# Molten Globule State of Protein Molecules in Heat-Induced Transparent Food Gels

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The microstructures of transparent gels from ovalbumin, hen egg white lysozyme, and bovine serum albumin were examined by high-resolution scanning electron microscopy. Three transparent gel matrices were clearly found to be composed of a common structural unit in which heat-denatured molecules formed fine networks of linear aggregates. The molecular conformation of heat-denatured proteins in linear aggregates produced after heat treatment in the absence of salt was studied by measuring circular dichroism spectra, intrinsic tryptophan fluorescence, and adsorption of the dye 1-anilinonaphthalene-8-sulfonate. These experiments showed that all three heat-denatured proteins did not take on a random-coiled state but rather remained partially folded, with some hydrophobic regions becoming exposed to the solvent environment. This conformation could thus be categorized as the "molten globule" state.

**Keywords:** *Transparent gel; linear aggregate; heat-denatured globular protein; molten globule state; partially folded conformation*

## INTRODUCTION

Gelation is pictured as the formation of a coherent, continuous, and well-defined network assembled from particles or polymers, this network being embedded in a water-based solvent. A variety of food gels with different molecular structures have been reported. Gels from polysaccharides and gelatin, which are commonly utilized in food applications, have a transparent appearance, and this property is attributed to the formation of fine networks of fibrous chains with "point contacts" or "crystalline" junction zones. Globular proteins such as ovalbumin, hen egg white lysozyme, and bovine serum albumin are also known to possess good gelling properties upon heating (Hegg *et al.*, 1979; Hegg, 1982; Clark, 1992; Hayakawa and Nakamura, 1986). The ability to form gels is affected by the pH, ionic strength, and protein concentration of the medium and the heating procedure. Manipulating these factors mainly produces two types of heat-induced protein gels: a transparent gel and a turbid gel (Egelandsdal, 1980; Hatta *et al.*, 1986; VanKleef, 1986). Electron microscopy is a powerful technique for studying the architecture of solid gels (Barbu and Joly, 1953; Clark *et al.*, 1981; Nakamura *et al.*, 1984; Hermansson, 1988). This technique reveals that a transparent gel is composed of a network of linear aggregates of heat-denatured molecules. This fact suggests that transparent gels of some globular proteins have a common property with polysaccharide and gelatin gels in terms of a three-dimensional network of linear polymers. On the other hand, a turbid gel is made up of heat-denatured molecules gathered into random agglomerates (Tani *et al.*, 1993). There is an alternative explanation which suggests that turbid gels are particulate and that the sizes of the particles are uniform (Barbut and Foegeding, 1993). These findings support previous results by Tombs stating that the difference between turbid and transparent gels can be attributed to the difference in the arrangements of heat-denatured molecules (Tombs, 1974). Dolgikh *et al.* (1981) report that heat-denatured α-lactalbumin takes on a compactly folded conformation, although globular proteins are transformed to completely unfolded chains in the presence of a high concentration of chemical denaturant. However, detailed knowledge about the conformation of globular protein molecules in heat-induced gels at the molecular level and whether the heat-denatured globular protein molecules share common features among different proteins beyond their species and origins remains scant.

Conditions for the formation of a transparent gel usually fall between a narrow range of pH and ionic strength in the medium. This range can be broadened using a two-step heating procedure (Kitabatake *et al.*, 1987). The initial heating of an ovalbumin solution produces a clear sol under salt-free conditions. When this sol is reheated after mixing with salt, the second heating yields a transparent gel even at high salt concentrations, whereas a turbid gel is formed after only a single heating. The basic unit of a transparent gel in a clear sol was found to be linear aggregates (Koseki *et al.*, 1989a). Similar phenomena have been observed in the gel formation in two common food proteins, bovine serum albumin, and hen egg white lysozyme (Murata *et al.*, 1993; Tani *et al.*, 1993). Using the two-step heating method, the range in salt concentration required for a transparent gel formation has been extended and applied to the formation of gels in bovine serum albumin. Under similar conditions, the hardness of clear lysozyme gels was enhanced. Linear aggregates were also detected in both clear sols after the first heating at low ionic strength. The fact that many food

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proteins form a transparent gel upon heating and that the heat-denatured protein molecules orient in a linear fashion in a transparent gel lattice leads to the hypothesis that, beyond their species and origins, different heat-denatured protein molecules may share certain conformational features.

Various optical methods, including UV differential spectra, circular dichroism, and fluorescence spectra, are often employed for analyzing protein structure. Because of high protein concentrations, gels from heat-denatured proteins are not suitable candidates for the techniques mentioned above. However, with the advent of the two-step heating procedure, a linear aggregate can be obtained in a soluble state as the intermediate product of a transparent gel. Because a linear aggregate produced at low ionic strength is a particle small enough to scatter light least, this material is the most suitable form for elucidating the conformation of heat-denatured protein molecules, assuming that the linear orientation of heat-denatured molecules can be regarded as an ordered structure.

In this paper, we first reconfirmed the identity of the microstructures of three transparent gels, ovalbumin, hen egg white lysozyme, and bovine serum albumin gels, by high-resolution scanning electron microscopy. Second, with the linear aggregates, the conformation of heat-denatured molecules was studied by measuring circular dichroism spectra, intrinsic tryptophan fluorescence; and adsorption of fluorescent dye as a marker of surface hydrophobicity.

## MATERIALS AND METHODS

**Proteins.** Ovalbumin was prepared by crystallization from fresh hen egg white in a half-saturated ammonium sulfate suspension. Crystallization was performed five times. Sodium azide (0.02%) and EDTA (0.1 mM) were added throughout the preparation of ovalbumin to prevent microbial contamination and the potential oxidation of free sulfhydryl groups. Hen egg white lysozyme and bovine serum albumin (BSA, fraction V) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan) and Sigma Chemical Co. (St. Louis, MO), respectively.

**Transparent Gel Preparation.** Before use, proteins were exhaustively dialyzed against distilled water containing 0.02% $NaN_3$ and 0.1 mM EDTA. The protein concentration was determined by absorbance at 280 nm, based on the following extinction coefficients: $E^{1\%}_{280nm}$ = 7.12 $cm^{-1}$ for ovalbumin, 26.35 for lysozyme, and 6.67 for BSA. Ovalbumin (5%) and lysozyme (5%) solutions were adjusted to pH 7 by adding 1 N HCl or 1 N NaOH. BSA (6%) solution was adjusted to pH 7.5. Heating temperatures for preparing gels were 80 °C for ovalbumin and lysozyme and 85 °C for BSA. All proteins were heated for 20 min. The ovalbumin solution was first heated in the absence of salt. After cooling to room temperature, the solution was heated again using 150 mM NaCl. Dithiothreitol was added to the lysozyme solution at a concentration of 7.5 mM throughout the procedure. The solution was first heated in the absence of salt and then treated with 50 mM NaCl upon the second heating. For the BSA gel, the first heating was made using 10 mM NaCl, followed by a second heating in the presence of 200 mM NaCl.

**Production of Linear Aggregates.** Ovalbumin linear aggregates were prepared by heating a 0.1% protein solution in 10 mM phosphate buffer (pH 7) at 80 °C for 10 min. A 0.1% lysozyme solution (pH 7) with 0.15 mM dithiothreitol was heated at 80 °C for 20 min. The polymerization of BSA was conducted by heating of 0.5% BSA in 20 mM phosphate buffer (pH 7.5) at 85 °C for 20 min.

**Scanning Electron Microscopy.** Transparent gels were each sliced into small pieces with a razor blade. Each sample was fixed in 2% glutaraldehyde for 2 h at room temperature and then treated with 2% tannic acid in 2% glutaraldehyde for 6 h. After several washings in phosphate buffer, the samples were post-fixed in 2% osmium tetraoxide for 2 h and then washed in the phosphate buffer. The fixed samples were dehydrated in consecutive steps, whereby the ethanol concentration was increased by 10% starting at 50% until 100%. The preparations were next immersed in isoamyl acetate for 30 min, followed by critical point drying in liquid $CO_2$. The gel specimens were broken and mounted on a copper specimen plate with aluminum oxide paint and coated with approximately 40 Å of platinum. Micrographs were taken with a Hitachi S-900 or S-4000 scanning electron microscope at an accelerating voltage of 5 kV.

**Circular Dichroism.** Linear aggregates were used as samples. Circular dichroism (CD) spectra were recorded at 25 °C with a J-500C spectropolarimeter (Jasco). The far-UV CD spectra were measured at concentrations of 0.5 mg/mL for ovalbumin and lysozyme and 0.6 mg/mL for BSA, with a 0.2 mm cell, from 190 to 250 nm. The near-UV CD spectra of each sample were measured at 1.0 mg/mL, with a 10 mm cell, from 245 to 310 nm. The far-UV CD spectral data were expressed as mean residue ellipticity (deg·$cm^2$/dmol) and the near-UV CD spectra in molar ellipticity.

**Tryptophan Fluorescence.** Fluorescence spectra were measured at 25 °C and recorded on a Hitachi F-3000 fluorescence spectrophotometer. The sample concentrations tested were 0.15 mg/mL for ovalbumin and lysozyme and 0.3 mg/mL for BSA. The excitation wavelength was 295 nm, and the emission wavelength was from 305 to 400 nm.

**1-Anilinonaphthalene-8-sulfonate Fluorescence.** Various forms of native or denatured proteins were incubated at 25 °C for 5 min with a 20-fold molar excess of 1-anilinonaphthalene-8-sulfonate (ANS) at a concentration of 1 μM. The excitation wavelength was 390 nm, and the emission wavelength was 470 nm. Emission spectra were corrected for background fluorescence caused by ANS in reactions lacking protein.

## RESULTS

**Gel Microstructure Observed with Scanning Electron Microscopy.** Using high-resolution scanning electron microscopy (SEM), we examined whether transparent gels from three food proteins shared common structural characteristics. During sample preparation, the double-fixation method was used with glutaraldehyde and osmium tetraoxide, effectively preserving the integrity of the intrinsic structure of the transparent gels (Figure 1). Panels A–C in Figure 1 show the SEM photographs of an ovalbumin transparent gel composed of fine networks of well-ordered linear aggregates of heat-denatured molecules. Very similar networks are also in the SEM photographs of lysozyme gel (Figure 1D–F) and BSA transparent gel (Figure 1G–I). Such results indicate that linear aggregates of heat-denatured molecules are the structural scaffolding in heat-induced transparent gels in globular proteins, irrespective of their species and structural specificity. Image analysis estimates the diameter of the ovalbumin string to be approximately 100 Å. The diameters of lysozyme and BSA linear polymers, or strings, were observed to be of almost the same magnitude as that of ovalbumin.

**Circular Dichroism Measurement.** Figure 2 presents the circular dichroism (CD) spectra in the far-UV region, showing the change in secondary structure. All protein molecules denatured in guanidinium hydrochloride did not possess ordered secondary structures but rather were in a random-coiled state. In the case of ovalbumin (Figure 2A) and lysozyme (Figure 2B), the heat-denatured molecules took on almost the same secondary structures as the native molecules. Heat-denatured BSA molecules were found to retain a significant degree of secondary structure (Figure 2C).

Figure 3 shows the profiles of the near-UV CD spectra, showing microenvironmental changes around

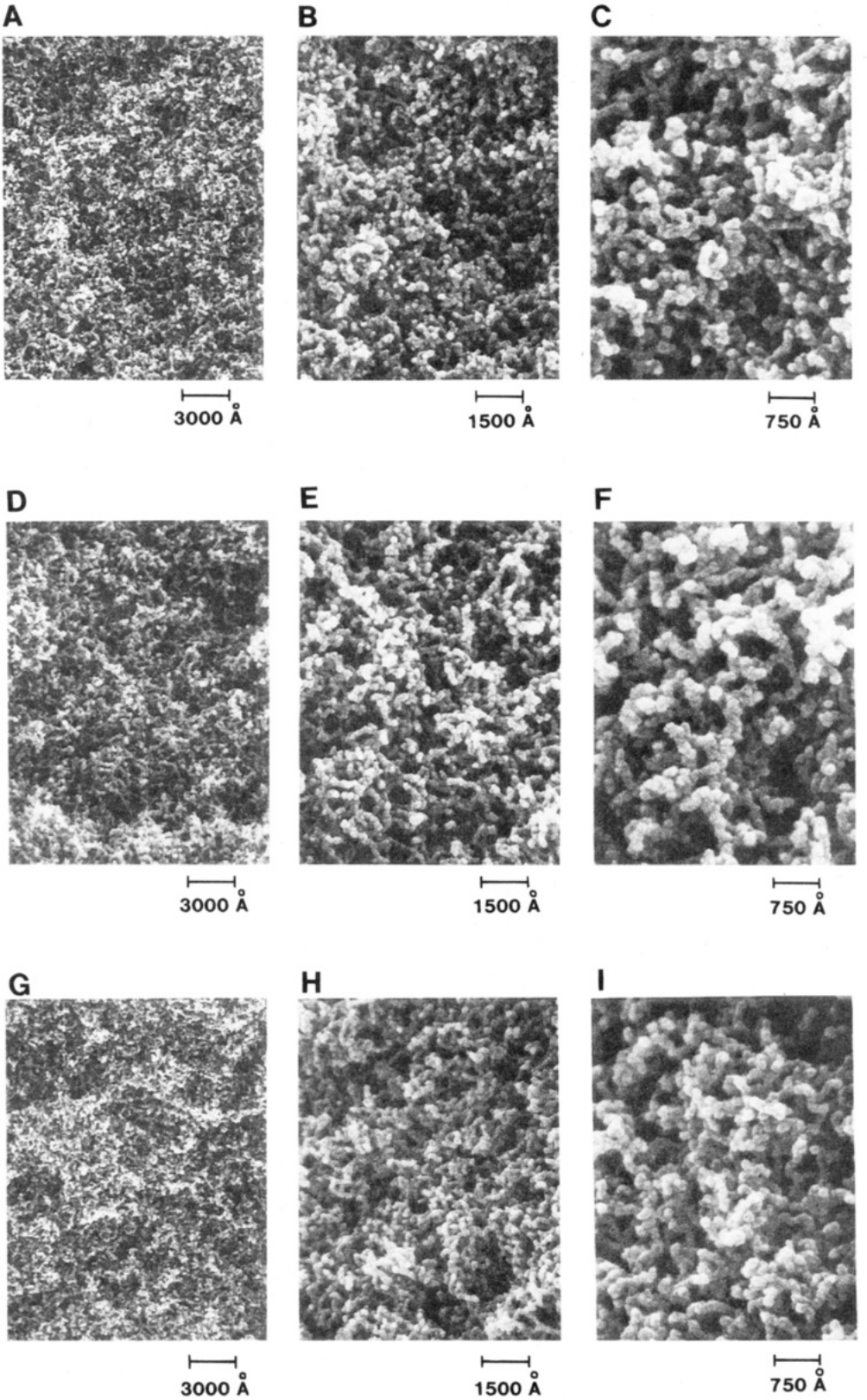


**Figure 1.** Microstructure of food protein gels by scanning electron microscopy. Photographs show linear polymerization of heat-denatured protein molecules in the transparent gels of ovalbumin (A–C), lysozyme (D–F), and bovine serum albumin (G–I). Resolution was performed at a magnification of ×50 K for (A), (D), and (G), ×100 K for (B), (E), and (H), and ×200 K for (C), (F), and (I). (The figure is reproduced here at 50% of the original.)

the aromatic amino acids within the protein molecule. The CD spectra reflect unique structural characteristics for each protein molecule. The molecules fully denatured by guanidinium hydrochloride treatment did not have any characteristic CD spectrum in the near-UV region tested, suggesting that the configuration of aromatic amino acid residues within each native molecule is disrupted and randomized in the molecule denatured by guanidinium hydrochloride. The integrity of the tertiary structure in native ovalbumin was completely lost in the heat-denatured molecules (Figure 3A). The CD profile of heat-denatured lysozyme was

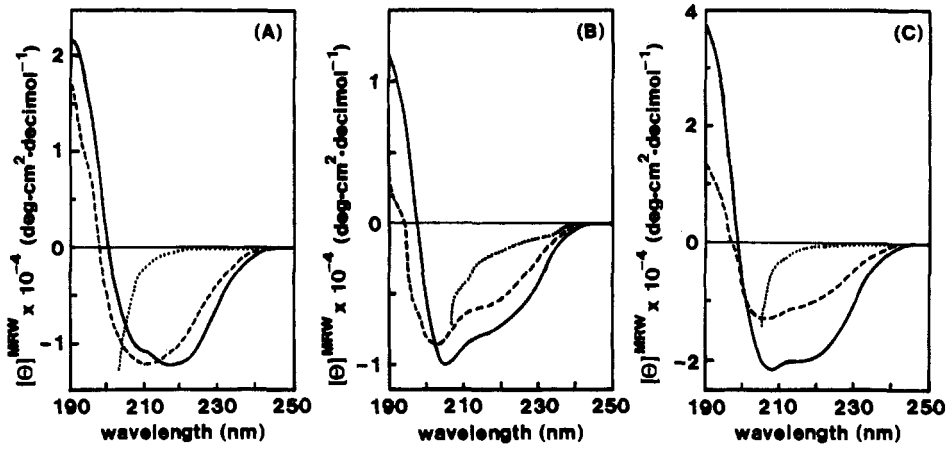


**Figure 2.** Far-UV CD spectra of different forms of protein molecules. The native form (solid line), the heat-induced form (dashed line), and the completely unfolded by 6 M guanidinium hydrochloride form (dotted line) were analyzed for ovalbumin (A), lysozyme (B), and bovine serum albumin (C), respectively.

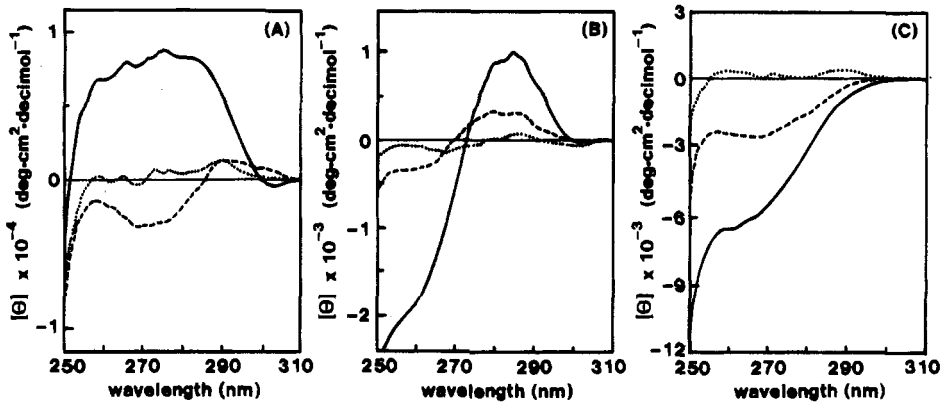


**Figure 3.** Near-UV CD spectra of different forms of protein molecules. The native form (solid line), the heat-induced form (dashed line), and the completely unfolded by 6 M guanidinium hydrochloride form (dotted line) were analyzed for ovalbumin (A), lysozyme (B), and bovine serum albumin (C), respectively.

more similar to that of the completely denatured form than to the native CD profile (Figure 3B). The tertiary structure observed in heat-denatured BSA molecules was found to be the same as the intermediate between the native state and the random-coiled state (Figure 3C).

**Intrinsic Tryptophan Fluorescence.** The microenvironmental changes around tryptophan residues were also examined by measuring the intrinsic fluorescence of the three molecular states, native, heat-denatured, and chemically denatured, as an index for the fluctuation of tertiary structure (Figure 4). A fluorescence emission maximum was found at 336 nm in the native ovalbumin molecule (Figure 4A). When ovalbumin was treated with the denaturant guanidinium hydrochloride, the emission maximum shifted from 336 to 352 nm, leading to a 64% decrease in the relative fluorescence intensity. Heated ovalbumin possessed the same emission maximum of 336 nm as native ovalbumin, but its relative intensity lay between those of the native and chemically denatured molecules. A fluorescence emission maximum was observed at 338 nm in native lysozyme (Figure 4B). The fluorescence changes markedly when the protein is fully unfolded; the emission maximum shifts from 338 to 350 nm accompanied by a roughly 35% increase in the fluorescence intensity. The red shift of fluorescence reflects transfer of tryptophan residues to a more polar environment. The emission maximum of the heat-denatured lysozyme was 343 nm, halfway between the fluorescence maxima of the native and fully unfolded proteins, with an approximately 15% increase in relative fluorescence intensity. The relative fluorescence intensity was also approximately halfway between those of the native and chemically unfolded molecules. This observation implies that, relative to the native protein, the tryptophan residues in heated lysozyme are exposed to a slightly more hydrophilic environment, as indicated by a 50% red shift emission. Conversely, the tryptophan residues in the heat-denatured molecules, relative to the chemically denatured proteins, are in a more hydrophobic environment, as indicated by a 50% blue shift emission. The molecular properties of heated BSA molecules were surprisingly unlike those of the heat-treated lysozyme molecules (Figure 4C). Upon heat denaturation, its fluorescence maximum blue shifts from 342 to 334 nm with a 34% decrease in relative fluorescence intensity. Complete unfolding induced a major red shift of emission maximum from 334 to 351 nm followed by a further decrease of fluorescence intensity. This demonstrates that the accessibility of solvent to the tryptophan residues is greater in the native conformation than in the heat-denatured conformation.

**1-Anilinonaphthalene-8-sulfonate Fluorescence.** The surface hydrophobicity of heat-denatured proteins was measured using a chromophore probe, ANS. The probe tests whether normally buried hydrophobic regions are exposed to the solvent environment after the protein is heat-denatured. As shown in Figure 5, all three proteins exhibited no fluorescence after denatur-

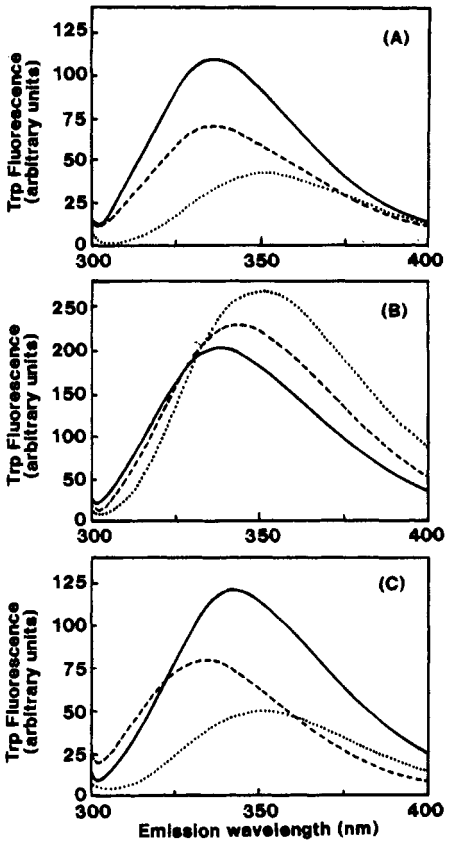


**Figure 4.** Tryptophan fluorescence emission spectra of different forms of protein molecules. The native form (solid line), the heat-induced form (dashed line), and the completely unfolded by 6 M guanidinium hydrochloride form (dotted line) were analyzed for ovalbumin (A), lysozyme (B), and bovine serum albumin (C), respectively. The numbers on the left denote arbitrary units.

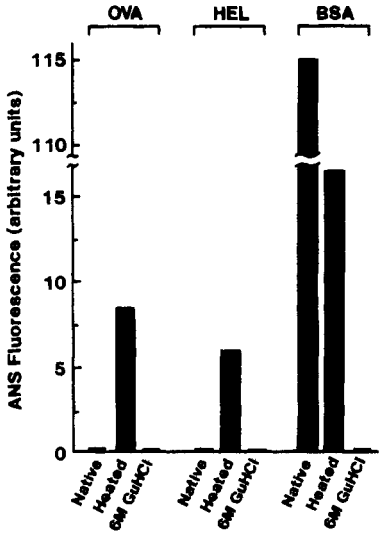


**Figure 5.** ANS fluorescence of different forms of protein molecules. The native form (native), the heat-induced form (heated), and the completely unfolded by 6 M guanidinium hydrochloride (6 M GuHCl) form were analyzed for ovalbumin (OVA), lysozyme (HEL), and bovine serum albumin (BSA), respectively. The numbers on the left denote arbitrary units.

ation with guanidinium hydrochloride. However, all three proteins exhibited significant fluorescence after heat denaturation. Neither native ovalbumin nor lysozyme exhibited any fluorescence. Native BSA, unlike native ovalbumin and lysozyme, exhibited high fluorescence.

## DISCUSSION

The double-fixation method with glutaraldehyde and osmium tetraoxide enabled us to visualize reasonably the apparent microstructures of all transparent gels from globular proteins. Our results indicate that for all three sample proteins the formation of linear aggregates, after heat treatment under selective conditions, shows that they possess a common characteristic structure, regardless of their origins and species.

A growing interest in the occurrence of the molten globule state in globular proteins has produced vast amounts of data and new information (Kuwajima, 1989; Ptitsyn, 1992). Ohgushi and Wada first proposed the name "molten globule" state for a non-native state of cytochrome *c* under acid perturbation (Ohgushi and Wada, 1983). This state is formed as a unique kinetic intermediate in the reversible interconversion between the native and denatured states in a protein molecule, as observed under equilibrium conditions at low pH or in the presence of a moderate concentration of a denaturant (Ptitsyn, 1987). The molten globule intermediate is widely characterized by the following: (1) the protein molecule in this state has native-like features in its secondary structural organization, whereas in its tertiary structure, the movement of side chains is greatly increased as compared to the native state; (2) the compactness of the molecule is "loosened" somewhat in the molten globule state, but its compactness is more coordinated than in the fully denatured state; and (3) some hydrophobic clusters are exposed, as reflected by the change in intrinsic tryptophan fluorescence and in increased binding of a hydrophobic fluorescent dye.

Data on CD spectra in the far-UV region show that the secondary structures were retained, to a large extent, in all heat-denatured protein molecules. CD profiles in the near-UV region, however, indicate that the characteristic tertiary structures of ovalbumin and lysozyme were largely disrupted by heat treatment. Native tertiary structure was, to some extent, damaged in the heat-denatured BSA molecule. These results suggest that heat-denatured protein molecules take on a compact and partially folded conformation distinguished from both the native and the fully denatured states, although they appear more or less different in degree. That they are transformed to an intermediate state during the conversion between the native and the completely denatured states by heat treatment is consistent with previous reports by other investigators (Burke and Rougvie, 1972; Egelandsdal, 1986; Wang and Damodaran, 1991). This satisfies the first criterion described above.

Koseki *et al.* (1989b) described an ovalbumin linear aggregate model as a simple worm-like chain of cylinders. They observed that these cylinders had a diameter of 120 Å in a polymer chain, consistent with our electron microscopic observations by high-resolution SEM that give values between 100 and 150 Å along the contour of the linear ovalbumin aggregates. According to recent analyses of thermally induced linear aggregates of ovalbumin by dynamic light scattering, a dimer model explains the formation mechanism of linear aggregates (Nemoto *et al.*, 1993; Doi, 1993). This model proposes that two heat-denatured ovalbumin molecules join together to form a dimer with a major axis of 100–120 Å and a minor axis of 50–60 Å. Dimers then collide and join together, with the two axes crossing at right angles, alternately. Relative to the estimated diameter of 56 Å in native ovalbumin, the diameter of heat-

denatured ovalbumin molecules is calculated to be almost the same, assuming the molecules are spherical. Clearly, the speculated model is compatible with the second criterion described above. However, this evidence is indirect, and the diameter should be studied on other heat-denatured proteins. It would be more direct and convincing to evaluate the diameter of heat-denatured proteins in a "monomeric" state by small-angle X-ray scattering and viscosity measurement.

Using intrinsic tryptophan fluorescence and surface hydrophobicity, we confirmed that all three heat-denatured proteins met the third criterion. Tryptophan fluorescence curves of heat-denatured ovalbumin and lysozyme molecules, with respect to both fluorescence intensity and emission wavelength, show maxima which lie between corresponding values of the native and completely denatured proteins, respectively. ANS bound only to the heat-denatured molecules of ovalbumin and lysozyme, suggesting that some portions of hydrophobic regions, which had been buried within the native molecule, are exposed upon heat denaturation. Unlike ovalbumin and lysozyme molecules, the tryptophan residues in BSA molecules were less accessible to the solvent in the heat-denatured state than in the native state, although a maximum fluorescence intensity of the heat-denatured BSA molecules lies between those of the native and chemically denatured states. Thus, the surface hydrophobicity of BSA is greater in the native state, as some literature would indicate (Damodaran, 1986). Compared to fully denatured BSA molecules, heat-denatured molecules bind a significant amount of ANS, suggesting that the heat-denatured molecules assume the intermediate state. However, the anomalous behavior of BSA can be accounted for. Because, physiologically, BSA is an important carrier of fatty acids and aliphatic compounds, some hydrophobic regions are exposed to the solvent in the native state. The fluorescence and CD spectra obtained by Lee and Hirose (1992) of disulfide-reduced human serum albumin correspond to the fluorescence and CD spectra we obtained from our analysis of the heat-denatured molecules. These facts support heat-denatured BSA molecules sharing common properties with ovalbumin and lysozyme molecules as intermediates between the native and chemically denatured states. It is likely that the transitional conformation upon heat denaturation of protein molecules is a molten globule state.

The concept of a molten globule state has aided in the understanding of the physiological functions of some globular proteins in cell biology and protein chemistry. On the basis of the data of van der Goot *et al.*, Hirose reviewed the possible involvement of molten globule state in exerting protein functionality during food processing, in which this state is also assumed to have an important role in emulsification, foaming, and gelling (van der Goot *et al.*, 1991; Hirose, 1993). It is also likely, therefore, that globular proteins in the molten globule state mediate food protein functionality, more so than the native or denatured states.

In conclusion, transparent gels can be made from many types of materials, such as polysaccharides, gelatin, and myofibrillar proteins, and some globular proteins. In our experiment, heat-induced transparent gels from three globular proteins, ovalbumin, lysozyme, and BSA, are composed of the matrices of networks of linear aggregates, in which heat-denatured molecules are aligned in a highly ordered fashion, regardless of protein species and origins. The conformation of heat-denatured molecules could be elucidated by analyzing linear polymers. Heat denaturation involves the transition of protein molecular conformation from the native state to a molten globule state, followed by the association of heat-denatured molecules either with a highly ordered manner in a transparent protein gel or with random scattering in a turbid gel. This finding clearly supports the gelation models proposed by Barbu and Joly (1953) and Tombs (1974). Protein gels can also be formed under various conditions other than heat, exemplified by rennet gels of casein micelles and transglutaminase gels. The problem remains open whether the concept of a molten globule state could be applicable to many types of protein gel formation. However, in the future, a better understanding of the molten globule state will shed light on the field of food science and technology and enhance the development of new food materials based on scientific speculation rather than on trial-and-error manipulations.

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