Intermediates in Denaturation of a Small Globular Protein, Recombinant Human Stefin B*

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Guanidinium HCl (GdmHCl), pH, and heat denaturation of the recombinant human stefin B, a low molecular weight protein inhibitor of cysteine proteinases, has been followed by circular dichroism. From the noncoincidence of the transitions in the near and far UV, the existence of stable intermediate states possessing few persistent tertiary interactions but most of the native-like secondary structure, was inferred. These intermediate states exist at equilibrium under various conditions, namely, state G at 1.7 M GdmHCl (pH 8, 25 °C), state A at pH 4 (0.6 M GdmHCl, 25 °C) and state T above 68 °C. By size exclusion chromatography, their apparent compactness was determined. The intermediate states A, T, and G were compact and are therefore classified as "molten globule" states.

It has been widely accepted that folding and denaturation of small, globular proteins may be approximated by a two-state equilibrium. Nevertheless, in several small globular proteins during the course of denaturation, states of intermediate conformation have been detected. When transitions were measured by different spectroscopic probes, equilibrium intermediates have been found for the following proteins: bovine and human carbonic anhydrase B (1, 2), \( \beta \)-lactamase from Staphylococcus aureus (3, 4), \( \beta \)-lactamase from Bacillus cereus (5), tryptophan synthase \( \alpha \)-subunit (6), bovine growth hormone (7), a chemically modified form of human growth hormone (8), \( \alpha \)-lactalbumin (9), and glutamine synthase II isoform (10).

In some cases (11–13), these states have been described as so called "molten globules" (MG) for which two main characteristics are recognized. First, they show native-like secondary structure, was inferred. These intermediate states exist at equilibrium under various conditions, namely, state G at 1.7 M GdmHCl (pH 8, 25 °C), state A at pH 4 (0.6 M GdmHCl, 25 °C) and state T above 68 °C. By size exclusion chromatography, their apparent compactness was determined. The intermediate states A, T, and G were compact and are therefore classified as "molten globule" states.

As a consequence, these structures exhibit a greater exposure to solvent of hydrophobic groups (15) and exchange hydrogens faster than the native state (13, 16). More important, molten globule intermediates have been demonstrated widely as kinetic intermediates during folding (15–18), and it is proposed that they play a general role in the folding of globular proteins (19).

Further criteria that have been used to characterize the MG states include the fact that they do not exhibit a change in enthalpy on heating and their unfolding has been reported as being a noncooperative process (17).

Evidence provided by the use of a variety of different techniques has established that the folding pathway involves a number of species of intermediate conformation, i.e.

\[
U \rightarrow X_1 \rightarrow X_2 \rightarrow \ldots \rightarrow X_n \rightarrow I \rightarrow N
\]

where \( I \) is the kinetic molten globule intermediate and \( I \rightarrow N \) is the rate-determining step (reviewed in Ref. 14).

This simple, sequential scheme becomes more complicated if dimerization and aggregation are involved. That molten globular intermediates are prone to dimerization was shown for carbonic anhydrase B (20), and protein concentration effects have been observed in some other cases (8, 10).

With human stefin A, human stefin B constitutes the stefin family of the cysteine proteinases inhibitors (21). Its primary structure has been determined (22, 23), as has the three-dimensional structure of human stefin B in the complex with papain (24). Together with chicken cystatin (25), it serves as a model for the interaction of cysteine proteinases with their inhibitors (see review in Ref. 26).

Its structure being known, stefin B represents a rather simple model for protein folding studies. It is a small protein of M, 11,000, and there are no disulfide bonds in the molecule. It has been cloned and expressed in Escherichia coli in a soluble form (27). To prevent covalent dimer formation (23), a recombinant form of human stefin B, where Cys\(^5\) has been replaced by Ser, was used throughout the present study. Various other mutations have been produced (28).

The unfolding transitions induced by GdmHCl, pH, and heat are presented. The intermediate states that appear at medium GdmHCl concentrations, at acidic pH, and at a higher temperature have been characterized by CD and by size exclusion chromatography (SEC).

**MATERIALS AND METHODS**

Cloning and Isolation of the Protein—Recombinant human stefin B was prepared as described (27). It was expressed intracellularly in E. coli, using the pKP 1500 expression vector. It was purified by a two-step procedure, comprising affinity chromatography on papain-Sepharose and ion-exchange chromatography on a MonoQ column (fast protein liquid chromatography system) (27), a slight modification to the previous procedure (23).

A value \( A_{594}^\text{th} = 6.8 \) was used for determining the concentration of stefin B from its absorbance at 280 min.

**Chemicals**—Ultra-pure GdmHCl was from GIBCO-BRL. Concentration was determined by refractive index measurements (29). Buffer was 0.02 M Tris, 0.13 M NaCl, pH 8.0, or in a few experiments, 0.02 M HEPES, 0.1 M NaCl, pH 7.0.
Fluorescence—A Perkin-Elmer LS-3 spectrophotometer was used to measure tyrosine emission at 303 nm, with the excitation wavelength set at 280 nm. Bandwidth was 10 nm, throughout. The cell was thermostatted. Fluorescence intensity was expressed as relative fluorescence I/I₀, where I₀ is the corresponding value for the denatured state.

Circular Dichroism—A DICHROGRAPH III (Jobin Yvon) was used to measure circular dichroism in the near (260–320 nm) and the far (200–250 nm) ultraviolet regions. The cell was thermostatted. Representative wavelengths of 277 and 222 nm were chosen, respectively, as indicators of tertiary and secondary structure of the protein. For calculating the mean residue ellipticity in deg cm²/dmol, a mean weight of 112/residue (MRW) was used.

Preparation of the Protein Solutions—For denaturation by pH, the samples were prepared by dialyzing the protein against 0.02 M acetate buffer, 0.6 M GdmHCl, or against 0.6 M GdmHCl in pure water. Initial pH values of the protein solutions were 7.5 and 7.1 (±0.2), respectively. Concentrated acetic acid or 0.1 M Tris buffer was added to achieve the required pH values. After equilibrating for 20–30 min at each pH value, the ellipticities at 277 and 222 nm were measured in 2- and 0.1-cm cells, respectively. After the equilibrium measurements, the samples with pH 3.5 or 10.5 were diluted to pH 8.0 and dialyzed against Tris buffer, pH 8.0, 0.6 M GdmHCl, in the cold. The reversibility was around 80%.

For denaturing the protein by heat, the solution was gradually heated in the thermostatted block of the CD spectrometer. The temperature in a 2-cm cell filled with water was measured by a thermocouple, and a calibration curve between the temperatures in the cell and the temperature of the water bath was drawn. At each 3–5 °C interval, the protein was left to equilibrate for 10–15 min, and the CD at 277 and 222 nm was measured in the cuvettes of 2 or 0.1 cm of length, respectively. Separate solutions of the same concentration were used for the measurement at 277 or 222 nm. Some aggregation was observed on heating the sample until above the transition temperature. Around 90% reversibility could be shown if the protein that was heated to 70 °C for 15 min was slowly cooled and filtered before repeating the experiment.

Size Exclusion Chromatography at Equilibrium—A thermostatted size exclusion Superose 12 column (Pharmacia LKB Biotechnology Inc.) was connected to a fast protein liquid chromatography system. The technique of denaturant gradient size exclusion chromatography was introduced by Endo et al. (30) and later applied to 17 globular proteins (31). Shalongo et al. (32, 33) have studied the folding of thioredoxin and ribonuclease A in this manner.

Our procedure differed somewhat from the denaturant gradient one (30), as equilibrated samples were applied on the column, precolumn equilibrated with the same GdmHCl concentration. The volume of elution is inversely proportional to the Stokes radius (34). Therefore, longer retention is usually connected to higher compactness. This holds only for proteins of the same size and shape that do not interact with the column. In such a case, different conformations or associated states of the protein in question can be detected.

**RESULTS AND DISCUSSION**

GdmHCl Denaturation Followed by Spectroscopy—GdmHCl denaturation of the recombinant human stefin B was followed at pH 8.0 by three spectroscopic probes: near UV CD, far UV CD, and tyrosine emission (Fig. 1). From the data at 25 °C, the fractions of the native state were calculated as defined:

\[ f_n = (x - x_0)/(x_N - x_0) \]  

where \( x \) is the value for a spectroscopic parameter and \( x_0 \) and \( x_N \) are the values for the native and denatured states, respectively.

The choice of base lines is critical for the evaluation of experimental data. For the fluorescence data, the dependence on GdmHCl was extrapolated to the transition region. As the same slope is observed with either native or denatured states, this may be prescribed to a nonspecific (solvent) effect of GdmHCl on protein fluorescence. Dependence of CD at 222 nm on GdmHCl in the denatured base-line region was sometimes linearly extrapolated to the transition region (6, 10). Anyhow, our data points of \( [\theta]_{222} \) from 3 to 4.5 M GdmHCl, which are prone to a high relative error of around 30%, do not permit such an extrapolation. We rather took a constant value of (−500 ± 200) deg cm²/dmol for the totally denatured state at 4.0 M GdmHCl.

Fractions of the native state against GdmHCl concentration are given in Fig. 2 for the three probes. The curves from the tyrosine fluorescence and the near UV CD experiments both exhibit transition midpoints at 1.6 M GdmHCl. The curve from the far UV CD is reproducibly shifted to a higher transition midpoint at 1.75 M GdmHCl and is also less cooperative. In Fig. 2, in addition to spectroscopic results, SEC data are shown, which are discussed in the following section.

Denaturational transitions at 25 °C followed by multiple probes have shown that the unfolding equilibria for stefin B are more complex than two-state, and that states of conformation intermediate between the native and fully unfolded are significantly populated at intermediate concentrations of denaturant. The protein could be placed into group C₁, as classified by Saito and Wada (31), even though no clear cut intermediates at equilibrium exist.

The near and far UV CD spectra at some GdmHCl concentrations are shown in Fig. 3. The results of the CONTIN analysis (35, 36), the fractions of \( \alpha, \beta \), and the remainder (R) secondary structure are shown in Table I and compared with the known secondary/tertiary structure of the native stefin B (24) and to the same analysis of stefin A.

GdmHCl Denaturation Followed by SEC—Denaturation of stefin B was monitored by SEC at 2 and 25 °C. SEC elution profiles are shown in Figs. 4 and 5.

At 2 °C, the exchange between unfolded and folded population was slowed down, and separate peaks were observed. The fraction of the native state was assumed to equal the area of the peak corresponding in elution volume to the folded protein divided by the total area and is presented as an inset to Fig. 4. The triangles in the inset to Fig. 4 were obtained from the far UV CD measurement performed at 2 °C. The above results indicate that the protein behaves in a two-state...
Intermediates in Denaturation of a Globular Protein

**Fig. 2.** Fraction of the native state ($f_n$) against GdmHCl (GuHCl) molarity at 25 °C. - - - , Tyr emission at 303 nm; — — , CD at 277 nm; - - - , CD at 222 nm; ----- , volumes of elution (SEC).

**Fig. 3.** CD spectra of the recombinant human stefin B in the near and far UV. All of the spectra were measured at 15 °C, pH 8.0. a, native protein; b, 1.53 M GdmHCl; c, 2.1 M GdmHCl; c', 1.9 M GdmHCl. MRW, mean residue weight.

**Fig. 4.** Size exclusion chromatography at 2 °C. SEC elution profiles of the stefin B, which were equilibrated in GuHCl concentrations as indicated. The column was Superose 12 (Pharmacia LKB Biotechnology Inc.). It was thermostatted to 2 °C by a fitted coat. Protein concentration was around 0.3 mg/ml. In the transition region of denaturation, apparently two peaks are in equilibrium. The native peak (a) has a $V_e$ of 16 ml, and the denatured-like peak (b) has a $V_e$ of 14.8 ml. The fraction of the native state, which is shown in the inset (O), was calculated by dividing the area of the native peak by the total area. For comparison, data from CD at 222 nm (A) are given. GuHCl, guanidinium hydrochloride.

**Table I**

<table>
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<tr>
<th>Protein/form</th>
<th>%α</th>
<th>%β</th>
<th>%R</th>
<th>Method</th>
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<tbody>
<tr>
<td>Stefin B/native</td>
<td>20</td>
<td>39</td>
<td>41</td>
<td>X-ray (24)</td>
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<td>30</td>
<td>45</td>
<td>Max α</td>
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<td>8</td>
<td>39</td>
<td>53</td>
<td>Chosen*</td>
</tr>
</tbody>
</table>

* The author of Ref. 35 suggests that the solution of highest α value should be considered if it can still adequately fit the experimental data. In such cases, it should be given preference to the “chosen solution.” R, remainder.

manner at 2 °C, changing from an unfolded to a native state in a cooperative fashion. This behavior is similar to that for stefin A under similar conditions (37, 38).

At 25 °C, fractions of the native state were calculated by the usual equation (Equation 1), where $x$ represents volumes of elution. The results are presented in the inset to Fig. 5, together with the far UV CD data. As stated in the previous section when discussing the spectroscopic results (Fig. 2), the protein does not behave in a two-state manner. SEC data at 25 °C (also presented in Fig. 2) confirm that a compact state exists from 1.6 to 1.8 M GdmHCl, which has no persistent tertiary interactions but much of the native-like secondary structure. This permits a conclusion that the major secondary structure change (total unfolding) takes place from within a compact intermediate with a $V_e$ of 15.7 ± 0.2 ml. The $V_e$ of 15.7 ml is close to the $V_e$ of the native, dimeric state.

It was possible to show that the state with a $V_e$ of 16.0 ± 0.2 ml represented the native state dimer (see Table II). Authentic human stefin B forms a dimer that is disulfide-bonded (23). Even though the cysteine has been replaced by serine in the recombinant form of the protein, the tendency to associate noncovalently would still be expected.

The elution volumes listed in Table II show that native stefin B at pH 8.0 elutes with $V_e$ = 16.0 ± 0.2 ml, smaller than expected for the molecular weight. This value is constant over a protein concentration ranging from 0.6 down to 0.1 mg/ml and at salt concentrations of 0.13 and 0.25 M. At pH 7.0 and 5.3, the value is increased to 17.2 ± 0.2 ml. These
point of stefin B is around pH 7.8 suggests that the electrostatic repulsion (which is shielded at high salt) plays a role in breaking the monomer association.

From Fig. 2, the major unfolding with loss of secondary structure occurring at higher denaturant concentration than the disruption of structure associated with aromatic residue ellipticity indicates an equilibrium of the form $N_2 = G_2 = 2U$, where each of the states is significantly populated over most of the transition. $G_2$ indicates the equilibrium, dimeric MG intermediate.

Size exclusion chromatography was performed under other solvent conditions where the equilibrium intermediates have been detected. Their elution volumes were compared with that of the native state (Table II).

**Acid-induced Denaturation**—Unfolding of stefin B was followed by ellipticity at 277 and 222 nm as a function of pH in the presence of 0.6 M GdmHCl (Fig. 6). At both high (pH 10) and low (pH 4) pH, states are populated that possess substantial secondary structure but virtually no tertiary structure, as judged by aromatic environment. The secondary structure of the high pH state B is closer to native than state A (Fig. 7). The latter elutes with $V_e = 18.2$ ml on gel exclusion (Table II), suggesting a compact state coupled with a degree of column interaction. When state A is heated to 70 °C, no change in the spectrum is detected (Fig. 7), indicating equivalence of states A and T (to be described below).

Acid-denatured states of some other proteins were found to have characteristics of molten globule intermediates. A well

**TABLE II**

<table>
<thead>
<tr>
<th>State/conditions</th>
<th>Temperature $V_e$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>°C</td>
</tr>
<tr>
<td>Native (dimer), pH 8.0, 0.02 M Tris, 0.25 M NaCl</td>
<td>2</td>
</tr>
<tr>
<td>Native (dimer), pH 7.0, 0.02 M HEPES, 0.1 M NaCl</td>
<td>25</td>
</tr>
<tr>
<td>Thermally denatured, T (15 min at 70 °C), pH 8.0, 0.02 M Tris, 0.2 M NaCl</td>
<td>30</td>
</tr>
<tr>
<td>Native (dimer), pH 5.3, 0.02 M acetate, 0.6 M GdmHCl</td>
<td>2</td>
</tr>
<tr>
<td>Acidic (monomer), pH 4.0, 0.02 M acetate, 0.6 M GdmHCl</td>
<td>25</td>
</tr>
<tr>
<td>Native (dimer), pH 7.0, 0.02 M HEPES, 0.8 M GdmHCl</td>
<td>25</td>
</tr>
<tr>
<td>State at 1.7 M GdmHCl, G (0.02 M Tris, pH 8.0, 0.02 M HEPES, pH 7.0)</td>
<td>2</td>
</tr>
<tr>
<td>State in 4 M GdmHCl, U (0.02 M Tris, pH 8.0)</td>
<td>25</td>
</tr>
</tbody>
</table>

observations can be explained with a dimer to monomer transition. Monomers exist below 0.6 M GdmHCl, at pH 5.3 and pH 7.0 (Table II). At pH 7.0, dimers are present from 0.8 to 1.8 M GdmHCl (see Fig. 5). The fact that the isoelectric

**FIG. 5.** Size exclusion chromatography at 25 °C. SEC elution profiles, i.e., absorption at 280 (A$_{280}$) against volume of elution ($V_e$) are given. The column was equilibrated at GdmHCl molarity as indicated. $V_e$ of 17.2 ml ($a'$) represents monomeric native state, with the dimeric native state ($a$) at 16.0 ml. From 1.8 M GdmHCl, this gradually changes to a $V_e$ of 14.6 ml at 4.2 M GdmHCl ($b$), which represents the monomeric unfolded state. Protein concentrations were 0.3 mg/ml. The fraction of the native state from SEC (0) is compared with the CD data at 222 nm (Δ) in the inset. It was calculated as described in the text. GuHCl, guanidinium hydrochloride.

**FIG. 6.** Acid-induced denaturation. Samples were prepared as described under "Materials and Methods." $a$, CD at 277 nm; $b$, CD at 222 nm. CD is expressed as mean residue ellipticity ($θ$)$_{MW}$ in the usual units, i.e., deg-cm$^2$/dmol. The reversibility was around 80%, as shown by the triangle in $a$. MRW, mean residue weight; Den, denatured state.

**FIG. 7.** Far UV CD spectra of intermediates in stefin B denaturation. $a$, acid state (pH 4.0; 0.6 M GdmHCl); $b$, basic state (pH 10.5; 0.6 M GdmHCl). State A spectrum did not change from 2 to 70 °C, so the two states, A and T, must have similar far UV CD.
Fig. 8. Thermal denaturation. Samples were processed as described under "Materials and Methods." The buffer was 0.02 M Tris, 0.13 M NaCl, as usual. a, CD at 277 nm; b, CD at 222 nm. Again, the CD js recalculated into the mean residue ellipticity (θ)_{mean} in the units deg·cm$^2$/dmol. The reversibility is shown by the triangles, which were obtained after slowly cooling the sample. MRW, mean residue weight.

Fig. 9. Comparison of the volumes of elution ($V_e$) of recombinant human stefin B in its native and thermally denatured state. The Superose 12 size exclusion column was used for measuring the volumes of elution, as described under "Materials and Methods." a, column heated to 66 °C. Heat-denatured state T, (protein heated for 15–20 min on 65–70 °C) was applied. The main peak is centered at 17.2 ml, which is characteristic for the native monomeric state. b, column heated to 30 °C. The first peak with a $V_e$ of 16.4 ml was observed when the heat-denatured sample (heated for 20 min to 70 °C), was applied. The second peak with a $V_e$ of 16.3 ml was obtained when the native state was injected.

studied example is α-lactalbumin (39). The volumes of elution of transient folding intermediates in β-lactamase and carbonic anhydrase B refolding from state A were found equal to the volumes of elution of state I, i.e. the molten globule folding intermediate (19).$^{3}$

Denaturation by Heat—The effect of temperature on the unfolding of stefin B is shown in Fig. 8. The CD at 222 nm (Fig. 8b) remains practically unchanged up to 75 °C, whereas the CD at 277 nm (Fig. 8a) melts between 63 and 68 °C. Some aggregation was observed under these conditions. After slowly cooling the sample and filtering off the aggregate, a second transition was nearly superimposable on the first, as shown in Fig. 8a (triangles). An appreciable increase in CD at 277 nm was observed below 22 °C (Fig. 8a), which might have arisen from an increase in rigidity of the aromatic side chains.

It can be concluded that no cooperative change of [θ]$_{222}$ takes place. An absolute value of [θ]$_{222}$ above 60 °C, however, is not very reliable. A separate measurement of the spectrum of stefin B at 70 °C (not shown), has shown that the [θ]$_{222}$ amounts to $-3500$ deg·cm$^2$/dmol. Similar is the value in the spectrum of state A when heated to 70 °C (Fig. 7). In any case, the single value of [θ]$_{222}$ cannot be taken as a global measure of the secondary structure. For example, CONTIN analysis of a deletion mutant of stefin B, [θ]$_{222}$ = $-3600$, gave the native-like fractions of the secondary structure.$^3$

To probe the compactness of the thermally denatured state, the size exclusion chromatography on the Superose 12 column was performed. The column was heated to 66 °C, and pre-heated protein was applied directly (Fig. 9a). There was no direct control, as the native state is not stable at 66 °C. Nevertheless, if one assumes that the properties of the column do not change with increasing temperature, the $V_e$ of 17.2 ml is similar to that of the native, monomeric state at 25 °C (see Table II). This seems to be in a relatively slow exchange with a smaller peak centered at 18.4 ml (similar to state A). When the heat-denatured state was applied to the column at 30 °C (Fig. 9a), the majority of it eluted at the same position as that for the native, dimeric state (Fig. 9b). Size exclusion chromatography has thus shown that the thermally denatured state, T, is essentially as compact as the native state, providing evidence that it takes the form of a molten globule state. This is direct evidence for the existence of the molten globule state, as defined by accepted criteria (14), resulting from thermal denaturation (40).

REFERENCES


$^3$ E. Žerovnik and R. H. Pain, unpublished results.
Intermediates in Denaturation of a Globular Protein

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