A Circular Dichroism Study on Thermal Denaturation of a Dimeric Globular Protein, *Streptomyces* Subtilisin Inhibitor

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Thermal denaturation of *Streptomyces* subtilisin inhibitor was studied by means of circular dichroism (CD) measurements in the far-UV and near-UV regions. The denaturation was found to be largely reversible; the partial irreversibility was associated with a slight loss of the inhibitory activity. Difference CD spectra in the far-UV region clarified the existence of two distinct steps in the thermal transition of the secondary structure. The first step below 80°C is attributable to a partial conformational change in the α-helix portion, whereas the second step between 80°C and 94°C is attributable to a major conformational change involving the β-sheet portion. On the assumption that the major denaturation involves dissociation of the SSI into its subunits, the enthalpy and entropy changes were determined to be 216 kcal·mol⁻¹ and to be 603 cal·deg⁻¹·mol⁻¹, respectively.

*Streptomyces* subtilisin inhibitor (SSI) is a globular protein consisting of two identical subunits of molecular weight 11,500. The subunit contains two α-helices, five-stranded β-sheet and two disulfide bonds (1–3). The β-sheet forms the subunit-subunit interface, and thus is located at the central portion of the protein, whereas the α-helices are at the peripheral region. Among aromatic residues contained in the subunit (3 Phe, 3 Tyr, and 1 Trp), the single Trp (residue 86) located in the βα-strand (2) shows the most prominent signal in the near-UV region. Absorption (4), fluorescence (5), and NMR (6) methods have been used to monitor environmental changes of Trp 86 upon thermal denaturation of SSI.

To obtain information about the global conformational change associated with thermal denaturation, we measured the CD spectra of SSI in the far-UV region at various temperatures. Near-UV absorption spectra were also measured at various temperatures to examine the change in microenvironment of the aromatic residues particularly that of Trp 86. From the profile of the changes of CD spectra with temperature, we concluded that the conformational changes of SSI occur in two steps: the first step involves loosening of the α-helix, while the second step is the major denaturation step involving dissociative unfolding concomitant with the unfolding of the hydrophobic core.

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Abbreviation: SSI, *Streptomyces* subtilisin inhibitor.
MATERIALS AND METHODS

SSI isolated from culture broth of *Streptomyces albogriseolus* s-3253 was generously supplied by Prof. Murao and was purified according to the method of Murao and Murao (7). Gly-L-Phe-Gly, Gly-L-Tyr-Gly, and Gly-L-Trp-Gly purchased from Vega Biochemicals were mixed at a ratio equivalent to the composite ratio in SSI as a model system for the near-UV spectra of SSI. Buffer solution for SSI and the model system was 0.01 M phosphate buffer, the ionic strength of which was adjusted to 0.1 by NaCl. We checked the purity of SSI by measuring the relative ratio of CD intensity to absorbance at 280 nm. This ratio is a reliable conventional measure of a purity of protein sample. The ratio shown by the purified SSI was \(2.56 \times 10^{-4}\). We assayed the inhibitory activity of SSI against caseinolysis by subtilisin BPN’ according to the method developed by Murao and Sato (8). Harimarstein casein and Folin-Ciocalteu reagent were purchased from Wako Chemicals. We measured the inhibitory activity of SSI before and after thermal denaturation.

Absorption and CD spectra were recorded on a Hitachi 340 spectrophotometer and a JASCO J-40 circular dichroism spectrometer at various temperatures, monitored by thermocouples fixed on the surface of cells. To avoid evaporation of the solvent, stoppered cells were used. The changes in the absorption spectra were recorded as difference absorption spectra relative to the initial one at 20°C. Difference absorption observed for SSI was corrected by taking account of spectral changes due to temperature effect on aromatic chromophores based on the results for model systems. The protein concentration and the light path length in the near-UV range were 1.15 mg/ml (5.00 \(\times\) 10^-5 M) and 10 mm, respectively, while below 250 nm the light path length was 0.1 mm. The magnitudes of CD were expressed in terms of \(\Delta \varepsilon\) and mean residue ellipticity \(\Theta\) (deg-cm²·dmol⁻¹) for the former and the latter wavelength ranges, respectively. \(\Theta\) can be expressed in terms of \(\Delta \varepsilon\) as follows:

\[
\Theta = \frac{32.98 \times \text{MRW} \times c \text{ (mol/liter)}}{100 \times l \times c \text{ (g/ml)}} \Delta \varepsilon
\]

MRW, \(l\) and \(c\) are the mean residue weight, the optical path length (in dm) and the concentration of the protein, respectively. CD spectra sensitive to secondary structure were analyzed by using a flexible least-squares method developed by Provenccher and Glöckner (9). The general Fortran regularization package is available on request and was applied to SSI without any changes. The sensitivity of our circular dichroism spectrometer was normalized to the reference data (10) at 1-nm intervals from 190 to 240 nm with a standard lysozyme solution in phosphate buffer (pH 7).

To compare the denaturation temperature with those obtained by other workers (6, 11), we also studied the thermal denaturation in D_2O at pH 7 (\(\mu=0.1\)).

RESULTS

Changes in the CD Spectrum in the Far-UV Region—Figure 1a shows changes in the CD spectrum in the far-UV region, which is sensitive to change in the secondary structure, during thermal denaturation. Secondary structure fractions determined by analysis of the CD spectrum at 20°C (native state) were 24% \(\alpha\)-helix, 36% \(\beta\)-sheet, and 40% remainder. The term remainder covers \(\beta\)-turn, random coil structure and nonpeptidic chromophores. The secondary structure determined from the crystallographic study comprises 12\% \(\alpha\)-helix, 21\% \(\beta\)-sheet, and 67\% random coil structure (2).

There was no striking change between room temperature and 60°C. Above 80°C, a remarkable decrease in CD intensity occurred and an isodichroic point appeared at about 209 nm. The presence of the isodichroic point indicates that the conformational transition above 80°C is a two-state transformation. Figure 1b shows difference CD spectra observed in the low (20-80°C) and in the high (80-94°C) temperature regions. The two difference CD spectra differ in profile and show two stages of unfolding processes. Plots of relative CD intensities at 218 nm versus temperature shown in Fig. 2 give a denaturation temperature, i.e., the temperature of half conversion (T_1/2), of 87°C for the major process. However, the CD spectrum at 94°C shows a residual ellipticity of about 50\% of the original, suggesting that the protein retains a kind of ordered structure in the
thermally denatured state. This interpretation is supported by a further reduction in the CD intensity which is induced by denaturants such as urea or guanidine hydrochloride (--- in Fig. 1a). In D$_2$O the denaturation temperature was found to be higher by about 5°C than in H$_2$O. Thermal denaturation was largely reversible as shown in Figs. 1a and 2. At higher concentrations, larger ellipticities were found for the renatured SSI than for the native protein, possibly indicating the presence of a small portion of aggregated species. The inhibitory activity of renatured SSI was reduced to about 95% of that of the native SSI. The reduction of the inhibitory activity was concentration-dependent and showed a gradual increase during repeated cycles of denaturation-renaturation.

**Changes in the Absorption and CD Spectra in the Near-UV Region**—Figure 3a shows the absorption spectrum of SSI at 20°C and Fig. 3b shows difference absorption spectra at various temperatures relative to the initial spectrum at 20°C. Negative peaks at 292 nm and 284 nm are attributable to a blue shift of the absorption spectra of tryptophan and tyrosine residues. In the longer-wavelength region, a weak positive difference absorption appeared with increasing temperature, with a red shifted component in the absorption spectrum in addition to the main blue shifted one. Positive peaks in the difference absorption of the model system, shown in Fig. 3c (curves 9-13), suggest that the appearance of the weak positive component in SSI spectra as due to the temperature effect on exposed aromatic residues. The model system itself shows a significant spec-
Fig. 3. (a) Absorption spectra of SSI and the model system at 20°C and difference absorption spectra ((b) and (c)) at various temperatures relative to the initial spectrum at 20°C in 0.01 M phosphate buffer at pH 7.0 (μ=0.1, NaCl). The optical path was 1 cm. Protein concentration was 5.00 x 10⁻⁵ M on a dimer basis. SSI: (a) 1, the initial absorption spectrum at 20°C; (b) temperatures are 2, 50; 3, 65; 4, 75; 5, 85; 6, 90; 7, 20 (renatured) °C. The model system: (a) 8, the initial absorption spectrum at 20°C; (c) temperatures are 9, 40; 10, 60; 11, 70; 12, 80; 13, 90°C.

Central change on heating. On the assumption that the same spectral change is included in the spectral change observed for SSI, the contribution of the model system was subtracted from the SSI spectra, and the resultant difference absorbances at 292 nm are also plotted against temperature in Fig. 3. The midpoint transition of Fig. 3 gives a denaturation temperature of 86°C.

Figure 4 shows the temperature dependence of CD intensities in the aromatic region. Above 80°C a remarkable decrease occurs and at 90°C we obtained only a diffuse spectrum. The inset of Fig. 4 shows the temperature dependence of the aromatic CD for the model system. CD intensities of SSI at 285 nm are plotted against temperature in Fig. 2 without correction for the CD changes in the model system. The plot gives a denaturation temperature of 85°C, in agreement with the denaturation temperature of 86°C determined from the absorption in the near-UV region. These spectral changes show a nearly complete loss of the native environment of the aromatic side chains. Furthermore, the spectral change in the far-UV CD also occurs at a similar temperature (87°C). The latter finding shows that the complete loss of the microenvironmental structure around aromatic residues occurs simultaneously with the denaturation of the secondary structure, although a rather extensive secondary structure is still maintained in the denatured state.

DISCUSSION

Two types of difference CD spectra shown in Fig. 1b (curves 1 and 2) discriminate two different denaturation stages below and above 80°C. This discrimination, which has not been made with other methods, was clearly apparent in the CD spectra which are sensitive to the secondary structure. Hence, thermal transition of SSI can be
CD STUDY ON THERMAL DENATURATION OF SUBTILISIN INHIBITOR

written as

\[ N \xrightarrow{\text{step I}} N' \xrightarrow{\text{step II}} D, \]

Scheme 1

where \( N, N' \), and \( D \) represent the native, the intermediate, and the denatured forms of the inhibitor, respectively.

The first difference CD (curve I) is similar to the CD of an \( \alpha \)-helix with double minima (10), indicating that the first step (step I) may involve the structural change of an \( \alpha \)-helix. If the decrease in CD intensity in step I is assumed to arise from the unfolding of an \( \alpha \)-helix alone and if the standard ellipticity \([\theta]\) of an \( \alpha \)-helix (11) applied, then the change in CD in step I corresponds to the unfolding of about three residues in an \( \alpha \)-helix. It appears likely that this conformational change in step I is attributable to the unfolding of the \( \alpha_\beta \)-helix (residue 100–107), which has been defined as being loosely helical by an X-ray study (2).

Kainosho and Tsuji have monitored the thermal denaturation of SSI by measuring the \( ^{13} \)C NMR peak of three Met residues in which the carbonyl carbons were labeled with \( ^{13} \)C (11). Met 103 is located in the \( \alpha_\beta \)-helix. The low-field shift of the carbonyl carbon resonance of Met 103 demonstrated the hydrogen-bond formation in the helix. The resonance showed a slight high-field shift with increasing temperature up to 85°C in \( D_2 \)O, suggesting that the \( \alpha_\beta \)-helix did not break down but loosened below 80°C in \( H_2 \)O. The relatively low extent of decrease (about three residues) of \( \alpha \)-helix content reflects a delicate change in conformation in step I.

The enthalpy change (\( \Delta H \)) for step I is obtained from the slope of the plots of log\((f/(1-f))\) versus reciprocal temperature using the van't Hoff relation;

\[
\Delta H = -R \frac{d \ln K}{d (1/T)}
\]  (1)

where \( K \) is the equilibrium constant between the native (N) and the intermediate (N') states and \( f \) stands for the denatured fraction and can be read from the transition curves shown in Fig. 2. In the analysis of the transition curve we used the dashed straight line segments (Fig. 2) as base lines for the native, the intermediate, and the denatured states. The existence of a plateau in the transition curve obtained for the difference absorbance makes it possible to read the denatured fraction below 80°C. From plots of difference absorbances at 292 nm below 80°C, we tentatively estimated \( \Delta H \) for step I to be about 13 kcal-mol\(^{-1}\). This value of \( \Delta H \) seems consistent with the loosening of the \( \alpha_\beta \)-helix portion. However, conformational loosening at various sites of the protein, as indicated by the intensity decrease in the near-UV CD (see Fig. 4), would contribute to the total value of \( \Delta H \).

The second difference CD (curve 2 in Fig. 1b) in step II has a minimum at 218 nm and a crossoverpoint at 207 nm, both of which are characteristics of a \( \beta \)-sheet (13). If the observed difference CD is assumed to arise from the change at the \( \beta \)-sheet portion, it corresponds to the loss of about 22% of the total of 113 residues, indicating a significant loss (about 60%) of the \( \beta \)-content in the native molecule. A large decrease in \( \beta \)-sheet portion is a reasonable candidate for the major process of denaturation of SSI, as the \( \beta \)-sheet comprises a major portion of the secondary structure of SSI, and plays an important role in maintaining the hydrophobic core of the subunit as well as maintaining the subunit-subunit interface. The present finding is in conflict with that obtained from H-D exchange IR spectroscopy that the \( \alpha \)-helices are more stable than the \( \beta \)-sheets in SSI (4).

Since SSI is a dimeric protein, two models of the major denaturation need to be considered: (i) the protein unfolds while retaining the dimeric form and (ii) the protein undergoes a dissociative transition to give two denatured monomers. In the dissociative case, D in Scheme 1 must be replaced by 2D as follows;

\[ N \xrightarrow{\text{N}} N' \xrightarrow{} 2D. \]

Scheme 2

Discrimination between Schemes 1 and 2 cannot be achieved by the present spectroscopic method. However, the results of differential scanning calorimetry (DSC) favor the dissociative scheme for thermal denaturation of SSI (14). Based on the DSC study, we assume that step II follows Scheme 2. Furthermore, \( K \), the equilibrium constant between the intermediate (N') and the denatured (D) states in step II of thermal denaturation of SSI, is given as follows;
TABLE I. Summary of the thermal denaturation parameters of SSI studied by CD and absorption methods.

<table>
<thead>
<tr>
<th>Step</th>
<th>Transition Type</th>
<th>Partial transition</th>
<th>Dissociative transition involving entire molecule</th>
<th>$T_n$ (°C)</th>
<th>$\Delta H$ (kcal-mol$^{-1}$)</th>
<th>$\lambda$ (nm)</th>
<th>Portions responsible for spectral changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step I</td>
<td>Partial transition</td>
<td>$&lt;80$</td>
<td>87</td>
<td>13</td>
<td>218</td>
<td>285</td>
<td>$\alpha$-helix</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>86</td>
<td>218±10</td>
<td>285</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step II</td>
<td>Dissociative transition involving entire molecule</td>
<td>87</td>
<td></td>
<td>216±10</td>
<td>218</td>
<td>285</td>
<td>mainly $\beta$-sheets</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>86</td>
<td></td>
<td>285</td>
<td></td>
<td>all aromatic sites</td>
</tr>
</tbody>
</table>

Fig. 5. The van't Hoff plots for the major process of thermal denaturation of SSI obtained by CD and absorption spectrosopies. $\log (f^4/1-f)$ is plotted against reciprocal temperature, assuming that SSI undergoes a dissociative transition. $\bullet$, CD at 218 nm; $\bigcirc$, CD at 285 nm; $\square$, difference absorbances at 292 nm.

$K = \frac{[D]^4}{[N]^4} = \frac{4f^4}{1-f} C_0$  \hspace{1cm} (2)

where $C_0$ is the total protein concentration. We obtained $\Delta H$ for step II from the temperature dependence of $K$ following the van't Hoff relation (Eq. 1); $f$ was obtained from the transition curves by a procedure similar to that previously described for analysis of step I.

A straight line was obtained from the average plots of $\log K$ against reciprocal temperature for the far-UV CD at 218 nm, the near-UV CD at 285 nm and the near-UV absorption at 292 nm, as shown in Fig. 5. The average value of $\Delta H$ was determined to be $216 \pm 10$ kcal-mol$^{-1}$. The corresponding average value of $\Delta S$ was calculated to be $600 \pm 28$ cal-deg$^{-1}$. Takahashi and Sturtevant have determined the van't Hoff enthalpy of dissociative unfolding of SSI to be $202$ kcal-mol$^{-1}$ by scanning calorimetry (14).

The coincidence between $\Delta H$ obtained from the present CD study and that (about $202$ kcal-mol$^{-1}$) obtained by the calorimetry based on the dissociative scheme (14) supports the present dissociative process for the major thermal denaturation. Dissociation into subunits would simultaneously cause destruction of the $\beta$-sheet because the subunit-subunit interaction is maintained by the $\beta$-sheet. This is in accordance with the $\beta$-like profile of the difference CD spectrum observed for step II.

Thermal denaturation parameters obtained from CD and absorption studies of SSI are summarized in Table I.

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CD STUDY ON THERMAL DENATURATION OF SUBTILISIN INHIBITOR


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