The Role of the Disulfide Bridge in the Stability and Structural Integrity of Ovalbumin Evaluated by Site-Directed Mutagenesis

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To provide a molecular explanation of the role of the disulfide (SS) bridge in the thermostability and structural integrity of ovalbumin (OVA), we prepared SS-mutated OVAs in which SS-forming residues were replaced by Ala or Ser (C73A, C73S, C120A, and C73/120A), and compared the conformation, thermostability, susceptibility to elastase, and formation of heat-stable OVA (S-OVA) with those of the wild-type. The circular dichroism (CD) and tryptophan fluorescence spectra revealed that the SS-mutated OVAs assumed a native-like conformation similar to the wild-type. The thermal denaturation temperature for the SS-mutated OVAs was significantly lower than that for the wild-type. C73S, C120A, and C73/120A mutants converted to S-OVA on alkaline treatment. Analyses for elastase digestion fragments showed that a non-native SS bridge was generated in all SS-mutated OVAs, but non-native SS-pairing did not contribute to thermostability. Hence, we concluded that the presence of the original SS bridge in OVA contributes to conformational stability but is not directly related to the conversion to S-OVA.

Key words: ovalbumin; disulfide bridge; conformational stability; S-ovalbumin

Hen ovalbumin (OVA) has unique structural characteristics and contains a single disulfide (SS) bridge (Cys73-Cys120) and four free thiol (SH) groups (Cys11, Cys30, Cys367, and Cys382) in a single polypeptide chain of 385 amino acid residues.1,2) The role of the SS bridge in OVA has not been well-established in relation to stability and conformation. Takahashi et al. studied the effects of DTT reduction on the stability and conformation of OVA.3) Considering data from the CD spectrum, resistance to protease, and reactivity of SH groups, they found that the reduced form of OVA retained a native-like conformation but was less stable by 6.8°C than intact OVA, suggesting that the SS bridge is closely implicated in the thermostability of OVA.

OVA is known to convert to a heat-stable form of OVA (S-OVA) under alkaline conditions. Although the stabilization mechanism is not clear, Takahashi et al. found that SS-reduced and carboxymethylated (RCM) OVA was not converted to the S-OVA after alkaline treatment for S-OVA production, and concluded that the SS bridge of OVA is requisite for conversion to S-OVA.4) Hence, it has been speculated that the SS bridge of OVA plays important roles in thermostability and conversion to S-OVA. In their experiments, however, chemical modification using the reducing reagent and SH-reactant was employed. Chemical modification frequently affects the structure of a protein, depending on the size and properties of the SH-reactant introduced.5,6) Hence we prepared SS-mutated OVAs in which either or both of the SS-forming residues were replaced by Ala or Ser (C73A, C73S, C120A, and C73/120A), using an Escherichia coli expression system, because it has been reported that the expressed OVA using this system assumed almost the same conformation and SS structure to hen OVA.7,8) We compared the structure, thermal denaturation, susceptibility to elastase, and S-OVA formation of the SS-mutated OVAs with those of wild-type OVA, and attempted to determine whether the presence of the original SS bridge in OVA is essential for structural stability and conversion to S-OVA.

Materials and Methods

Materials. Restriction enzymes, ligase, and polymerase were purchased from Takara Bio (Otsu, Japan). Oligonucleotide primers and porcine pancreatic elastase (EC 3.4.21.11) were from Sigma-Aldrich Japan (Tokyo). A DNA sequencing kit was from MS Technosystems (Osaka, Japan). Carbencillin, isopropyl-β-d-thiogalactopyranoside (IPTG), and phenylmethylsulfonyl fluoride (PMSF) were from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were of analytical grade.

Strains and plasmids. RNA extraction and cloning of the OVA gene were performed as described in our previous paper.8) E. coli strains XL1-Blue and BL21 were used as host cells in cloning experiments and for OVA expression respectively. pT7 Blue T vector was used in the subcloning of OVA cDNA, and was from Novagen Merck (Darmstadt, Germany). Expression plasmid pET-22b(+) containing the IPTG-inducible T7 promoter and the ampicillin resistance gene, was from Invitrogen (Carlsbad, CA, USA). The signal sequence pelB reader in pET-22b(+) was removed because hen OVA contains a native secretion signal sequence.9)

Plasmid construction and protein expression. The SS-mutated proteins, Ala/Ser substitutes for the SS-forming residues of OVA, were constructed by PCR using synthetic oligonucleotide primers:
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5'-GAAGCTCACGCTGCACATCG-3' (sense) and 5'-CAGATGTGCACGCTGACATCTG-3' (antisense) for C73A, and 5'-ACTGTGCAAGGCTGAAAGGA-3' (sense) and 5'-TCTCTCACAGGCCGC-GAAT-3' (antisense) for C120A and 5'-GAAGCTCAGTGCGGACATCTG-3' (sense) and 5'-CAGATGGCCAGCTGACATCTG-3' (antisense) for C73S (underlining indicates mutation sites). The plasmid for C73/120A OVA was constructed with the template plasmid containing the sequence C73A OVA and the primers employed in constructing C73A OVA by the QuickChange method (Stratagene, La Jolla, CA). The SS-mutated OVAs were expressed using an E. coli expression system.7) The purification procedures were the same as those used previously.5) To visualize proteins, SDS–PAGE was done using 10% polyacrylamide gels under both reducing and non-reducing conditions by the standard method of Laemmli,10) and were stained with Coomassie Brilliant Blue R-250.

Measurements of CD. CD spectra were recorded with a model J-600 spectrometer (Jasco, Hachioji, Japan), which was continuously purged with nitrogen. Far-UV CD spectra were recorded at a protein concentration of 0.25 mg/mL in 10 mM phosphate buffer (pH 7.0) with a 1-mm cell at wavelengths from 260 to 200 nm at 25 °C. The data were collected in triplicate and expressed as mean residue ellipticity.

The unfolding transition curves were obtained by monitoring the ellipticity at 222 nm (θ222) as a probe for conformational changes between the native (N) and the unfolding (U) state of the protein in the thermal-denaturation process. The water-jacket cell (1.0-mm pathlength) containing the sample (0.1 mg/mL protein solution) was heated at a linear rate of 1 °C/min from 25 °C to 90 °C with a thermostatically regulated circulating water bath, Thermo Supplier EZL-80F (Taitec, Koshigaya, Japan). The apparent fraction of thermostatically regulated circulating water bath, Thermo Supplier EZL-80F (Taitec, Koshigaya, Japan). The apparent fraction of unfolding (Fapp) was obtained by (θther est - θ)(θn - θu), where θu and θn are the values of the native and the unfolding state, respectively, and θ is the observed value of θ222 at a given temperature. The value of Fapp was plotted as a function of temperature. Assuming that the unfolding equilibrium followed a two-state mechanism, the thermal denaturation temperature (Tm) of unfolding, a mid-transition temperature, was determined from the Fapp unfolding curves.

Measurement of fluorescence. Tryptophan fluorescence spectra were recorded with a model FP-6300 fluorescence spectrophotometer (Jasco, Hachioji, Japan) at 25 °C at a protein concentration of 0.25 mg/mL in 10 mM phosphate buffer (pH 7.0). The excitation wavelength was 295 nm and the emission wavelength ranged from 300 to 400 nm.

Alkaline treatment for S-OVA formation. For conversion to a heat-stable form of OVA, alkaline treatment was conducted with some modifications, as reported by Yamanoto et al.11) Briefly, a protein sample was dissolved in 0.1 M glycine–NaOH buffer (pH 9.9) containing 0.02% sodium azide at a protein concentration of 1.0 mg/mL and incubated at 50 °C for 24 h. The alkaline-treated sample was then passed through a Sephadex column (NAP-10, GE Healthcare Japan, Hino) equilibrated with 10 mM phosphate buffer, pH 7.0.

Pancreatic elastase digestion, and N-terminal analysis and molecular weight of the proteolytic fragments. The proteins were incubated at 1 mg/mL with 10 μg/mL of pancreatic elastase in 50 mM Tris–HCl buffer (pH 8.0) at 25 °C for 1 h.12) Digestion was stopped by the addition of 0.1 mM PMSF. The samples were subjected to TOF-MS and SDS–PAGE by the standard method of Laemmli.10) In TOF-MS analysis for the molecular weight measurement, the protein samples (0.025% protein) were mixed with a matrix, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) dissolved in a solution containing equal volumes of acetonitrile and 0.1% trifluoroacetic acid. Spectra were obtained using a Voyager™ RP mass spectrometer (PerSeptive Biosystems, Framingham, MA).

After SDS–PAGE, protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) with a model AE-6675 electroblot apparatus (Atto, Tokyo, Japan). The proteins transferred to the membrane were stained with 0.1% Ponceau S solution. After washing with a 1% acetic acid solution and distilled water, the membrane was dried. The protein bands were cut and subjected to sequence analysis with a model PPSQ-21A protein sequencer (Shimadzu, Kyoto, Japan).

Preparation of the DTT-reduced form of OVA. The reduced form of OVA was prepared under non-denaturing conditions by incubating the intact protein at 1.0 mg/mL with 5 mM DTT at 37 °C for 1 h in 50 mM Tris–HCl buffer (pH 8.2) containing 1 mM EDTA by the procedure described by Takahashi et al.3) DTT in the reduced OVA solution was removed on a gel filtration column (PD-10, GE Healthcare Japan, Hino) prior to CD measurements.

Results and Discussion

Structural properties

Previous papers indicate that the conformation and SS structure of recombinant OVA from the E. coli expression system are essentially identical to those of hen OVA, except for a lack of post-translational modifications.7) In the present study, recombinant OVAs (wild-type, C73A, C120A, and C73/120A) were isolated from the periplasmic fraction of E. coli cells and then purified by ion-exchange chromatography. The homogeneity of these protein preparations was examined by SDS–PAGE in the presence of 2-ME (Fig. 1). The wild-type and SS-mutated OVAs displayed a single band on the SDS–PAGE gel, demonstrating their homogeneity and the success of purification.

The structural integrity of recombinant OVA proteins was evaluated by measuring their far-UV CD and intrinsic tryptophan fluorescence spectra. The CD spectra for the SS-mutated OVAs C73A, C120A, and C73A/C120A were similar to each other, and also close to that of the wild-type (Fig. 2A).

In addition, the conformational states of the recombinant proteins were analyzed by the tryptophan fluorescence spectra. As shown in Fig. 2B, almost the same fluorescence spectra were observed for the C120A, C73/120A, and wild-type OVAs. In contrast, C73A OVA showed a fluorescence spectrum that had a peak at the same wavelength but with slightly decreased intensity, as compared with the other SS-mutated and wild-type OVAs. Considering the data from CD and tryptophan fluorescence spectra, SS-mutated OVA proteins appeared to assume substantially a native-like conformation similar to the wild-type.

![Fig. 1. SDS–PAGE Profiles under Reducing Conditions for Wild-Type and Mutant OVAs.](image-url)
Thermal denaturation temperature

The thermal denaturation temperature of the SS-mutated OVA was examined by monitoring CD ellipticity at 222 nm observed at different temperatures as a conformation probe as compared with that of the wild-type (Fig. 3). The $T_d$ value of denaturation was deduced from the $F_{\text{app}}$ unfolding curves, assuming a two-state transition for unfolding. The $T_d$ obtained is summarized in Table 1. $T_d$ for the wild-type OVA was 69.8°C, the same as the previous datum. 8) On the other hand, the $T_d$ values for the C120A and C73/120A OVAs were 64.6°C and 66.0°C, lower by 5.2°C and 3.8°C than the wild-type OVA respectively, and was the decreased $T_d$ value reported for DTT-reduced form of hen OVA. 3) In contrast, the $T_d$ value for C73A OVA dramatically decreased, to 57.0°C. As described above, a significant decrease in tryptophan fluorescence intensity was observed for C73A OVA. 3) Hence, based on the crystallographic data for hen OVA, 2) we attempted to determine the effects of the mutation of Cys73 with Ala on the environment surrounding these tryptophan residues, but since the distance between these tryptophan residues and Cys73 was too distant to form interactions, the hydrophobicity of the side-chain of Ala73 might alter the environment surrounding tryptophan residues. The local alteration in the C73A OVA might cause destabilization against thermal denaturation. It appears that the original SS bridge of OVA contributes directly to structural stability against thermal denaturation.

Recently, Sun and Hayakawa found that OVAs from five avian species contained a single SS bridge in common, as well as hen OVA. 13) This strongly confirms that the SS bridge plays a substantial role in the thermostability and structural integrity of OVA.

Formation of S-OVA

OVA is known to convert to heat-stable S-OVA upon alkaline treatment. 14,15) A study using RCM-OVA by Takahashi et al. indicated that the presence of the SS bridge (Cys73-Cys120) is crucial for conversion to S-OVA. 9) Hence, we expected that SS-mutated OVA is not converted to a heat-stable form by alkaline treatment. The $T_d$ values of the alkaline-treated protein are

<table>
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<tr>
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<th>Intact (I)</th>
<th>Alkali-treated (A)</th>
<th>A-I</th>
<th>DTT-reduced (R)</th>
<th>I-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>69.8</td>
<td>78.7</td>
<td>8.9</td>
<td>64.0</td>
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<td></td>
<td></td>
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<td>C120A</td>
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<td>74.2</td>
<td>9.6</td>
<td>65.0</td>
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<tr>
<td>C73/120A</td>
<td>66.0</td>
<td>75.4</td>
<td>9.4</td>
<td>64.6</td>
<td>1.4</td>
</tr>
<tr>
<td>C73S</td>
<td>63.4</td>
<td>70.7</td>
<td>7.3</td>
<td>64.4</td>
<td>-1.0</td>
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Table 1. Thermal Denaturation Temperatures ($T_d$, °C) of Intact, Alkaline-Treated, and DTT Reduced Forms for Wild-Type and Mutant OVAs
shown in Table 1. After alkaline treatment, the treated wild-type OVA showed a dramatically increased $T_d$ value, by 8.9 °C, which is consistent with previous data. To our surprise, the $T_d$ for the C120A and C73/120A OVAs also increased, by 9.6 °C and 9.4 °C, after alkaline treatment, respectively. The $T_d$ value for C73A OVA could not be determined because the protein aggregated during alkaline treatment. Hence we prepared the Ser mutant (C73S) as a substitute for C73A and characterized it. The C73S mutant displayed far-UV CD and tryptophan fluorescence spectra identical to wild-type OVA (Fig. 2A and B), and its $T_d$ value was 63.4 °C (Fig. 3 and Table 1), suggesting that this mutation does not induce further conformational changes, except for the impact of the SS cleavage. The increment of the $T_d$ value for the alkaline-treated C73S mutant was 7.3 °C higher than for the intact one. These data indicate that cleavage of the original SS bridge in OVA did not affect the formation of a heat-stable form under alkaline treatment. This result is in conflict with that reported by Takahashi et al. It is possible that a bulky carboxymethyl moiety in RCM-OVA interferes with the conversion of OVA into a heat-stable form under alkaline conditions. We concluded that the presence of the original SS bridge is not required for conversion to a heat-stable form of OVA upon alkaline treatment.

**Susceptibility of the fragments to pancreatic elastase and N-terminal analysis**

It is known that special proteases such as subtilisin and elastase hydrolyze restrictedly a native hen OVA. Takahashi et al. and Yun et al. have reported that a DTT-reduced form of hen OVA had the additional cleavage site at the N-terminal side of Cys73, in addition to the well-known cleavage sites in plakalbumin, the digest of native OVA by subtilisin. Such a susceptibility of DTT-reduced OVA to subtilisin might be related to its highly fluctuating conformation induced by SS reduction. Likewise, porcine pancreatic elastase cleaves selectively a native hen OVA at only the P1-P1’ site (Ala352-Ser353). It was expected, therefore, that the elastase hydrolysis profile for SS-mutated OVA would differ from that for the wild-type OVA. The SS-mutated OVAs and the wild-type as control were incubated with elastase, and then the SDS–PAGE patterns for their digests were compared (Fig. 4A). As expected, the hydrolysis profile for the SS-mutated OVAs was apparently different from that for the wild-type OVA. For each SS-mutated OVA, two typically large fragments, of 39.5 and 31.5 kDa, were detected, while only a 39.5 kDa fragment was observed for the wild-type OVA. The SS-mutated OVA proteins appeared to have at least one additional cleavage site, in addition to the canonical cleavage site of elastase, indicating that the conformation of the SS-mutated OVA is less stable than that of the intact OVA (the wild-type). In sum, the original SS bridge apparently contributes to the conformational stability of OVA.

To determine the additional cleavage site, N-terminal sequencing was performed for the two fragments. The N-terminal sequences were as follows:

- A 39.5 kDa fragment: GSIGA
- A 31.5 kDa fragment: QAGTS for C73A, C73A/C120A, and C73S; GTSVN for C120A

In light of the established primary structure and the approximate molecular weight estimated by TOF-MS analysis, the 39.5 kDa fragment corresponded to Gly1-Ala352, a peptide fragment, generated from the cleavage at the P1-P1’ site. The 31.5 kDa fragment corresponded to a peptide fragment Gln72-Ala352 for C73A, C73/120A, and C73S OVAs, and for C120A OVA the peptide fragment corresponded to Gly74-Ala352. Similarly to the susceptibility of DTT-reduced OVA to subtilisin, SS-mutated OVA appeared to be susceptible to elastase in the vicinity of Cys73, which is included in SS bridge of OVA, suggesting that SS-mutated OVA assumed a less stable and more fluctuating conformation than the SS-bonded OVA (the wild-type). This indicates again that the SS bridge of OVA is significantly related to conformational stability.

**SDS–PAGE analysis of the elastase digests**

SDS–PAGE analysis of the elastase digests was also carried out under non-reducing conditions (Fig. 4B). The mobility of the 39.5 kDa fragment was much faster under the non-reducing condition, as compared to the mobility of the corresponding 39.5 kDa fragment on SDS–PAGE under the reducing condition, which sug-
suggests the presence of an SS bridge in the fragment. In addition, a possible SS bridge in the fragment appeared to be formed at a different position from the wild-type OVA, as judged by the difference in mobility of the 39.5 kDa fragment on SDS–PAGE under the non-reducing condition. In contrast, no difference in the electrophoretic mobility of the 31.5 kDa fragment was observed as between the presence and the absence of 2-ME. It is possible that the 39.5 kDa fragment forms an intrachain SS bridge, because the peptide fragment contains two SH groups at Cys11 and Cys30 in common. Indeed, it has been reported that regeneration of the SS bridge occurred when the DTT-reduced form of hen OVA was incubated under the alkaline conditions used to prepare S-OVA. Further, the formation of non-native SS isomers as a folding intermediate occurs upon refolding of heat-denatured hen OVA.

To check the presence of an intrachain SS bridge in SS-mutated OVA, SDS–PAGE analysis under the non-reducing condition was carried out as well as of the wild-type OVA as a control (Fig. 5), and then its profile was compared with the SDS–PAGE profile under the reducing condition, as shown in Fig. 1. The differences in electrophoretic mobility of the SS-mutated OVA proteins as between the non-reducing and the reducing condition of SDS–PAGE indicated the presence of an intrachain SS bridge in their proteins, as well as the wild-type OVA. In addition, SS-mutated OVA displayed smear bands besides a clear band, suggesting the possibility of the formation of non-native SS isomers.

**Effect of non-native SS bridge on thermal stability**

It was found that each SS-mutated OVA probably contained a non-native SS bridge. The effect of the non-native SS bridge on the thermostability of SS-mutated OVA was investigated by measuring the $T_d$ value after DTT reduction. The wild-type OVA as a control was also reduced under the same conditions. The formation of a reduced form of SS-mutated OVA and of the wild-type by DTT was confirmed by SDS–PAGE under the non-reducing condition (Fig. 6). The $T_d$ value of the DTT-reduced wild-type OVA was 64.0°C, a decrease of about 5.8°C (Table 1). Contrast, the $T_d$ values for SS-mutated OVA proteins C73A, C120A, C73/120A, and C73S was retained even after DTT reduction. Hence, it appeared that the non-native SS bridge in the SS-mutated OVA proteins does not contribute to structural stability against thermal denaturation.

**Conclusions**

To clarify the role of the SS bridge in the thermostability and structural integrity of OVA, SS-mutated OVA proteins, in which the original SS-forming residues were replaced by Ala or Ser, were prepared using an E. coli expression system and were compared with the wild-type OVA. In view the data from the far-UV CD spectra and tryptophan fluorescence spectra, the SS-mutated OVA proteins assumed a native-like conformation. The data on thermal denaturation and susceptibility to elastase revealed that the SS bridge in OVA contributed to thermostability and structural integrity.

Upon alkaline treatment for S-OVA formation, the SS-mutated OVAs (C73S, C120A, and C73/120A) converted to a heat-stable form, as well as the wild-type OVA. Hence, it was concluded that the presence of the original SS bridge in OVA is not required for the conversion of OVA into S-OVA.

It was suggested that each SS-mutated OVA protein contains a non-native SS bridge in the molecule, as judged from peptide mapping and SDS–PAGE analysis of elastase digests. The non-native SS bridge in the SS-mutated OVA protein, however, did not contribute to thermostability. Although the reason SS-mutated OVA proteins form a non-native SS bridge is unclear, the formation of a non-native SS bridge may be necessary at the initial stage of protein folding.

**References**