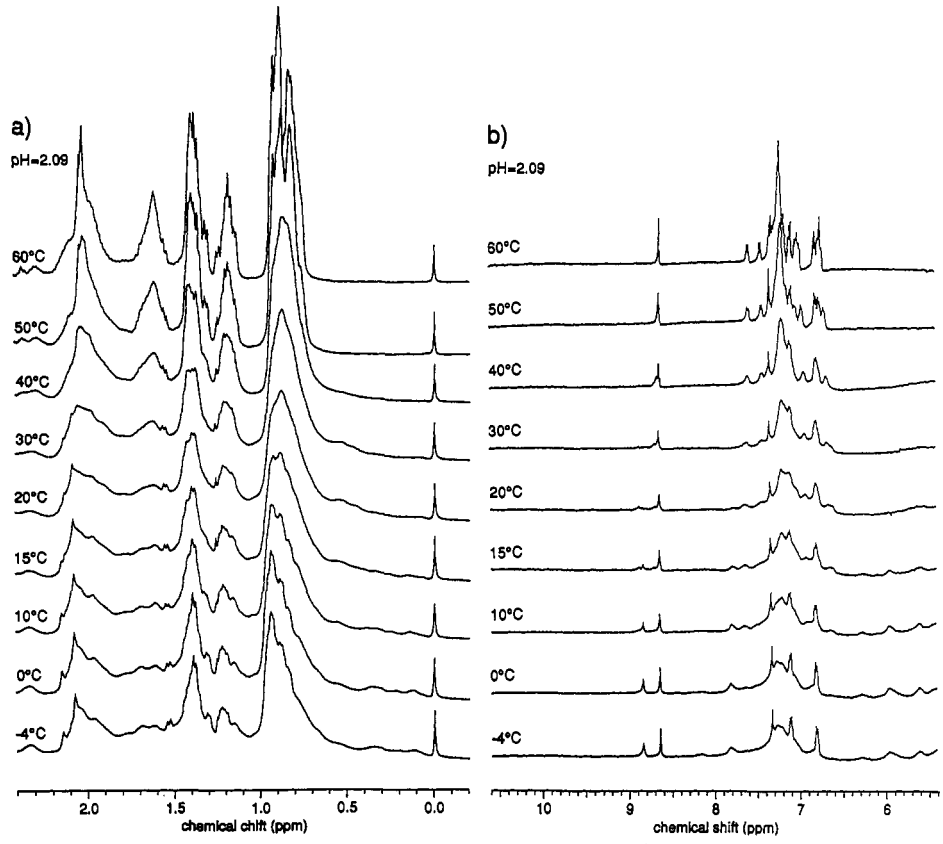

FIGURE 7: Temperature dependence of ^{1}H NMR spectra of SSI at pH 2.09 (a) in the aliphatic region and (b) in the aromatic region.

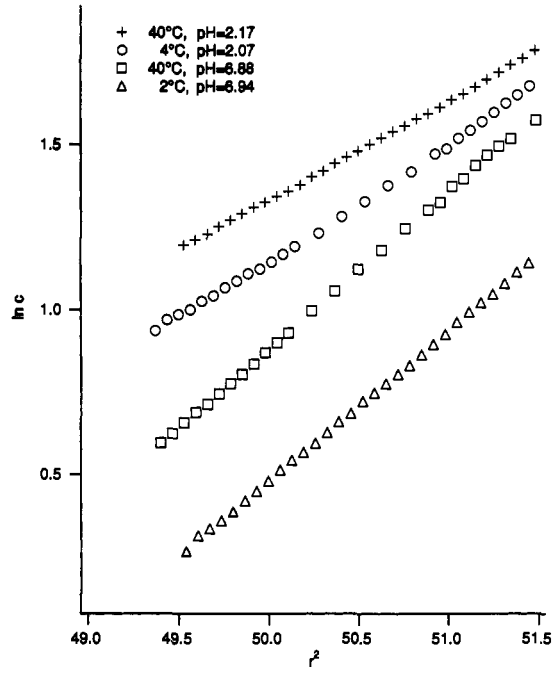

FIGURE 8: Plot of the logarithm of concentration against the square of the radius of gyration (cm^2) in the sedimentation equilibrium experiment: (+) pH 2.17, 40 °C, protein concentration 2.72 mg/mL; (○) pH 2.07, 4 °C, 2.25 mg/mL; (□) pH 6.88, 40 °C, 2.02 mg/mL; (△) pH 6.94, 2 °C, 2.05 mg/mL.

amine the possibility of formation of the tertiary structure by intersubunit interaction between subunits, e.g., aggregation, the effective molecular weights of the SSI in the native, cold-denatured, and heat-denatured states were determined by sedimentation equilibrium experiments. Figure 8 shows the plot of the logarithm of concentration versus radius of gyration. From the slope of the plot, effective molecular weights were determined to be 21 000 (pH 7, 40 °C; N form), 12 000 (pH 2, 40 °C; D form), 22 000 (pH 7, 2 °C; N form), and 13 000 (pH 2, 4 °C; D′ form). The determined effective molecular weight of SSI in the D′ form is close to that of the formula value of the monomeric form of SSI (11 500), indicating that the two subunits of SSI are dissociated, and hence the tertiary structure of the D′ form is of intrasubunit origin. It also denied the possibility that the broadness of some of the ^{1}H NMR signals of SSI in the D′ form (e.g., Phe) was caused by aggregation of the subunits. The result also confirmed the earlier suggestion from the DSC studies (Takahashi & Sturtevant, 1981; Tamura et al., 1991b) that the subunits are dissociated upon heat denaturation.

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Dissociation of Calcium from the Phosphorylated Calcium-Transporting Adenosine Triphosphatase of Sarcoplasmic Reticulum: Kinetic Equivalence of the Calcium Ions Bound to the Phosphorylated Enzyme†

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ABSTRACT: The internalization of ^{45}Ca by the calcium-transporting ATPase into sarcoplasmic reticulum vesicles from rabbit muscle was measured during a single turnover of the enzyme by using a quench of 7 mM ADP and EGTA (25 °C, 5 mM $MgCl_2$, 100 mM KCl, 40 mM MOPS·Tris, pH 7.0). Intact vesicles containing either 10–20 μM or 20 mM Ca^{2+} were preincubated with ^{45}Ca for ~20 s and then mixed with 0.20–0.25 mM ATP and excess EGTA to give 70% phosphorylation of E_{tot} with the rate constant k = 300 s^{-1}. The two ^{45}Ca ions bound to the phosphoenzyme (EP) become insensitive to the quench with ADP as they are internalized in a first-order reaction with a rate constant of k = ~30 s^{-1}. The first and second Ca^{2+} ions that bind to the free enzyme were selectively labeled by mixing the enzyme and ^{45}Ca with excess ^{40}Ca, or by mixing the enzyme and ^{40}Ca with ^{45}Ca, for 50 ms prior to the addition of ATP and EGTA. The internalization of each ion into loaded or empty vesicles follows first-order kinetics with k = ~30 s^{-1}; there is no indication of biphasic kinetics or an induction period for the internalization of either Ca^{2+} ion. The presence of 20 mM Ca^{2+} inside the vesicles has no effect on the kinetics or the extent of internalization of either or both of the individual ions. The Ca^{2+} ions bound to the phosphoenzyme are kinetically equivalent. A first-order reaction for the internalization of the individual Ca^{2+} ions is consistent with a rate-limiting conformational change of the phosphoenzyme with k_c = 30 s^{-1}, followed by rapid dissociation of the Ca^{2+} ions from separate independent binding sites on E~P·Ca_2; lumenal calcium does not inhibit the dissociation of calcium from these sites. Alternatively, the Ca^{2+} ions may dissociate sequentially from E~P·Ca_2 following a rate-limiting conformational change. However, the order of dissociation of the individual ions can not be distinguished. An ordered-sequential mechanism for dissociation requires that the ions dissociate much faster ($k \geq 10^5$ s^{-1}) than the forward and reverse reactions for the conformational change (k_{-c} = ~3000 s^{-1}). Finally, the Ca^{2+} ions may exchange their positions rapidly on the phosphoenzyme ($k_{mix} \geq 10^5$ s^{-1}) before dissociating. A Hill slope of n_H = 1.0–1.2, with $K_{0.5}$ = 0.8–0.9 mM, for the inhibition of turnover by binding of Ca^{2+} to the low-affinity transport sites of the phosphoenzyme was obtained from rate measurements at six different concentrations of Mg^{2+}.

The calcium-transporting ATPase of sarcoplasmic reticulum (SR)[1] couples the chemical free energy released from the hydrolysis of ATP to the vectorial transport of two calcium ions into the lumen of the SR (de Meis & Vianna, 1979; Jencks, 1980; Inesi, 1985). Scheme I describes a simple catalytic cycle for the coupled reaction in which the different states of the enzyme are defined by their chemical composition, rather by numbers or signs (Makinose, 1973; Pickart & Jencks, 1984).

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[1] Abbreviations: CaATPase, calcium-transporting ATPase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EP, phosphoenzyme; MOPS, 4-morpholinepropanesulfonic acid; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; SR, sarcoplasmic reticulum; SRV, sarcoplasmic reticulum vesicles.

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