Formation of Intermolecular β-Sheet Structure during Heat Denaturation of Ovalbumin

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CD spectra of heat-treated ovalbumin were measured to elucidate the mechanism of the aggregate formation. The change in CD spectra during and after the heat treatment was interpreted as the result of increase in β-sheet structure in sacrifice of helical structure. The partially unfolded form of heat-denatured ovalbumin was predicted from the cooperativity of the urea denaturation curves. The formation of β-sheet structure of heat-denatured ovalbumin increased in higher protein concentration and salt concentration in which the intermolecular interaction was enhanced. In addition, the formation of β-sheet structure increased in proportion to an increase in the molecular weight of heat-denatured ovalbumin aggregates. It was concluded from these results that heat-induced ovalbumin aggregates or gels were built from the partially unfolded molecules with a significant amount of secondary structure and cross-linked with the intermolecular β-sheet structure strengthening by the exposed hydrophobic residues.

Although various modes of protein denaturation have been extensively studied (Kauzmann, 1959; Tanford, 1968, 1970; Privalov, 1979; Lapanje, 1978), their mechanisms are not well understood. Especially, thermal denaturation of proteins is least understood, because aggregate formation accompanying thermal denaturation makes characterization by conventional physicochemical techniques difficult. Furthermore, thermal denaturation is often irreversible. For further understanding of thermal denaturation, it seems pertinent to understand the pathway and mechanism of the aggregate formation on thermal denaturation. Particularly, it is important to make clear what kind of interaction is responsible for the initial stage of the aggregate formation. Combination of information thus obtained with that for chemical modification at the later stage of thermal denaturation (Klibanov, 1983; Ahern and Klibanov, 1985; Zale and Klibanov, 1986) will provide knowledge necessary to understand changes in protein structure on heat treatment. Since ovalbumin forms soluble aggregates when its aqueous solution at neutral pH is heated, ovalbumin has been studied as a model of irreversible denaturation (Kato et al., 1983; Egelandsdal, 1986; Kato and Takagi, 1987). It has been reported in the previous papers (Kato et al., 1983; Kato and Takagi, 1987) that the soluble ovalbumin aggregates are formed predominantly by hydrophobic interaction without disulfide bond and a kind of regular structure is formed during the progress of the aggregation. This suggests that the ordered structure between unfolded molecules is formed during the irreversible thermal denaturation of ovalbumin.

Painter and Koenig (1976) reported that the formation of intermolecular β-sheet structure during thermal denaturation of various egg white proteins was observed by Raman spectroscopy. Clark et al. (1981) also reported a similar observation by the infrared and laser Raman spectroscopic study during the heat-induced gelation of a number of globular proteins. However, these proposals of the formation of β-sheet structure are based on qualitative data. We wanted information not about the presence or absence of a particular secondary structure but about the change in the content of such a structure to elucidate the mechanism of the intermolecular interaction between unfolded molecules. The circular dichroic analysis is particularly suited for such a purpose. At present, circular dichroism is a powerful technique for studying protein conformation in solution. Chang et al. (1978) have recently developed the CD analysis by introduction of the nonlinear least-squares curve-fitting program for the estimation of helix, β-sheet, β-turn, and unordered form. Therefore, this method was introduced in this study to follow quantitatively the changes in the secondary structure during the thermal denaturation of ovalbumin.

MATERIALS AND METHODS

Ovalbumin was crystallized with sodium sulfate from fresh egg white and recrystallized five times (Kekwick and Cannan, 1936). Thermal denaturation of ovalbumin was carried out in 67 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.05% sodium azide, or in 6.7 mM sodium phosphate buffer, pH 7.0, containing 0.01 M NaCl and 0.05% sodium azide. Five milliliters of 0.1% ovalbumin solution was put in a test tube with an internal diameter of 15 mm, immersed in a water bath kept at 30 °C, and then heated at a rate of 3 °C/min. As soon as the solution was heated to a given temperature, the test tube was dipped into an ice-water mixture, and CD measurement was subsequently carried out at 25 °C immediately after cooling.

CD measurements were carried out with a Jasco J-500 recording spectropolarimeter equipped with a data processor (Model DP-501). CD spectra were measured at protein concentrations of 0.28–1.11 mg/mL using a cell with 0.090-mm light path in the wavelength range of 190–260 nm. CD spectra were expressed in terms of mean residue ellipticity (deg cm^2 $dmol^{-1}$). The accuracy of the data was improved by averaging 16 scans integrated with the data processor. CD curves thus obtained were simulated by a nonlinear least-squares curve-fitting program that was written essentially according to the method of Chang et al. (1978). The estimation of α-helix, β-sheet structure, β-turn, and unordered form was carried out in the computed curves giving the best fitting. Reversibility of the heat denaturation of ovalbumin was examined by comparing the CD spectra of ovalbumin before, during, and after heat treatment up to 80 °C. CD measurement was carried out not only at 25 °C but also in a cell holder circulated with hot water of 80 °C. The effect of urea on the secondary structure of heat-denatured ovalbumin was studied as follows: 1–9 M urea was added to a heat-denatured ovalbumin solution cooled to 25 °C after heating

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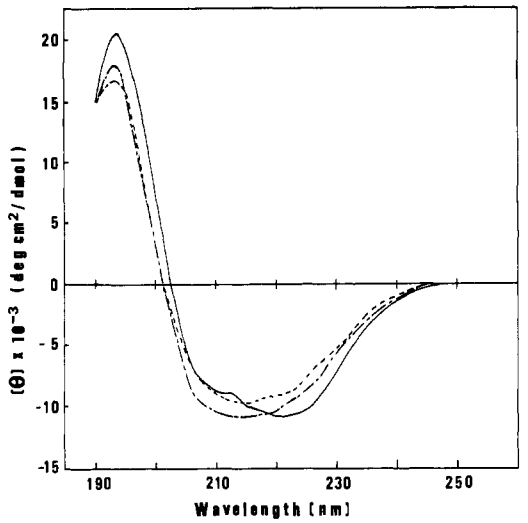

Figure 1. Circular dichroism spectra of native and denatured ovalbumin: —, native ovalbumin; - - -, ovalbumin heated at 80 °C; -·-, ovalbumin cooled at 25 °C after heating at 80 °C.

at 80 °C, and then the CD spectrum was measured.

The molecular weights of heat-denatured ovalbumin were determined by the low-angle laser light scattering technique described by Takagi and Hizukuri (1984). Ovalbumin solutions were applied to high-performance gel chromatography on a TSK G3000SW column (Toso Co.; 0.75 × 60 cm) at a flow rate of 0.3 mL/min. Elution from a column was monitored with a low-angle laser light scattering photometer (LS-8, Toso) and then with a precision differential refractometer (RI-8, Toso). The weight-average molecular weights of heat-denatured ovalbumin were determined by

$$M_w = k(\text{area})_{LS}/(\text{area})_{RI}$$

where k is a constant depending on the instrumental and experimental conditions and $(\text{area})_{LS}$ and $(\text{area})_{RI}$ are the total areas in the elution peak of LS photometer and the refractometer, respectively.

RESULTS

Figure 1 shows typical examples of the CD spectra of ovalbumin before, during, and after heat treatment up to 80 °C. A minimum and a shoulder were observed for native ovalbumin at 222 and 210 nm, respectively. When ovalbumin was heated to 80 °C, the amplitude at 222 nm was attenuated and the shoulder at 210 nm was slightly amplified. Standing at 25 °C after the heat treatment significantly amplified the negative deflection in the region between 210 and 220 nm. The constant CD curves were drawn during the standing time at 25 °C. The change in CD spectra suggests that the heat denaturation of ovalbumin progresses irreversibly without renaturation. From the survey of CD spectra of model peptides and proteins of known three-dimensional structures, the helical structure gives minima in CD spectra at 222 and 208 nm and the β-sheet structure gives minimum at 218 nm. Therefore, at a glance, the changes in CD spectra of ovalbumin during heat denaturation suggest that the β-sheet structure increases with heat denaturation in sacrifice of helical structure and the change is irreversible. To get more quantitative insight into the change in CD spectra of ovalbumin during heat denaturation, it was attempted to simulate the CD spectra according to the procedure of Chang et al. (1978), which gives the contents of helix, β-sheet, β-bend, and unordered structures. As shown in Table I, a marked increase in the β-sheet structure in sacrifice of helical structure was observed during heat denaturation of ovalbumin. The tendency was further promoted by cooling at 25 °C after heating at 80 °C. This result suggests that the ovalbumin molecule transforms with significant amount of β-sheet structure during and after such heat treatment.

Table I. Secondary Structure Contents Calculated from CD Spectra of Native, Heat-Denatured, and Cooled Ovalbumin

ovalbumin	fractions of secondary structure			
	helix	β-sheet	β-turn	unordered
native	0.49	0.13	0.14	0.24
heat-denatured[a]	0.16	0.36	0.15	0.33
cooled[b]	0.14	0.46	0.09	0.31

[a] CD spectrum was measured in a cell holder at 80 °C after 10-min heating at 80 °C. [b] CD spectrum was measured in a cell holder at 25 °C after cooling the heat-denatured ovalbumin in *a*.

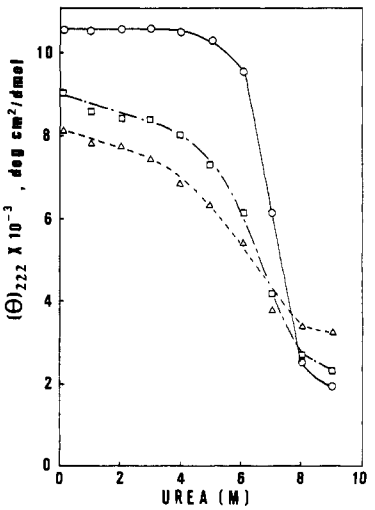

Figure 2. Urea denaturation curves of native and heat-denatured ovalbumin: ○—○, native ovalbumin; △- - -△, ovalbumin heat treated at 80 °C; □-·-□, ovalbumin cooled at 25 °C after heating up to 80 °C.

Figure 2 shows the urea denaturation curves of ovalbumin before, during, and after heat treatment up to 80 °C. The extent of denaturation was determined by measuring the ellipticity at 222 nm, since the negative CD value at 222 nm is characteristic of ordered structure. The ellipticity of native ovalbumin did not change up to 5 M urea, while that of denatured ovalbumin gradually lowered even in the range 0–4 M urea. The urea denaturation curves of heat-treated ovalbumin also indicated that alternative regular structure was formed by the heat denaturation.

Figure 3 shows the effect of heating temperature on the formation of secondary structures of heat-denatured ovalbumin at various temperatures ranging between 72 and 84 °C. The contents of helical, β-sheet, and β-bend structures were estimated from the best fittings in the simulation of the CD spectra of ovalbumin at 25 °C after heating to various temperatures. It is shown that ovalbumin begins to denature when heated to 76 °C and the denaturation proceeds as the temperature increase. The helix content decreased to less than half, while the β-sheet content increased to more than 3-fold. The β-turn of ovalbumin also decreased with heat denaturation. Thus, a marked increase of β-sheet structure in sacrifice of helical structure was observed during heat denaturation of ovalbumin.

To elucidate whether β-sheet structure is formed intramolecularly or intermolecularly, changes in the β-sheet content of heat-denatured ovalbumin were followed in various conditions. Figure 4 shows the effect of protein

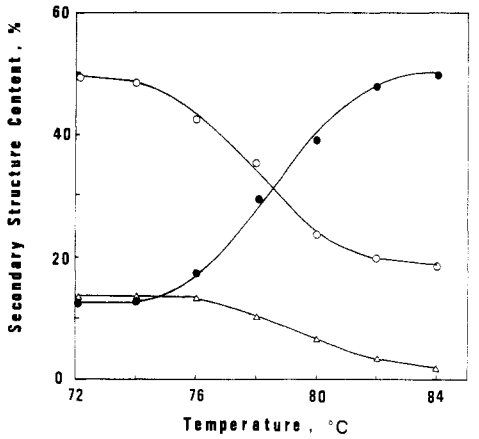

Figure 3. Changes in the contents of the secondary structure of ovalbumin during heat denaturation. Conditions: 0.1% ovalbumin solution in 67 mM phosphate buffer, pH 7.0, containing 100 mM NaCl was heated to a given temperature and then immediately cooled to 25 °C. Key: O—O, α-helix; ●—●, β-sheet structure; △—△, β-turn structure.

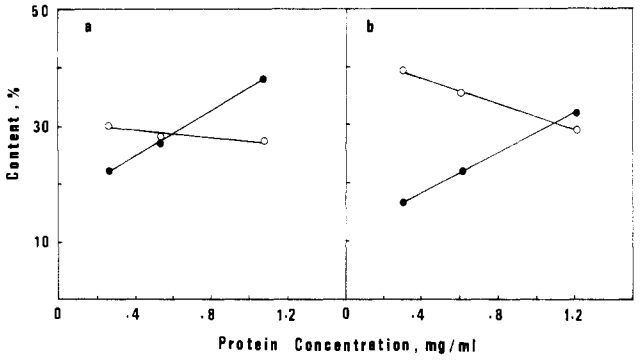

Figure 4. Effects of protein concentration on the contents of β-sheet structure by heat denaturation of ovalbumin: a, in 67 mM phosphate buffer, pH 7, 0, containing 100 mM NaCl; b, in 20 mM phosphate buffer, pH 7.0. Key: ●—●, β-sheet; O—O, α-helix.

concentration on the formation of β-sheet structure of ovalbumin at 25 °C after heating up to 80 °C. The contents of β-sheet structure of heat-denatured ovalbumin increased with the increase in protein concentration both in high (a) and low (b) salt concentrations of phosphate buffer solution, pH 7.0. On the other hand, the α-helix content slightly decreased with the increase in protein concentration. Figure 5 shows the effect of the salt concentration of buffer solution on the formation of β-sheet structure in ovalbumin at 25 °C after heating up to 80 °C. The formation of β-sheet structure of heat-denatured ovalbumin is increased in higher salt concentration of buffer solution where the protein–protein interaction of heat-denatured molecules is enhanced. On the other hand, α-helix content was decreased with the increase in the salt concentration. Figure 6 shows the relationship between the molecular weight and the β-sheet content of heat-denatured ovalbumin. A good correlation was observed between the molecular weight and the β-sheet content. The correlation coefficient was 0.89 and significant ($p < 0.05$). This directly indicates that the heat-induced ovalbumin aggregates are formed by intermolecular β-sheet structure.

DISCUSSION

CD spectra were measured during and after mild heat treatment of ovalbumin. The nonlinear least-squares curve-fitting method was applied to examine changes in the secondary structure of ovalbumin during and after heat denaturation. In early studies, changes in the helix content of proteins were followed by monitoring the ellipticity at

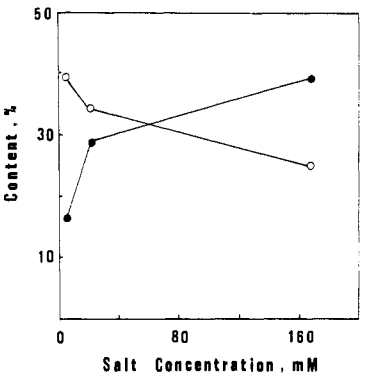

Figure 5. Effects of salt concentration on the contents of β-sheet structure by heat denaturation of ovalbumin. Conditions: 0.1% ovalbumin solution in various concentration of phosphate buffer was heated to 80 °C and then immediately cooled to 25 °C. Key: ●—●, β-sheet; O—O, α-helix.

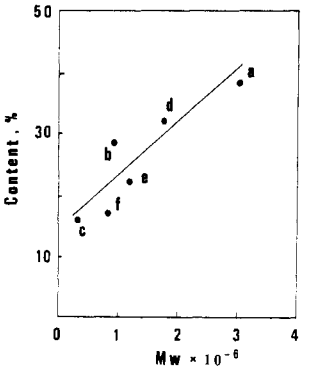

Figure 6. Relationship between the molecular weight and the content of β-sheet structure of heat-induced ovalbumin aggregates: a, 0.1% ovalbumin solution in 67 mM phosphate buffer, pH 7.0, containing 100 mM NaCl cooled at 25 °C after heating to 80 °C; b, 0.1% ovalbumin solution in 20 mM phosphate buffer, pH 7.0, cooled at 25 °C after heating up to 80 °C; c, 0.1% ovalbumin solution in 2 mM phosphate buffer, pH 7.0, cooled at 25 °C after heating to 80 °C; d–f, 0.12%, 0.06%, and 0.03% ovalbumin solution in 20 mM phosphate buffer, pH 7.0, cooled at 25 °C after heating to 80 °C, respectively.

222 nm. As shown in Figure 1, the changes in the separate ellipticity are small in the heat-denatured ovalbumin. Therefore, the ambiguous conclusion may be reached on the basis of the changes in the ellipticity at a single wavelength. Actually, the helix content of ovalbumin was reduced by the heat denaturation to half of its native value on the basis of the curve-fitting analysis, while it was reduced to less than 20% on the basis of the changes in the ellipticity at 222 nm. The change in CD spectra during and after the heat treatment was interpreted as a result of an increase in β-sheet structure at the sacrifice of helical structure. Egelandsdal (1986) has reported the same conclusion using the curve-fitting analysis of CD spectra of mildly heat-treated ovalbumin. However, he did not describe the role of β-sheet structure in the formation of aggregates or gels. We have brought into focus this point. Another characteristic of the heat denaturation of ovalbumin is irreversibility. This was confirmed from CD spectra (Figure 1) of ovalbumin during and after heat treatment at 80 °C. The CD spectrum did not recover but amplified the difference between native and denatured forms by standing at 25 °C after the heat treatment of ovalbumin. It is interesting that the urea denaturation curves of heat-treated ovalbumins indicate the cooperativity, although it is lower than that of native ovalbumin. This suggests that the partially unfolded stable structure

is formed by the heat treatment of ovalbumin.

The rapid increase in β-sheet structure was quantitatively observed during heat denaturation of ovalbumin. The formation of β-sheet structure of heat-denatured ovalbumin increased in higher protein concentration and salt concentration in which the intermolecular interaction was enhanced. It seems likely that protein interactions would be primarily of a hydrophobic nature under high-salt concentration. β-Sheet is generally thought to be stabilized by free energy gain from increased hydrogen-bond formation. It seems possible that the increased β-sheet structure was stabilized by hydrophobic interaction.

In addition, the content of β-sheet structure was increased in proportion to the molecular weight of the heat-denatured ovalbumin aggregates. Therefore, it is apparent that the β-sheet is intermolecularly formed between unfolded molecules. It is probable that the partially unfolded form with a significant amount of secondary structure is stabilized by the intermolecular interaction. Thus, the irreversibility of the heat denaturation of ovalbumin is derived from the intermolecular interaction formed by β-sheet structure. Taking into account the result that the exposed hydrophobic residues on the molecular surface remarkably increased with the heat denaturation of ovalbumin (Kato et al., 1983), the β-sheet structure may be liable to form in the hydrophobic environment. That is, the intermolecular β-structure may be strengthened by the exposed hydrophobic residues that exclude the water in the space between unfolded molecules. Thus, it seems reasonable to conclude that ovalbumin aggregates or gels are built from the partially unfolded molecules with a significant amount of secondary structure and cross-linked with the network of the intermolecular β-sheet structure strengthening by the exposed hydrophobic residues.

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Ability of L-Canavanine To Support Nitrogen Metabolism in the Jack Bean, *Canavalia ensiformis* (L.) DC.

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The ability of L-canavanine, a nonprotein amino acid of certain leguminous plants, to support the nitrogen metabolism of jack bean, *Canavalia ensiformis* [Leguminosae], was assessed by administration of L-[*guanidino*-N^3-^{15}N]arginine, L-[*guanidinooxy*-N^3-^{15}N]canavanine, or L-[*guanidinooxy*-N^1-^{15}N]canavanine into the cotyledons of 9-day-old plants. A strikingly similar pattern of ^{15}N assimilation into de novo synthesized amino and imino acids resulted from feeding L-[*guanidino*-N^3-^{15}N]arginine and L-[*guanidinooxy*-N^3-^{15}N]canavanine. Glutamic acid plus glutamine and alanine were the most heavily labeled of the detected compounds. Some transfer of ^{15}N from L-[*guanidino*-N^3-^{15}N]arginine to canavanine was noted. This may occur by a transamidination reaction between L-canaline and L-arginine. L-[*guanidinooxy*-N^1-^{15}N]Canavanine also supported amino and imino acid biosynthesis in this plant, but much more alanine and less glutamic acid and glutamine were labeled. These experiments provide substantive experimental evidence for the long-reputed hypothesis that canavanine functions as a nitrogen-storing metabolite.

L-Canavanine, the 2-amino-4-(guanidinooxy)butyric acid structural analogue of L-arginine, is distributed throughout the Lotoideae (Fabaceae), a major group of leguminous plants, and is found in such agronomically important plants as clover, alfalfa, trefoil, and Lespedezas (Bell et al., 1978; Rosenthal, 1979). Canavanine is frequently the principal nonprotein amino acid of the seed and often accounts for more than 3% of the dry matter (Van Etten et al., 1961, 1967). There is considerable evidence that this potentially toxic secondary metabolite functions in higher plant chemical defense against herbivores, particularly insects (Rosenthal, 1979; Rosenthal and Bell, 1979). In contrast, its putative role as a nitrogen-storing metabolite is far less firmly established experimentally. The storage of appreciable canavanine in the seed, which is precipitously lost during germination; its facile translocation from the cotyledons to the growing parts of the plant; its high

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