

Crystal Structure of S-ovalbumin as a Non-loop-inserted Thermostabilized Serpin Form*

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Ovalbumin, a non-inhibitory member of serine proteinase inhibitors (serpin), is transformed into a heat-stabilized form, S-ovalbumin, under elevated pH conditions. The structural mechanism for the S-ovalbumin formation has long been a puzzling question in food science and serpin structural biology. On the basis of the commonly observed serpin thermostabilization by insertion of the reactive center loop into the proximal β -sheet, the most widely accepted hypothetical model has included partial loop insertion. Here we demonstrate, for the first time, the crystal structure of S-ovalbumin at 1.9-Å resolution. This structure unequivocally excludes the partial loop insertion mechanism; the overall structure, including the reactive center loop structure, is almost the same as that of native ovalbumin, except for the significant motion of the preceding loop of strand 1A away from strand 2A. The most striking finding is that Ser-164, Ser-236, and Ser-320 take the D-amino acid residue configuration. These chemical inversions can be directly related to the irreversible and stepwise nature of the transformation from native ovalbumin to S-ovalbumin. As conformational changes of the side chains, significant alternations are found in the values of the χ_1 of Phe-99 and the χ_3 of Met-241. The former conformational change leads to the decreased solvent accessibility of the hydrophobic core around Phe-99, which includes Phe-180 and Phe-378, the highly conserved residues in serpin. This may give a thermodynamic advantage to the structural stability of S-ovalbumin.

Ovalbumin is a major protein component of avian egg white with a molecular mass of ~45 kDa. During the storage of non-fertilized eggs, native ovalbumin is transformed via an intermediate state into a thermostabilized form, S-ovalbumin (ΔT_m , about 8 °C) (1, 2). This transformation is a serious problem for food processing, because egg white that includes S-ovalbumin forms a heat-induced gel with poor strength (3). S-ovalbumin is also generated during the development of fertilized eggs (4, 5). The conversion of native ovalbumin to S-ovalbumin has been accounted for by an elevation of egg white

pH due to the release of carbon dioxide through the eggshell, and it can be reproduced *in vitro* by an alkaline treatment (1, 4).

To understand the protein thermostabilization mechanism, the structural difference between native ovalbumin and S-ovalbumin has been extensively investigated by spectroscopic and biochemical approaches. The obtained data, however, have been consistent with a variety of models, including deamidation (6), surface hydrophobicity change (7), and secondary structure transition (8, 9) mechanisms. As for secondary structure change, studies using far UV CD and Raman spectroscopy have concluded that a small loss of α -helix content and a concomitant increase in β -structure participate in S-ovalbumin formation (8, 9), though a latter analysis from a different laboratory has been consistent with the absence of a secondary structure change (10). This conclusion about the secondary structure transition along with alternative evidence have led to the proposal of a widely accepted model for S-ovalbumin formation that includes a partial insertion of the reactive center loop into the proximal β -sheet (9, 11).

On the basis of structural similarity (12, 13), ovalbumin has been grouped as a member of the serine proteinase inhibitor (serpin) superfamily. Serpin is a protein with metastable conformation, which undergoes a unique conformational change upon exertion of the inhibitory activity (14–16); following the proteolytic cleavage at the P1-P1' site, the reactive center loop is inserted into the proximal β -sheet, and this insertion accompanies large thermostabilization of the protein (14). Although ovalbumin is a non-inhibitory serpin that lacks a loop insertion mechanism, its mutant, in which a hinge residue (Arg-339) for the motion of loop insertion is replaced by a less bulky one, is transformed into a significantly thermostabilized form following the canonical P1-P1' cleavage (17, 18). Furthermore, our crystallographic study has shown that the P1-P1'-cleaved and thermostabilized ovalbumin mutant assumes the fully loop-inserted conformation (18). The acquisition of the loop insertion ability by the single residue substitution implies that ovalbumin also has the serpin metastable nature in the native form.

The metastable nature of ovalbumin has appeared to support the partial loop-inserted model for the S-ovalbumin formation (9, 11). Some spectroscopic and biochemical studies have, however, strongly suggested the participation in S-ovalbumin formation of a structural mechanism other than the partial loop insertion. First, the results from ^{31}P -NMR have revealed the absence of large conformational change on the reactive center loop; the distances between the phosphate group of Ser-344 and an artificially associated Mn^{2+} differ by only 0.4 Å for native ovalbumin and S-ovalbumin (19). Second, the fully loop inserted, P1-P1'-cleaved ovalbumin mutant R339T is further thermostabilized by the alkaline treatment (20). The aim of the current study is to reach a crucial conclusion concerning S-ovalbumin structure by the use of crystallographic evidence.

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The atomic coordinates and structure factors (code 1UHG) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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EXPERIMENTAL PROCEDURES

Materials—Native ovalbumin was isolated from fresh eggs by crystallization in an ammonium sulfate solution (21). Egg white ovalbumin comprises the three components of A1-, A2-, and A3-ovalbumin, which contain two, one, and no phosphorus atoms, respectively (22). The major component, A1-ovalbumin, was isolated by ion-exchange chromatography as described (22). S-ovalbumin was prepared by incubation of A1-ovalbumin in 0.1 M glycine-NaOH buffer, pH 10, at 30 °C for 7 days. The sample was passed through a Sephadex column (NAP-10, Amersham Biosciences) equilibrated with sodium phosphate buffer, pH 6.0, and subjected to analysis by differential scanning calorimetry and crystallization for structural determination.

S-ovalbumin was also produced in eggs that had been stored at 30 °C for one month (1). S-ovalbumin was isolated from the stored eggs by crystallization in an ammonium sulfate solution in the same way. The isolation of an ovalbumin component by ion-exchange chromatography, however, did not work for S-ovalbumin. For thermostability analysis, therefore, the non-fractionated S-ovalbumin sample was used. Attempts to produce a fine crystal from the S-ovalbumin preparation were unsuccessful.

Differential Scanning Calorimetry—Native ovalbumin and S-ovalbumin samples were analyzed for their thermostability with a calorimeter (MCS-DSC, Micro Cal, Massachusetts). Ovalbumin samples were equilibrated with 10 mM sodium phosphate buffer, pH 6.0, and then degassed for 10 min under reduced pressure prior to calorimetric analysis.

Crystallization of S-ovalbumin—The solution in the crystallization drop was prepared by mixing 3 μ l of protein solution (90 mg/ml, 10 mM sodium phosphate buffer, pH 6.0) with 3 μ l of precipitant solution (48–50% ammonium sulfate filtered, 50 mM sodium acetate buffer, pH 6.0). The droplets were equilibrated against 1 ml of the precipitant solution at 30 °C. Crystals were obtained within 2–3 months. To confirm that the crystals were really from S-ovalbumin and not from native ovalbumin, a few of them were rinsed three times with 50 mM sodium acetate buffer (pH 6.0) containing 55% ammonium sulfate, dissolved in 10 mM sodium phosphate buffer (pH 6.0), and then subjected to analysis by differential scanning calorimetry.

Data Collection and Refinement—Diffraction data were collected at the SPring-8 BL44XU beamline (Hyogo, JAPAN) using a wavelength of 0.9 Å with an Oxford PX210 CCD detector. A total of 253,412 reflections was collected to 1.8 Å. The data were processed, merged, and scaled with the D*Trek program and truncated with the CCP4 program package (23). The precession images indicated that the crystal belongs to space group P1. Four molecules were included in an asymmetric unit. The initial model was made by the molecular replacement with the dimmer structure of native ovalbumin resolved at 1.95 Å (24, 25) using Amore in the CCP4 program (26). Several rounds of restrained least squares refinement was done at 1.9-Å resolution by simulated annealing with molecular dynamics in the CNS program package (27). To improve the model, manual model building was carried out using TURBO-FRODO. Eight hundred and seventy-one water molecules having more than 3σ on the $F_o - F_c$ map were then added to the model. The final model was determined with an R -factor value of 0.196 ($R_{\text{free}} = 0.247$) for 99,562 reflections with $F > 2\sigma(F)$ between 20–1.9-Å resolution. Solvent accessible area was calculated using the NACCESS program (28).

RESULTS

Thermostability of S-ovalbumin Utilized for Crystallization—A1-ovalbumin (native ovalbumin) prepared from fresh hen egg white displayed the denaturation temperature of 77.7 °C (Fig. 1, profile *a*). The ovalbumin sample from the egg white after storage in shell eggs at 30 °C for one month displayed a thermostability peak of S-ovalbumin with increased T_m value of 85.8 °C (Fig. 1, profile *b*). The isolation of A1-S-ovalbumin from this sample by conventional chromatography did not work. To prepare A1-S-ovalbumin, therefore, we incubated *in vitro* native A1-ovalbumin in an alkaline condition. The alkaline-treated ovalbumin sample also showed the increased T_m values of 85.5 °C (Fig. 1, profile *c*), which indicates total transformation of native ovalbumin into S-ovalbumin without the inclusion of the intermediate. It was confirmed that the crystal obtained from this protein sample is indeed that of S-ovalbumin; the solubilized protein crystal displayed

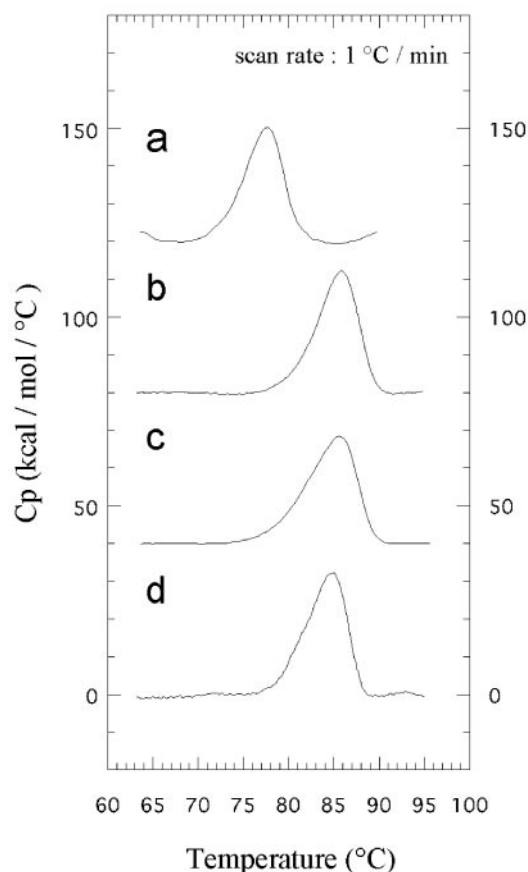


FIG. 1. **Thermostability of native ovalbumin and S-ovalbumin.** The protein thermostability for native ovalbumin (profile *a*), S-ovalbumin from stored egg (profile *b*), S-ovalbumin produced by alkaline treatment *in vitro* (profile *c*), and the solubilized S-ovalbumin crystal (profile *d*) was analyzed with a differential scanning calorimeter. The temperature was scanned at 1 °C min⁻¹, and the protein concentration was 0.5 mg/ml in sodium phosphate buffer at pH 6. Endothermic transition profiles are arbitrarily shifted on the ordinate scale for clarity.

essentially the same increased thermostability with a T_m value of 85.1 °C (Fig. 1, profile *d*).

Structural Determinations—The crystal structure of S-ovalbumin was determined at 1.9-Å resolution. The relevant refinement statistics and the final model data are shown in Table I and Table II, respectively. The mode of the crystal packing of the four molecules was very similar for S-ovalbumin and native ovalbumin (24, 25). The four molecules of S-ovalbumin were, therefore, assigned according to the native ovalbumin molecules. Molecule A consisted of 383 amino acid residues (positions 202 and 203, missing residues), 252 water molecules, two phosphoserine residues, two *N*-acetylglucosamine residues, and two sulfate molecules. Molecule B consisted of 385 amino acid residues, 220 water molecules, one phosphoserine residue, one *N*-acetylglucosamine residue, and two sulfate molecules. Molecule C consisted of 374 amino acid residues (positions 343–353, missing residues), 214 water molecules, no phosphoserine residues, two *N*-acetylglucosamine residues, and one sulfate molecule. Molecule D consisted of 385 amino acid residues, 185 water molecules, two phosphoserine residues, one *N*-acetylglucosamine residue, and two sulfate molecules. The C^α atoms of the S-ovalbumin molecules were superimposed well with those of native ovalbumin molecules; the root mean square deviation values estimated by TURBO/rigid2 were 0.326 Å for 374 C^α atoms of molecule A, 0.332 Å for 362 C^α atoms of molecule B, 0.295 Å for 360 C^α atoms of molecule C, and 0.288 Å for 377 C^α atoms of molecule D.

TABLE I
Summary of data collection and refinement

Data collection	
Resolution limit (Å)	1.8
Unit cell	
a	62.6
b	70.9
c	83.4
α	87.2
β	71.7
γ	75.9
Space group	P1
Total measurements	253,412
Unique measurements	122,497
Averaged redundancy	2.2
Completeness (%)	95.5
R_{sym} (%)	4.7
Refinement statistics	
Resolution range (Å)	20–1.9
Used reflections	99,562
Completeness (%)	96.1
R -factor (%)	19.6
R_{free} (%)	24.7
Root mean square deviation	
Bond length (Å)	0.01
Bond angles (degrees)	1.5

Fig. 2a displays the structure of molecule D that has the lowest B -factor value among the four molecules. One of the most important findings was that the reactive center loop of S-ovalbumin assumes almost exactly the same conformation as that of native ovalbumin (24, 25) without any insertion into the proximal β -sheet (Sheet A, Fig. 2a). This clearly excludes the previously hypothesized partial loop insertion mechanism for the S-ovalbumin formation (9, 11).

Comparison with the Native Ovalbumin Structure—When the overall structure is compared by the C^α traces, S-ovalbumin displays a very similar conformation to native ovalbumin except for the marked motion of preceding loop of strand 1A away from strand 2A (residues 125–128, Fig. 2b).

To our great surprise, Ser-236 was clearly detected as the amino acid residue with the D-configuration by both the $2F_o - F_c$ and omit maps; the residues exist on the turn from strand 2B to strand 3B, which is exposed to the solvent without making interactions with surrounding residues (Fig. 3a). In addition, Ser-164 and Ser-320 were detected as the D-amino acid residues; the $F_o - F_c$ map of the residues, though less clear than that of Ser-236, allowed the D-configuration only (Fig. 3, b and c).

Significant transitions of side-chain conformation were found in Phe-99 on strand 2A and in Met-241 on strand 3B (Fig. 2b). The χ_1 value of Phe-99 and the χ_3 value of Met-241 were decreased by 111° and 167.9° , respectively, in S-ovalbumin. In addition, slight changes of the side-chain conformation are observed in the residues that surround the side chain of Phe-99, as shown in Fig. 4. The surrounding residues include Ile-32 on helix B, Leu-87 and Ile-90 on helix D, Ile-178 on strand 3A, Met-239 on strand 3B, and Leu-377 on strand 5B. The sum of solvent accessibility of the residues (Ile-90, Thr-91, Tyr-97, Leu-101, Ile-178, Val-179, Phe-180, Met-239, and Phe-378) that make van der Waals contacts with Phe-99 is decreased by $29 \text{ \AA}^2$ according to the conversion from native ovalbumin to S-ovalbumin. In contrast, there are no significant changes in the surrounding residue conformations around Met-241.

DISCUSSION

The crystal structure, solved here, provides crucial information about the structural mechanism of the S-ovalbumin formation, which has been one of the major unsolved questions in the serpin structural biology and the food science of the egg white protein.

Structural Characteristics of S-ovalbumin—Serpins have been shown to increase their thermostability by the insertion of a reactive center loop into the proximal β -sheet; the typical serpin thermostabilization involves the full loop insertion in the P1-P1'-cleaved form (14, 18, 29) and a non-cleaved latent form (14, 30). The crystal evidence presented here clearly demonstrates that S-ovalbumin assumes an alternative class of the thermostabilized serpin structure.

The thermostabilized S-ovalbumin structure does not include any loop insertion. This unequivocally excludes the possibility of the previous structural model of S-ovalbumin in which a partial loop insertion is hypothesized to play a central role for the thermostabilization (9, 11). The overall structure, including the reactive center loop structure, is almost the same as that of native ovalbumin, except for the significant motion of the preceding loop of strand 1A away from strand 2A and the changes in the side-chain conformation of Phe-99 and Met-241 (Figs. 2 and 4). The most striking finding was that Ser-164, Ser-236, and Ser-320 take the D-amino acid residue configuration (Fig. 3).

Previous crystallographic and thermodynamic data on ovalbumin stress the crucial participation of a small P14 hinge residue for the loop insertion. The hinge residue corresponds to Arg-339 in wild-type ovalbumin, and loop insertion ability is gained by its replacement by a less bulky residue such as the serine (17) or threonine (18) residue. The native structural characteristic of Arg-339 is retained in the S-ovalbumin crystal structure (data not shown). The absence of the loop insertion in S-ovalbumin is consistent with the conclusion that the presence of a small P14 hinge residue is essential for loop insertion.

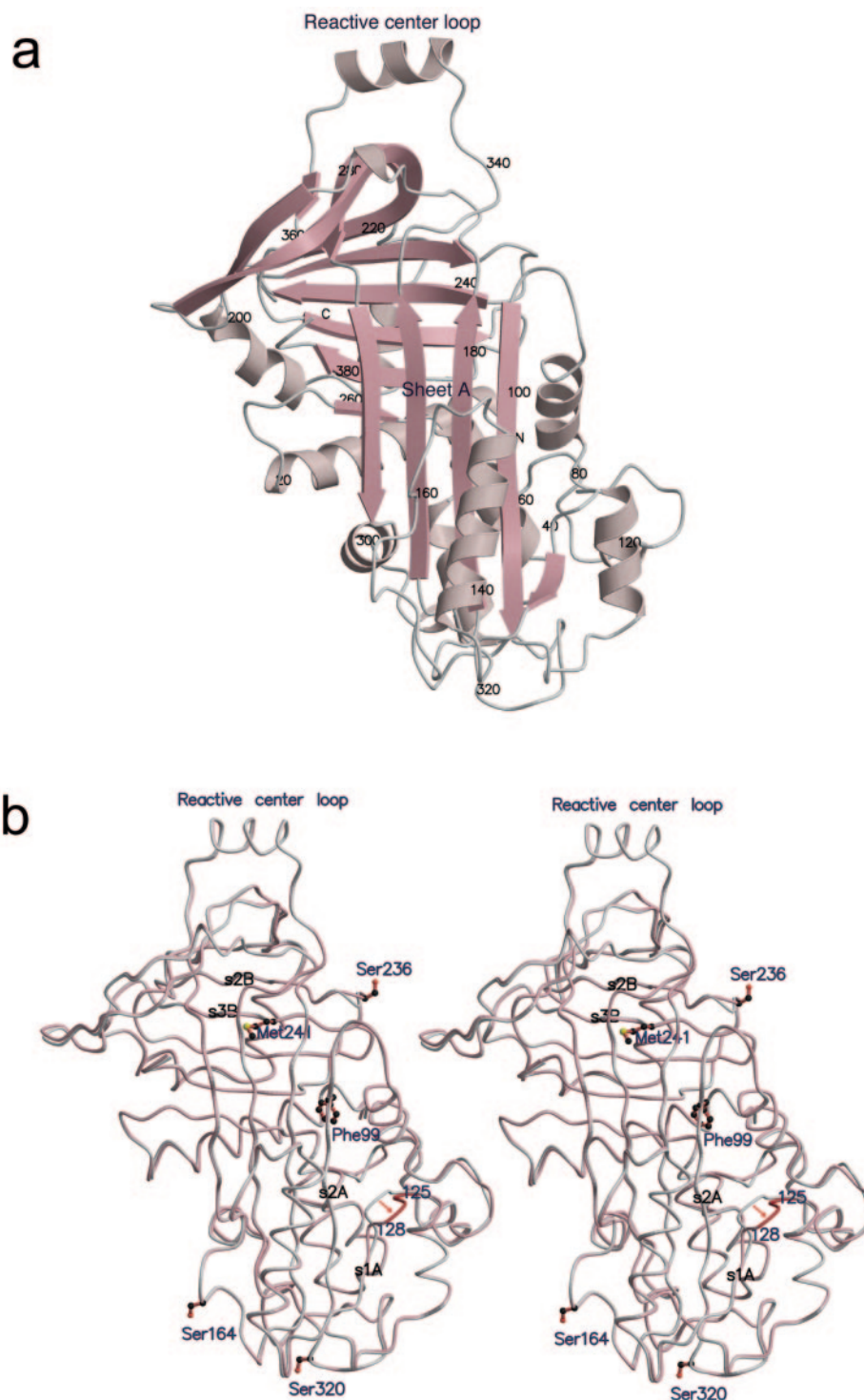
Configurational Inversions of Restricted Serine Residues—Under alkaline conditions, D-Ser can be generated in two ways; one way is through the dehydroalanine intermediate, and the other is through the C^α carbanion intermediate (31). The former intermediate is usually derived from a phosphoserine residue through dephosphorylation (31). The racemization through a dehydroalanine intermediate is, however, very unlikely, because Ser-68 and Ser-344 are confirmed to be phosphorylated in the present S-ovalbumin structure as in native ovalbumin (24, 25). The latter process depends on the electron-withdrawing and resonance-stabilizing capacities of amino acids (31). Among all the non-glycine amino acids, Ser has the highest electron withdrawing capacity and the fastest racemization rates (31). Therefore, it is very likely that D-Ser was generated through the carbanion intermediate during the alkaline treatment.

The production of S-ovalbumin depends on the elevation of pH of egg white in developing fertilized (4, 5) and stored unfertilized eggs (1, 2). Another major egg white protein, ovotransferrin, does not undergo any thermostabilization under the same egg white conditions (4). Among the 34 serine residues, 31 are confirmed to take the L-configuration; only Ser-164, Ser-236, and Ser-320, which assume the D-configuration in the native ovalbumin, are detected as the D-amino acid residues in the present S-ovalbumin structure (Figs. 2 and 3). The question then arises as to why the configurational inversion occurs on the restricted amino acid residues of ovalbumin. We speculate that the unique inversion is directly related to the serpin-metastable nature of ovalbumin. The native serpin structure has been considered as a kinetically trapped metastable intermediate during protein folding *in vivo* (32, 33). Serpins are considered to have many local strains in the native structure (32, 33) that provide, upon exertion of the serpin inhibitory activity, the driving force for the loop insertion. On the basis of the unique structural characteristics of serpins, it is reasonably assumed that some strained thermodynamic sit-

TABLE II
Final model of the four molecules in the asymmetric unit

Segment Identification	A	B	C	D
Modeled protein residues	1–201, 204–385	1–385	1–342, 354–385	1–385
Waters	252	220	214	185
Sulfates	2	2	1	2
Carbohydrates	2	2	2	1
Averaged <i>B</i> -factor ($\text{\AA}^2$)	29.7	29.1	28.1	27.4

FIG. 2. **Structural characteristics of S-ovalbumin.** *a*, the overall main-chain structure of S-ovalbumin. The α -helices and β -strands are shown in *rose* and *pink*, respectively. *b*, overall structural differences between native ovalbumin and S-ovalbumin. S-ovalbumin structure (*pink*) is superimposed on native ovalbumin structure (*white*, Protein Data Bank accession number 1OVA) in a C^α trace. The reactive center loop assumes almost the same conformation as that of native ovalbumin. Marked motion of the preceding loop of strand 1A away from strand 2A (125–128) is displayed with a *red arrow*. Three observed configurational inversions (Ser-164, Ser-236, and Ser-320) and two conformational transitions (Phe-99 and Met-241) of S-ovalbumin are shown in ball-and-stick form. The figures were produced with molecule D using MOLSCRIPT (48) and Raster3D (49).



uations in native ovalbumin are released by the configurational inversion, thereby increasing the overall stability of ovalbumin.

Mode of Ovalbumin Denaturation and Implications for Thermostabilization—Previous studies of ovalbumin denaturation

may provide important insights into the thermostabilization problem in S-ovalbumin. In the presence of a high concentration of urea, ovalbumin is transformed into a randomly coiled, fully denatured state (34); from this fully denatured state,

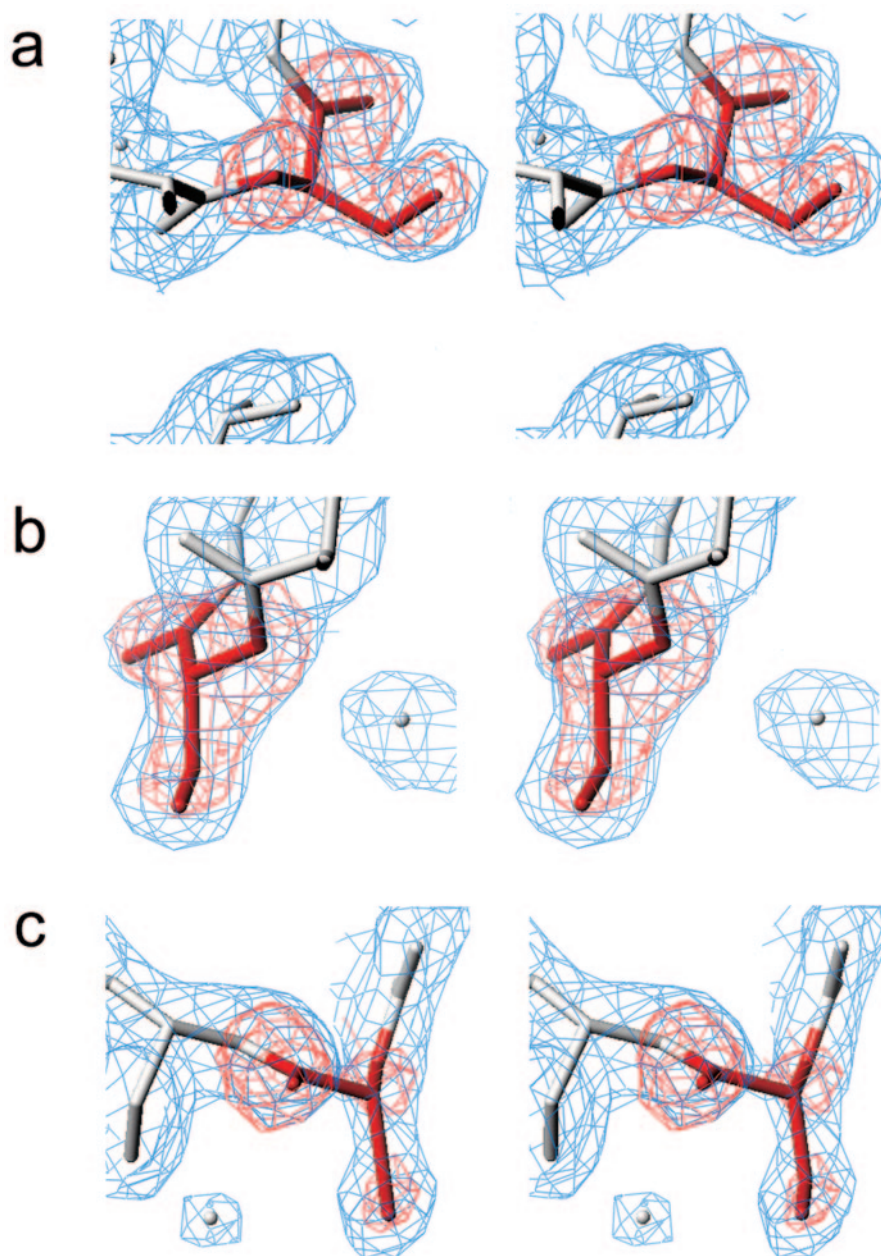


FIG. 3. Stereo views depicting the configurational inversions in Ser-164, Ser-236, and Ser-320. Electron density maps (blue, $2F_o - F_c$, contoured at 1σ) calculated for the residues Ser-236 (a), Ser-164 (b), and Ser-320 (c) fit well on the D-configuration as illustrated in red. The omit maps (magenta, $F_o - F_c$, contoured at 3σ) were obtained after the refinement of the model in which the amino acid residues Ser-164, Ser-236, and Ser-320 were excluded. The figures are produced with molecule C using TURBO-FRODO.

ovalbumin correctly refolds into the native form under native folding conditions (35–38). The conversion between the native and fully denatured states is, therefore, completely reversible in ovalbumin. Another important point is that, during this reversible interconversion, an intermediate state with partially folded molten globule-like conformation is produced (37, 38). The reversible interconversion is also confirmed for the thermal denaturation system (39). Unlike the urea-induced denaturation system, however, ovalbumin denatured at a high temperature assumes molten globule conformation, but the fully denatured one does not (40). These results highlight the importance of the free energy difference between the native and molten globule states as the determinant for ovalbumin thermal stability.

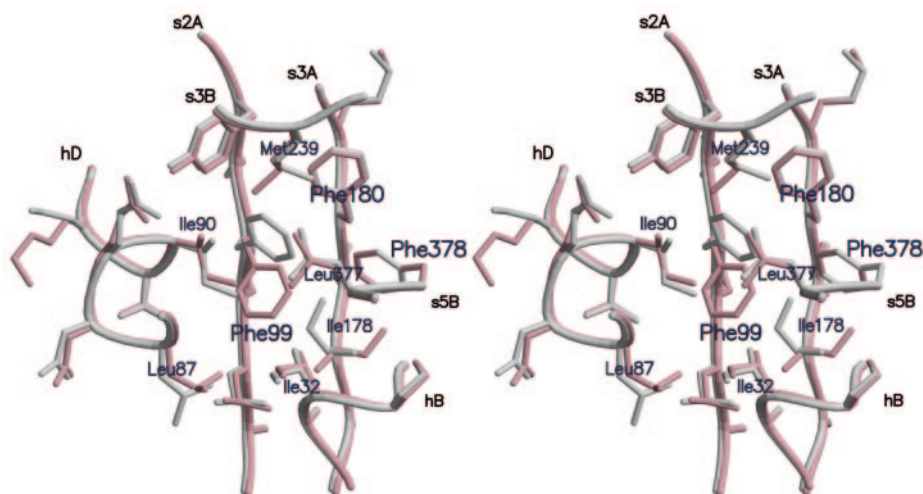
The free energy of a protein state depends on its entropy and enthalpy. Possible thermodynamic factors that work singly or in combination for thermal stabilization are the decreased enthalpy and increased entropy for the native state and the increased enthalpy and decreased entropy for the molten globule state. Some of the characteristics of the crystal structure

may be related to the decreased enthalpy of the native form of S-ovalbumin.

With respect to conformational transition, the motion of the preceding loop of strand1A away from strand2A and the changes in the side-chain conformation of Phe-99 and Met-241 are detected (Fig. 2b). The conformational change in the Phe-99 side chain induces a decrease in the solvent accessibility of the surrounding residues (Ile-90, Thr-91, Tyr-97, Leu-101, Ile-178, Val-179, Phe-180, Met-239, and Phe-378) by $29 \text{ \AA}^2$. The surrounding residues include Phe-180 and Phe-378, which are two of the 51 conserved residues in the serpin superfamily that are considered important for serpin structural stability (41). These strongly suggest that the rearrangement of the hydrophobic core around Phe-99 strengthens the packing of helix B, helix D, sheet A, and sheet B, which makes a prominent contribution to the structural stability of ovalbumin as a serpin (Fig. 4).

The conformational change that comprises the motion of the preceding loop of strand 1A away from strand 2A has also been observed for serpin 1K upon the formation of the Michaelis complex with trypsin (42). Although the thermostabilization by

FIG. 4. **Modulation in the hydrophobic interactions around Phe-99.** The side-chain conformation around Phe-99 is shown in *white* for native ovalbumin (Protein Data Bank accession number 1OVA) and in *pink* for S-ovalbumin. The stereo diagram was produced with molecule D using MOLSCRIPT (48) and Raster3D (49).



the complex formation has not been experimentally confirmed for serpin 1K, α_2 -antiplasmin is considered to exert the inhibitory activity by the structural stabilization upon the formation of the noncovalent Michaelis-like complex with a serine proteinase (16, 43). The motion of strand 1A might, therefore, correspond to a structural transition that is related to the structural relaxation in serpin.

In regard to the configurational inversions of the three serine residues, the present crystal structure cannot be directly related to the decreased enthalpy for the native form; all the Ser-164, Ser-236, and Ser-320 residues reside on the protein surface in either native ovalbumin or S-ovalbumin and do not have any direct interactions with surrounding residues. The molten globule state of ovalbumin is the protein state that has about half of the native secondary structure, some fluctuating nature, and a native-like compact conformation (38). The structural situation in which the D-amino acid residues reside on the polypeptide sequence in a non-localized way might result in a modulated non-native conformational state, which works as a thermostabilization factor by increasing the enthalpy and/or decreasing entropy in the molten globule state. The modulated molten globule state might be also related to the poor strength of the thermally induced S-ovalbumin gel (3).

Related Spectroscopic and Biochemical Observations—Since the discovery of the thermostabilized S-ovalbumin formation in stored eggs, the underlying structural mechanism has been extensively investigated by spectroscopic and biochemical analyses. The conclusions reached about the participation of a conformational alteration have been controversial.

As spectroscopic observations that support the conformational transition, a 2–5% loss of α -helix (9) and a 3–4% increased β -sheet content (8) have been detected by far UV CD and Raman spectroscopy analyses, respectively. An earlier study by the same Raman spectroscopy has, however, been consistent with the absence of a secondary structure change upon the formation of S-ovalbumin (10). The results from ^{31}P -NMR have also been consistent with the absence of large conformational change, at least on the reactive center loop; the distances between the phosphate group of Ser-344 and an artificially associated Mn^{2+} differ by only 0.4 Å for native ovalbumin and S-ovalbumin (19).

The conformational changes found in the crystal structure of S-ovalbumin are very limited and do not include any secondary structure segment. The small conformational change detected by the spectroscopic analyses (8, 9) cannot, therefore, be related to the conformational changes found in the crystal structure. Instead, we speculate that the spectroscopic changes are re-

lated to the inherent ovalbumin nature that tends to be transformed into the molten globule state under some non-physiological conditions. Most of the egg white ovalbumin molecules are transformed into S-ovalbumin under the commonly employed alkaline conditions (e.g. pH10, 55 °C, 16–24 h). Under the equivalent alkaline conditions for recombinant ovalbumin, however, a much decreased recovery of the S-ovalbumin production has been found (44), and a better recovery can be attained at a less drastic temperature of 50 °C (20). These results are probably due to the slightly destabilized nature of the recombinant protein because of the absence of post-translational modification such as phosphorylation, glycosylation, and N-terminal acetylation (44). Furthermore, the disulfide-reduced form of egg white ovalbumin, which assumes the native conformation with a slightly less stability (45), is almost totally transformed into the molten globule state by the alkaline treatment (46). The commonly employed alkaline condition may be therefore a marginal one for maintaining the conformational stability of native egg white ovalbumin. The non-native conformational state seriously loses the CD signal of α -helix in the far UV region (38) and tends to make intermolecular interactions through β -sheet formation (10). It seems reasonable to hypothesize that the spectroscopic observations of the 2–5% loss of α -helix (9) and 3–4% increase in the β -sheet content (8) come from a partial denaturation of native ovalbumin during the alkaline treatment. An alternative observation that S-ovalbumin displays a slightly higher binding capacity against a hydrophobic fluorescent dye, aniline-1-naphthalene-8-sulfonate (7), can also be accounted for by the partial denaturation because the molten-globule state of ovalbumin shows a much higher dye binding capacity than native and fully denatured ovalbumin (38).

The partially loop-inserted conformer model largely depended on the observation that S-ovalbumin received a cleavage by elastase with a 38-fold greater rate than did native ovalbumin (9, 11). This observation, however, was based on a commercially available, non-purified ovalbumin preparation in which the presence of protein components other than native ovalbumin was detected by differential scanning calorimetry (9). In our re-examination using highly purified ovalbumin, no significant increase in the elastase susceptibility has been detected for S-ovalbumin (20). Although the reason for this discrepancy about protease susceptibility is not clear, the egg white is a well known source for a variety of potent proteinase inhibitors, including ovomucoid, ovoinhibitor, and ovostatin (47). It would be possible that the apparent higher protease susceptibility of S-ovalbumin (9) depends on inacti-

vation of contaminated proteinase inhibitor(s) by the alkaline treatment.

In conclusion, the crystal structure solved in the present paper clearly demonstrates the participation in the S-ovalbumin formation of a structural mechanism other than the partial loop insertion. This is consistent with our previous observation that the P1-P1'-cleaved, fully loop-inserted ovalbumin mutant R339T is converted to a further thermostabilized form by an alkaline treatment (20). Despite extensive literature about the conversion from native ovalbumin to S-ovalbumin, no evidence has been shown for the occurrence of the reverse reaction. Furthermore, we observe that native ovalbumin, the intermediate form, and S-ovalbumin refold correctly from the fully denatured state into the corresponding original non-denatured forms as evaluated by a variety of probes, including a thermostability analysis.¹ This irreversible nature between native ovalbumin, the intermediate form, and S-ovalbumin upon unfolding/refolding transition strongly suggests the participation of a chemical stepwise process for the S-ovalbumin formation. The present finding of the multiple D-Ser residues in S-ovalbumin, therefore, leads us to hypothesize that the configurational inversion of the amino acid residues plays a central role for the formation of the thermostabilized ovalbumin. The configurational inversions may accompany the observed conformational changes, such as the motion of the preceding loop of strand 1A away from strand 2A and the changes in the side-chain conformation of Phe-99.

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REFERENCES

- Smith, M. B., and Back, J. F. (1962) *Nature* **193**, 878–879
- Smith, M. B., and Back, J. F. (1965) *Aust. J. Biol. Sci.* **18**, 365–377
- Shitamori, S., Kojima, E., and Nakamura, R. (1984) *Agric. Biol. Chem.* **48**, 1539–1544
- Hatta, H., Nomura, M., Takahashi, N., and Hirose, M. (2001) *Biosci. Biotechnol. Biochem.* **65**, 2021–2027
- Sugimoto, Y., Sanuki, S., Ohsako, S., Higashimoto, Y., Kondo, M., Kurawaki, J., Ibrahim, H. R., Aoki, T., Kusakabe, T., and Koga, K. (1999) *J. Biol. Chem.* **274**, 11030–11037
- Nakamura, R., Takemori, Y., and Shitamori, S. (1981) *Agric. Biol. Chem.* **45**, 1653–1659
- Nakamura, R., and Ishimaru, M. (1981) *Agric. Biol. Chem.* **45**, 2775–2780
- Kint, S., and Tomimatsu, Y. (1979) *Biopolymers* **18**, 1073–1079
- Huntington, J. A., Patston, P. A., and Gettins, P. G. W. (1995) *Protein Sci.* **4**, 613–621
- Painter, P. C., and Koenig, J. L. (1976) *Biopolymers* **15**, 2155–2166
- Huntington, J. A., and Stein, P. E. (2001) *J. Chromatogr.* **756**, 189–198
- Hunt, L. T., and Dayhoff, M. O. (1980) *Biochem. Biophys. Res. Commun.* **95**, 864–871
- Huber, R., and Carrell, R. W. (1989) *Biochemistry* **28**, 8951–8966
- Gettins, P. G. W. (2002) *Chem. Rev.* **102**, 4751–4803
- Whistock, J., Skinner, R., and Lesk, A. M. (1998) *Trends Biochem. Sci.* **23**, 63–67
- Silverman, G. A., Bird, P. I., Carrell, R. W., Church, F. C., Coughlin, P. B., Gettins, P. G. W., Irving, J. A., Lomas, D. A., Luke, C. J., Moyer, R. W., Pemberton, P. A., Remold-ODonnell, E., Salvesen, G. S., Travis, J., and Whistock, J. C. (2001) *J. Biol. Chem.* **276**, 33293–33296
- Huntington, J. A., Fan, B., Karlsson, K. E., Deinum, J., Lawrence, D. A., and Gettins, P. E. W. (1997) *Biochemistry* **36**, 5432–5440
- Yamasaki, M., Arii, Y., Mikami, B., and Hirose, M. (2002) *J. Mol. Biol.* **315**, 113–120
- Goux, W. J., and Venkatasubramanian (1986) *Biochemistry* **25**, 84–94
- Yamamoto, H., Takahashi, N., Yamasaki, M., Arii, Y., and Hirose, M. (2003) *Biosci. Biotech. Biochem.* **67**, 830–837
- Sørensen, S. P. L., and Höyrup, M. (1915) *C. R. Trav. Lab. Carlsberg* **12**, 12–67
- Kitabatake, N., Ishida, A., and Doi, E. (1988) *Agric. Biol. Chem.* **52**, 967–973
- Collaborative Computational Project, Number 4 (1994) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **50**, 760–763
- Stein, P. E., Leslie, A. G. W., Finch, J. T., Turnell, W. G., McLaughlin, P. J., and Carrell, R. W. (1990) *Nature* **347**, 99–102
- Stein, P. E., Leslie, A. G. W., Finch, J. T., and Carrell, R. W. (1991) *J. Mol. Biol.* **221**, 941–959
- Navaza, J. (2001) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **57**, 1367–1372
- Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **54**, 905–921
- Hubbard, S. J., and Thornton, J. M. (1993) *NACCESS Computer Program*, Department of Biochemistry and Molecular Biology, University College, London
- Kaslik, G., Kardos, J., Szabó, E., Szilágyi, L., Závodszy, P., Westler, W. M., Markley, J. L., and Gráf, L. (1997) *Biochemistry* **36**, 5455–5464
- Lawrence, D. A., Olson, S. T., Palaniappan, S., and Ginsburg, D. (1994) *Biochemistry* **33**, 3643–3648
- Bada, J. L. (1985) in *Chemistry and Biochemistry of Amino Acids* (Barrett, G. C., ed), pp. 399–414, Chapman and Hall, New York
- Lee, K. N., Park, S. D., and Yu, M.-H. (1996) *Nat. Struct. Biol.* **3**, 497–500
- Im, H., Ahn, H.-Y., and Yu, M.-H. (2000) *Protein Science* **9**, 1497–1502
- Tatsumi, E., Takahashi, N., and Hirose, M. (1994) *J. Biol. Chem.* **269**, 28062–28067
- Takahashi, N., and Hirose, M. (1992) *J. Biol. Chem.* **267**, 11565–11572
- Onda, M., Tatsumi, E., Takahashi, N., and Hirose, M. (1997) *J. Biochem.* **122**, 83–89
- Onda, M., Tatsumi, E., Takahashi, N., and Hirose, M. (1997) *J. Biol. Chem.* **272**, 3973–3979
- Onda, M., and Hirose, M. (2003) *J. Biol. Chem.* **278**, 23600–23609
- Tani, F., Shirai, N., Onishi, T., Venelle, F., Yasumoto, K., and Doi, E. (1997) *Protein Sci.* **6**, 1491–1502
- Tani, F., Murata, M., Higasa, T., Goto, M., Kitabatake, N., and Doi, E. (1995) *J. Agric. Food Chem.* **43**, 2325–2331
- Irving, J. A., Pike, R. N., Lesk, A. M., and Whistock, J. C. (2000) *Genome Res.* **10**, 1845–1864
- Ye, S., Cech, A. L., Belmares, R., Bergstrom, R. C., Tong, Y., Corey, D. R., Kanost, M. R., and Goldsmith, E. J. (2001) *Nat. Struct. Biol.* **8**, 979–983
- Shieh, B. H., Potempa, J., and Travis, J. (1989) *J. Biol. Chem.* **264**, 13420–13423
- Arii, Y., Takahashi, N., Tatsumi, E., and Hirose, M. (1999) *Biosci. Biotechnol. Biochem.* **63**, 1392–1399
- Takahashi, N., Koseki, T., Doi, E., and Hirose, M. (1991) *J. Biochem.* **109**, 846–851
- Takahashi, N., Tatsumi, E., Orita, T., and Hirose, M. (1996) *Biosci. Biotechnol. Biochem.* **60**, 1464–1468
- Saxena, I., and Tayyab, S. (1997) *Cell. Mol. Life Sci.* **53**, 13–23
- Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24**, 946–950
- Merritt, E. A., and Bacon, D. J. (1997) *Methods Enzymol.* **277**, 505–524

¹ N. Takahashi, M. Onda, K. Hayashi, M. Yamasaki, T. Mita, and M. Hirose, unpublished data.