Conformational State of Disulfide-Reduced Ovalbumin at Acidic pH

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Ovalbumin assumes a highly ordered molten-globule conformation at pH 2.2. To investigate whether or not such structural nature is related to the existence of an intrachain native disulfide bond, the structural characteristics of disulfide-reduced ovalbumin at the acidic pH were compared with those of the native disulfide-intact protein by a variety of analytical approaches. The disulfide-reduced protein was found to assume a partially denatured molten globule-like conformation similar to the disulfide-intact counterpart as analyzed by the CD and intrinsic tryptophan fluorescence spectra and by the binding of a hydrophobic probe of anilino-1-naphthalene-8-sulfonate. The results from size-exclusion chromatography also showed that the disulfide-reduced and disulfide-intact proteins have essentially the same compact, native-like hydrodynamic volume. The disulfide-reduced protein was, however, highly sensitive to proteolysis by pepsin at the acidic pH under the proteolytic conditions in which the disulfide-intact protein was almost completely resistant. Furthermore, on a differential scanning calorimeter analysis the disulfide-reduced protein had an endothermic transition at a much lower temperature ($T_m = 48.5^\circ C$) than the disulfide-intact protein ($T_m = 57.2^\circ C$). Taken together, we concluded that the intrachain disulfide bond should not be directly related to the highly ordered molten-globule conformation of ovalbumin, but that its conformational stability depends on the presence of the disulfide bond. 

Key words: ovalbumin; conformational state; conformational stability; molten globule; disulfide reduction

Globular proteins essentially assume a single conformation in their native state, but under some denaturing conditions they are transformed into a fully denatured state with random-coiled conformations. In addition to these two extreme conformational states, the occurrence of the molten globule state has been demonstrated with many instances of globular proteins, as a partially denatured state. Studies of the molten-globule state are important in both the basic and applied biosciences of proteins. The protein state is formed as a kinetic intermediate in the protein folding pathway and in thermodynamical equilibrium under some mild denaturing conditions. The molten globule states of several food proteins are closely related to their functionalities including gelling and emulsifying properties. The molten globule state assumes a compact and considerably native-like secondary structure, but it is differentiated from the native state by the absence of close packing throughout the molecule, as reflected in the absence of a cooperative temperature transition and in a slightly increased hydrodynamic radius. The state is characterized as a nonspecific assembly of secondary structure segments brought about by hydrophobic interactions; this results in the formation of a hydrophobic cluster, which can be detected as increased binding of a hydrophobic dye. In addition to such a classical molten globule, the occurrence of more ordered as well as more disordered states has been demonstrated. For example, equine lysozyme and cytochrome c, partly denatured under acidic conditions, retains some ordered conformation as detected by a clear cooperative temperature transition. On the other hand, as a more disordered state, the existence of the pre-molten globule has been found for the guanidine hydrochloride-induced unfolding intermediates of $\beta$-lactamase and carbonic anhydrase B, and acid-denatured cytochrome c in a low salt condition. 

Ovalbumin (OVA), a major egg white protein, is a member of the serpin superfamily. The protein consists of a single polypeptide chain of 385 amino acid residues that folds into a globular conformation with a high secondary structure content (30.6% $\alpha$-helix and 31.4% $\beta$-strand), as shown in Fig. 1. Previous studies have demonstrated that OVA assumes a unique partially denatured conformation at acidic pH and CD spectra have shown that the secondary structure content is almost exactly the same at pH 2 and pH 7, but that the native tertiary interactions are almost completely disrupted at the acidic pH. An increased binding of a hydrophobic dye to OVA at pH 2 is also consistent with the view that this egg white protein assumes the classical molten-globule state at the acidic pH. However, intrinsic viscosity and the sedimentation coefficient, $S_{20,w}$, which both depend on the protein hydrodynamic volume, are essentially the same at the two pH values. The protein has a clear endothermic transition during heat denaturation at acidic pH. Furthermore, the acid protein is highly resistant to proteolysis by pepsin at a high pepsin to OVA ratio, the acid ovalbumin undergoes a limited proteolysis at the N-terminal site of Ala351, which is close to the canonical serpin cleavage site.

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Abbreviations: ANS, anilino-1-naphthalene-8-sulfonate; OVA, ovalbumin; SH-OVA, disulfide reduced ovalbumin
formational stability is significantly decreased by the disulfide reduction.

Materials and Methods
Materials. OVA was purified from fresh egg white as described before.\textsuperscript{20} SH-OVA was prepared by incubating OVA at 1.0 mg/ml, 37°C for 2 h in TE buffer (50 mM Tris-HCl, pH 8.2/1.0 mM Na₂-EDTA) containing 15 mM dithiothreitol. The complete reduction of the native disulfide bond was confirmed by peptide mapping analysis as described previously.\textsuperscript{20} The acidic SH-OVA was prepared by passing the disulfide-reduced protein through a Sephadex G-25 column (Pharmacia Biotech Inc., NAP-25) equilibrated with Buffer A (0.1 M potassium phosphate-HCl, pH 2.2). Pepsin was purchased from Sigma Chemical Co. Other chemicals including dithiothreitol and urea were guaranteed grade from Nacalai Tesque.

CD spectra. The CD spectra were recorded at 0.1 mg/ml protein for the far-UV and at 1.0 mg/ml for the near-UV region in TE buffer or in Buffer A with a Jasco J-720 spectropolarimeter. A cuvette of 0.2-cm light path for far-UV or of 1-cm light path for near-UV CD spectra was used. The temperature was kept at 25°C with a circulating water bath. The data are given as the averages of triplicate measurements. The far-UV data are expressed as mean residue ellipticity (degree·cm²/demigram) using a value of 111 as the mean residue weight of the protein, and those for the near-UV CD spectra are expressed as molar ellipticity.

Intrinsic tryptophan fluorescence. The fluorescence spectra of disulfide-intact OVA and SH-OVA were measured with a Hitachi fluorescence spectrophotometer (Model F-3000). The intrinsic tryptophan residues in OVA were excited at 295 nm, and emission spectra were recorded at a wavelength range from 300 to 420 nm. All measurements were done at a constant temperature of 25°C.

Binding of a hydrophobic probe. The protein dissolved in TE buffer or in Buffer A was mixed with 0.025 volume of 0.8 M anilino-1-naphthalene-8-sulfonate (ANS), and the fluorescence emission spectra were measured at 25°C with a fluorescence spectrophotometer (Hitachi, F-3000) at an excitation wavelength of 350 nm. The final concentrations of the protein and ANS were 2 μM and 20 mM, respectively.

Size-exclusion chromatography. Analytical gel filtration chromatography of SH-OVA was done on a column of TSK-GEF G3000SWxL (Tosoh) jointed to a high HPLC apparatus (Shimadzu, LC-4A). SH-OVA (10 μg) was chromatographed in Buffer A containing 0.2 M NaCl at a flow rate of 0.5 ml/min at 25°C or at 37°C. Eluted protein was detected by absorbance at 220 nm. When the denatured protein was chromatographed, the elution buffer contained 8 M urea. The Stokes radius was measured in 50 mM Tris-HCl, pH 7.0 containing 1.0 mM Na₂-EDTA and 0.2 M NaCl by the method of
Corbett and Roche.\textsuperscript{39} The protein standards used were catalase (\(M, 232,000\), \(R_s = 52\)), lactate dehydrogenase (\(M, 140,000\), \(R_s = 43.9\)), transferrin (\(M, 81,000\), \(R_s = 36\)), bovine serum albumin (\(M, 66,700\), \(R_s = 33.9\)), and OVA (\(M, 43,500\), \(R_s = 31.2\)). The void volume and total solvent-accessible volume of the column were calculated based on the elution volumes of blue dextran and sodium azide, respectively.

\textbf{Pepsin digestion.} SH-OVA and the disulfide-intact protein control were incubated at 0.33 mg/ml with 0.44 mg/ml pepsin in Buffer A at 25°C. At various incubation times, proteolysis was stopped by addition of 0.1 volume of 1.0 M Tris-base and 0.37 volume of SDS buffer (0.25 M Tris-HCl, pH 7.0, 4% SDS, 40% glycerol, 80 mM 2-mercaptoethanol) to the reaction mixture. The samples corresponding to 6.1 \(\mu\)g OVA were treated in a boiling water bath for 2 min, electrophoresed on a 10% polyacrylamide gel by the standard method of Laemmlli,\textsuperscript{38} and then stained with Coomassie Brilliant Blue R-250.

\textbf{Differential scanning calorimeter analysis.} SH-OVA and the disulfide-intact protein control were analyzed with a differential scanning calorimeter (Micro Cal, MCS-DSC). The protein concentration was 0.5 mg/ml in Buffer A. The temperature was scanned at 1 K-min\(^{-1}\).

\section*{Results}

\textbf{CD-spectra}

The conformational state of SH-OVA and the disulfide-intact form of OVA was analyzed by CD spectroscopy at acidic and near neutral pH. Although some CD spectrum data have been published previously for disulfide-intact OVA,\textsuperscript{18} the spectra at pH 2.2 and pH 8.2 were reexamed under the current conditions and directly compared with those of SH-OVA. As shown in Fig. 2, almost exactly the same far-UV CD spectra were observed for SH-OVA and disulfide-intact OVA at pH 2.2 and 8.2. The protein may, therefore, have essentially the same native secondary structure contents in SH-OVA and disulfide-intact OVA at the two different pH values. In contrast, near-UV CD spectra were significantly variable: as in a previous report,\textsuperscript{18} the clear positive extrema in OVA observed at pH 8.2 were almost completely lost at pH 2.2. Although the differences in the extremum intensities at the two pH values were much less in SH-OVA than in disulfide-intact OVA, the sharp positive extremum at 287 nm observed under the near neutral conditions was almost lost in the acidic condition. These data strongly suggested some differential tertiary structures at the two pH for SH-OVA.

\textbf{Intrinsic tryptophan fluorescence}

Ovalbumin contains three tryptophan residues, Trp148 in helix F, Trp184 as the nearest neighbor residue of the C-terminus of strand 3A, and Trp267 in helix H.\textsuperscript{17} The conformational states of SH-OVA were analyzed at pH 2.2 and at pH 8.2 by the intrinsic tryptophan fluorescence spectrum. As shown in Fig. 3, the fluorescence emission spectrum was almost indistinguishable for SH-OVA and disulfide-intact OVA at pH 8.2; the native proteins showed an emission maximum at 338 nm. In the presence of 9 M urea at pH 8.2, either disulfide-intact OVA or SH-OVA showed a typical redshift spectrum of an unfolded protein: the emission maximum was shifted to a longer wavelength of 352 nm, and the fluorescence intensity at pH 8.2 was decreased to about 50% of the native form. At pH 2.2, disulfide-intact OVA and SH-OVA showed fluorescence spectra that had a peak at the same wavelength but with much decreased intensity (55%) as compared with the spectrum at pH 8.2. The tryptophan fluorescence data were consistent with the view that SH-OVA as well as disulfide-intact OVA assume differential conformations...
from either the native or fully denatured form.

**ANS binding**
The conformational state of SH-OVA was analyzed by ANS binding experiments at the two pH values and compared with that of disulfide-intact OVA. Figure 4 shows the emission spectra of ANS in presence of SH-OVA or disulfide-intact OVA. In the native state at pH 8.2, either SH-OVA or disulfide-intact OVA showed very low levels of ANS binding. At pH 2.2, however, greatly increased fluorescence emission with a peak at 472 nm was observed; the fluorescence intensity was very similar for SH-OVA and disulfide-intact OVA. In the presence of 6 M urea at pH 2.2, ANS fluorescence was almost undetectable for either SH-OVA or disulfide-intact OVA. These observations are consistent with a similar molten globule-like state for SH-OVA and disulfide-intact OVA at pH 2.2.

**Analysis by size-exclusion chromatography**
The hydrodynamic radius was measured by size-exclusion chromatography. In the gel filtration chromatography, SH-OVA as well as disulfide-intact OVA were eluted as a sharp single peak at pH 2.2 and pH 8.2. As summarized in Table 1, the Stokes radius (Rs) was essentially the same between SH-OVA and disulfide-intact OVA at either pH 2.2 or pH 8.2. When the hydrodynamic radius was compared between the two pH conditions, both the protein forms showed slightly smaller values at pH 2.2 (Rs: 28.0 Å for SH-OVA; 27.7 Å for OVA) than at pH 8.2 (Rs: 30.1 Å for SH-OVA; 29.9 Å for OVA), while the proteins demonstrated much increased radii in presence of 8 M urea (Rs: 59.1 Å at pH 2.2 and 58.4 Å at pH 8.2 for SH-OVA; 56.9 Å at pH 2.2 and 57.6 Å at pH 8.2 for OVA). These data indicate that either SH-OVA or disulfide-intact OVA assumes a compact conformation at the acidic pH.

**Resistance to pepsin digestion**
The disulfide-intact form of OVA has previously been shown to be highly resistant at pH 2.2 to proteolysis by pepsin. It was examined whether or not this resistant nature is retained in SH-OVA. SH-OVA and disulfide-intact OVA as a control were incubated with pepsin at pH 2.2 for various times and the amounts of the intact protein were measured. As shown in Fig. 5, SH-OVA underwent a significant proteolysis under the conditions in which disulfide-intact OVA was almost completely resistant to the proteolysis; for example, at 120 min of incubation the retained non-proteolyzed protein was 42.2% for SH-OVA and 99.8% for disulfide-intact OVA as determined from the band intensities of the intact proteins. This indicated a more fluctuating nature at the acidic pH of the polypeptide chain in SH-OVA than in disulfide-intact OVA.

**Differential scanning calorimeter analysis**
The structural stability was investigated by the differential scanning calorimeter analysis. Figure 6 demonstrates that disulfide-intact OVA denatured at 57.2°C with a clear endothermic transition. SH-OVA, however, showed an endothermic transition at a lower temperature of 48.5°C. These data indicate that the conformational stability of SH-OVA is much less at pH 2.2 than that of disulfide-intact OVA.

### Table 1. Hydrodynamic Radii for the Different Forms of OVA

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH</th>
<th>Urea</th>
<th>Rs</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH-OVA</td>
<td>2.2</td>
<td>-</td>
<td>28.0±0.3</td>
</tr>
<tr>
<td>SH-OVA</td>
<td>8.2</td>
<td>-</td>
<td>30.1±0.0</td>
</tr>
<tr>
<td>SS-OVA</td>
<td>2.2</td>
<td>-</td>
<td>27.7±0.2</td>
</tr>
<tr>
<td>SS-OVA</td>
<td>8.2</td>
<td>-</td>
<td>29.9±0.0</td>
</tr>
<tr>
<td>SH-OVA</td>
<td>2.2</td>
<td>+</td>
<td>59.1±0.1</td>
</tr>
<tr>
<td>SH-OVA</td>
<td>8.2</td>
<td>+</td>
<td>58.4±0.0</td>
</tr>
<tr>
<td>SS-OVA</td>
<td>2.2</td>
<td>+</td>
<td>56.9±0.0</td>
</tr>
<tr>
<td>SS-OVA</td>
<td>8.2</td>
<td>+</td>
<td>57.6±0.1</td>
</tr>
</tbody>
</table>

*SS-OVA represents the disulfide-intact form of OVA.*

*Presence (+) or absence (-) of 8 M urea in the size-exclusion chromatography.*

*Data are the averages for duplicate measurements.*
the selective modification of Cys367 indicates the presence of some ordered conformation in disulfide-intact OVA at the acidic pH. Furthermore, the acid protein is highly resistant to proteolysis by pepsin; at a high pepsin to OVA ratio, the acid ovalbumin undergoes a limited proteolysis at the N-terminal site of Ala351, which is close to the canonical serpin cleavage site Ala352-Ser353.

The data in this report demonstrate that SH-OVA assumes a similar molten globule conformation at the same acidic pH as evaluated by the CD (Fig. 2) and intrinsic tryptophan fluorescence (Fig. 3) spectra, by the binding of a hydrophobic probe of ANS (Fig. 4), and by the size-exclusion chromatography (Table 1). SH-OVA is, however, highly sensitive to proteolysis by pepsin at the acidic pH under the proteolytic conditions in which the disulfide-intact protein is almost resistant (Fig. 5). Furthermore, on a differential scanning calorimeter analysis, SH-OVA has an endothermic transition at a much lower temperature than OVA (Fig. 6). We therefore conclude that the conformational stability of the protein at the acidic pH is significantly decreased by the disulfide reduction.

The similar conformation and decreased stability of SH-OVA as compared to disulfide-intact OVA are consistent with previous data at near neutral pH. The results from CD spectra are consistent with almost the same conformation for SH-OVA and disulfide-intact OVA at pH 7.0. The trypsin-resistant nature of disulfide-intact OVA is also retained in SH-OVA. As evaluated by the CD spectrum and the trypsin-resistance, SH-OVA correctly refolds from the urea-denatured random coiled state. Upon proteolysis with subtilisin, however, the N-terminal side of Cys73 is cleaved in SH-OVA, but not in OVA. On a differential scanning calorimeter analysis at pH 7.0, SH-OVA shows a lower denaturation temperature by 6.8°C than OVA. Likewise, at the acidic pH SH-OVA undergoes a thermal denaturation at a lower temperature by 8.7°C than disulfide-intact OVA (Fig. 6). The decreased stability of SH-OVA may be related to its highly fluctuating nature at the acidic pH. An aromatic disulfide, 2,2'-dipiridyldisulfide, reacts specifically with Cys367 of the protein at pH 2.2 generating a mixed disulfide protein derivative. SH-OVA, but not disulfide-intact OVA, undergoes significant sulfhydryl/mixed disulfide exchange reactions at a physiological temperature, as measured by the release of 2-thiopyridone. A kinetic analysis for the generation of disulfide-forming cysteines with Cys367 at 37°C shows that the rate for the intrachain exchange reaction is quite different for the five cysteine sulfhydryls. The effective concentrations of the five cysteine sulfhydrlys relative to the Cys367-mixed disulfide are 11.4, 4.6, 15.2, 5.9, and 8.9 mM for Cys11, Cys30, Cys73, Cys120, and Cys382, respectively. If the accessibilities of the five cysteine residues to the mixed-disulfide were simply dependent on the sulfur atom to sulfur atom distances in the native protein topology, then the effective concentrations would have been in the order: Cys11 > Cys30 > Cys382 > Cys73 > Cys120. SH-OVA may, therefore, assume more fluctuating conformation than with the typical molten-globule state of disulfide-intact OVA at the acidic pH. However, the intrinsic viscosity and the sedimentation coefficient, $S_{20,w}$, are essentially the same at the acidic and neutral pH values. An aromatic disulfide reagent, 2,2'-dithiopyridine, reacts only with Cys367 among the four cysteine sulfhydryls of OVA (Cys11, Cys30, Cys367, and Cys382) at pH 2.2;
disulfide-intact OVA at the acidic pH, and the mode of the fluctuation may reflect a unique topological situation that is differentiated from the native state.

References


