Raman Spectroscopic Study of Thermally and/or Dithiothreitol Induced Gelation of Lysozyme

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Opaque gels were formed by heating lysozyme solutions (10%, pH 7.2, 50 mM NaCl) containing 10 or 100 mM dithiothreitol (DTT) at 37, 60, 80, or 100 °C but not at 37 or 60 °C in the absence of DTT. Gels formed with 100 mM DTT were soluble in 8 M urea, while gels formed with no or 10 mM DTT at 100 °C were insoluble. Raman spectral analysis indicated increased exposure of aromatic residues and a general decrease in α-helical and increase in β-sheet secondary structure fraction of molecules in gels compared to in solution. Chemical and Raman spectral analyses of sulfhydryl and disulfide groups suggested that reduction or interchange reactions involving intramolecular disulfide bonds of lysozyme were responsible for destabilizing tertiary structure, allowing conformational changes and exposure of hydrophobic groups which led to gel formation. Intermolecular disulfide cross-links were not necessary for gelation but could lead to more stable gels.

INTRODUCTION

The ability to form a stable gel is one of the most important functional properties of proteins in food systems, imparting characteristic textural qualities to the food products. However, the structural features of proteins which may control the ability to gel or macroscopic differences in the gel such as transparency vs opacity are not well understood.

Three major categories describing protein molecular properties have been recognized as hydrophobic, electrostatic, and steric parameters, but these parameters are interdependent. In particular, the molecular flexibility of a protein may be restricted by the strength of internal hydrophobic and electrostatic interactions which maintain the native structure, as well as by the presence of covalent disulfide intramolecular bonds. Lysozyme from hen egg white is an example of a protein whose tertiary structure, including a hydrophobic box positioned in the interior of a compact globular region, is stabilized by four intramolecular disulfide bonds (Blake et al., 1978). It has been suggested that many functional properties of food proteins depend on the exposure of hydrophobic groups on the surface of the molecule and the ensuing interactions between these hydrophobic groups with oil (in emulsions), with air (in foams), or with other protein molecules (in gels or coagula) (Nakai and Li-Chan, 1988). Since hydrophobic amino acid residues usually prefer to locate in the interior of globular protein molecules, the unfolding of the native structure during food-processing steps such as homogenization, whipping, or heating may be necessary to allow participation of these hydrophobic groups in intermolecular hydrophobic interactions.

Many proteins do not gel even at temperatures approaching their denaturation temperature. For example, lysozyme did not form gels when heated at 80 (Hayakawa and Nakamura, 1986) or 95 °C (Hegg, 1982), although the denaturation temperature for lysozyme solution at pH 7 was determined as 75 °C by differential scanning calorimetry (Donovan et al., 1975), while egg white did not gel at temperatures below 60 °C, which corresponds to the denaturation temperature of conalbumin, the most thermolabile component of egg white proteins (Hirose et al., 1986). However, it was observed that thiol reagents such as 2-mercaptoethanol and dithiothreitol (DTT) induced gel formation of lysozyme and conalbumin under mild heating conditions (Hayakawa and Nakamura, 1986; Oe et al., 1986, 1987).

In our laboratory, we recently showed that 5% lysozyme solutions formed gels in the presence of 7.0 mM DTT when incubated at either 37 or 80 °C (Li-Chan and Nakai, 1989). On the basis of the ability of the gels to be redissolved by incubating in various denaturing and/or reducing media for 22 h at 22 °C, it was suggested that noncovalent interactions, predominantly hydrophobic or hydrogen-bonding type, were the forces involved in the gel network structure. The gel formed at 80 °C was, however, more stable than that formed at 37 °C. Although both gels could be dissolved completely by 8 M urea, only the 37 °C gel was solubilized by 1% SDS. It is possible that in addition to noncovalent interactions, the 80 °C gel was further stabilized by intermolecular disulfide bonds and that the seemingly anomalous solubility in 8 M urea was in fact an artifact resulting from urea-induced SH–SS interchange reactions which have been reported to occur (Xiong and Kinsella, 1990).

To monitor the changes in sulfhydryl groups and surface-exposed hydrophobic regions resulting from addition of DTT followed by incubation at 37 or 80 °C, turbid solutions containing 0.5% lysozyme and 0.7 mM DTT were studied, rather than the opaque gels formed by using 5% lysozyme and 7 mM DTT. Under these conditions, it was shown that DTT alone caused little change but subsequent heating resulted in large increases both in sulfhydryl groups measured by Ellman's reagent and in hydrophobicity measured by aromatic and aliphatic fluorescence probes (Li-Chan and Nakai, 1989). However, due to interference of turbidity or insolubility on measurement by these spectroscopic methods, no data have been reported on these molecular parameters or on possible alteration of the secondary structure of the protein molecules in the thiol-induced gels themselves, i.e., at high protein concentrations. In this respect, Raman spectroscopy has an advantage over other spectroscopic methods of being applicable to molecules in aqueous solution as well as in solid form. Bands in the Raman spectrum arising from amide I, amide III, and skeletal stretching modes of peptides and proteins are useful in characterizing different backbone conformations, while bands attributed to stretch-

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ing or deformation (bending) vibrational modes of various functional groups of amino acid residues can be used to monitor the environment around these side chains (Przybycien and Bailey, 1989; Tu, 1986; Williams, 1983; Carey, 1982; Clark et al., 1981; Painter and Koenig, 1976). In particular, the Raman spectrum can provide valuable information on SS or SH groups of cystinyl or cysteinyl residues, on CH groups of aliphatic residues, and on aromatic rings of tryptophanyl, tyrosinyl, and phenylalanyl residues.

The objective of the present study was to apply Raman spectroscopy to investigate structural changes in lysozyme in gels formed by heating in the absence or presence of dithiothreitol.

MATERIALS AND METHODS

Lysozyme (Product No. L6876), DL-dithiothreitol (Product No. D0632), and Ellman's reagent or 5,5'-dithiobis(2-nitrobenzoic acid) (Product No. D8130) were products of Sigma Chemical Co. (St. Louis, MO), while deuterium oxide (Product No. MD-175, min 99.9 atom % D) was from MSD Isotopes (Montreal, Canada). Solutions [10 or 20% (w/v)] of lysozyme were generally prepared by dissolving in distilled water containing 50 mM NaCl, and the pH was adjusted to 7.20–7.25 by using 0.1 or 1.0 N NaOH; 10 or 100 mM DTT was incorporated by addition of aliquots of freshly prepared 1 M DTT stock solution prior to final adjustment of the total solution volume. In some studies, the solutions were prepared by dissolving in water or deuterium oxide without any adjustment of pH or addition of NaCl (final pH and apparent pD of 5.75 and 5.85, respectively). Protein concentration was calculated from absorbance at 280 nm of diluted aliquots of the solutions by using $A^{1\%}{}_{280}$ of 26.7.

Depending on the subsequent analysis to be performed, heat treatments of the samples were carried out under slightly different conditions. For colorimetric determination of sulfhydryl groups, 0.2-mL aliquots of 10% lysozyme solution were placed in 13 mm (i.d.) × 100 mm long test tubes topped with a glass marble; for Raman scattering measurement, lysozyme solution was drawn into hematocrit capillary tubes whose ends were then sealed with Parafilm. The samples were heated by immersion in water baths under the following conditions: 60 °C for 15 min; 80 °C for 12 min; 37 °C for 30 min; or 37 °C for 24 h. A steam bath was used for heating at 100 °C for 12 min. To improve the signal to noise ratio, a higher (20%) concentration of lysozyme was used for preparation of deuterated samples to be used for difference spectrum analysis; for these samples, the higher protein concentration resulted in gelation even after short heating times such as 40 °C for 10 min or 75 or 100 °C for 5 min.

Gelation was evaluated subjectively by visual observation. Assessment of gel formation and qualitative ranking of gel strength was performed by observing effects on integrity of the gel structure by inversion of the tube containing the sample and by mechanical agitation of the formed gel using a spatula, vortex action, or sonication.

Sulfhydryl group content of lysozyme solutions and gels was determined by colorimetric reaction with Ellman's reagent, after excess DTT was first removed by repeated precipitation of protein with 12% trichloroacetic acid and centrifugation, according to the method of Beveridge et al. (1974). For some gels, brief sonication (15 W, 10 s × 2, using the microtip probe of a Tekmar sonic disruptor) was required to disperse the sample in 0.05 M NaCl solution at pH 7.2, prior to TCA precipitation. After removal of DTT, the samples were dispersed in 8 M urea in Tris-glycine-EDTA buffer at pH 8 [as described by Beveridge et al. (1974)] and then incubated at room temperature for 30 min, prior to addition of Ellman's reagent. After the samples stood for another 30 min at room temperature, absorbance at 412 nm was measured.

Raman spectra were recorded on a JASCO Model NR-1100 laser Raman spectrophotometer with excitation from the 488-nm line of a Spectra-Physics Model 168B argon ion laser. The Raman scattering of samples in a transverse/transverse arrangement (capillary held horizontally and incident laser beam perpendicular to the capillary axis) was measured at ambient temperature under the following conditions: laser power, 200 mW; slit height, 2 mm; spectral resolution, 5.0 cm^{-1} at 19 000 cm^{-1}; sampling speed, 120 cm^{-1} min with data taken every cm^{-1}; 6–10 scans per sample. Background correction, normalization, smoothing, and difference spectrum computation of the recorded spectra were performed with the NR-1100 data station. Original recorded spectra in the 400–1800-cm^{-1} region were normalized to the intensity of the H-C-H deformation mode at 1445–1450 cm^{-1}, after baseline correction, as recommended by Chen et al. (1973). Raman spectra were plotted as intensity (arbitrary units) against shift in wavenumber (cm^{-1}) from the incident argon laser line at 488 nm or 20 492.8 cm^{-1}. Assignment of the bands observed in the spectra to vibrational motions of various side chains or peptide backbone was based on comparison to Raman spectral data reported in the early work of Lord and co-workers (Lord and Yu, 1970; Chen et al., 1973, 1974) and more recent reviews or monographs of Raman spectra of proteins [e.g., Carey (1982) and Tu (1986)].

Estimation of the secondary structure composition of the samples based on the Raman spectra in the amide I region was carried out on an IBM-AT computer, using the Raman spectral analysis package (RSAP) (version 2.1) described by Przybycien and Bailey (1989), which is based on the algorithms of Williams (1983) for least-squares analysis of the amide I band.

All analyses were performed at least in duplicate, and results are reported as the average of these replicates.

Table I. Visual Observations and Sulfhydryl Content of 10% Lysozyme Samples after Various Reducing Agent and Temperature–Time Treatments

treatment	observation[a]	sulfhydryl content, mol/mol of protein
lysozyme control		
no heat treatment	–	0.03
37 °C, 30 min	–	0.03
37 °C, 24 h	±	0.01
60 °C, 15 min	±	0.01
80 °C, 12 min	++[b]	0.00
100 °C, 12 min	++++[c]	0.06[d]
lysozyme + 10 mM DTT		
no heat treatment	±	0.01
37 °C, 30 min	±±[b]	0.19
37 °C, 24 h	++[b]	0.86
60 °C, 15 min	+[b]	1.3
80 °C, 12 min	+++[c]	0.54[d]
100 °C, 12 min	++++[c]	0.97[d]
lysozyme + 100 mM DTT		
no heat treatment	±±	0.14
37 °C, 30 min	+[b]	2.5
37 °C, 24 h	++[b]	6.0
60 °C, 15 min	++[b]	5.9
80 °C, 12 min	+++[b]	5.4
100 °C, 12 min	++++[b]	5.6

[a] Symbols for observations: – solution; ± slightly turbid; ±± turbid; + weak gel; ++ moderately strong gel; +++ strong gel; ++++ very strong gel. [b] Gel became dispersible after sonication. [c] Gel was not dispersible even after sonication. [d] A_{412} of supernatant only (some sample remained insoluble even in the urea-containing buffer used for Ellman's reaction).

RESULTS

Table I summarizes the effects of heat treatment of lysozyme solution, in the absence or presence of the reducing agent DTT, on gel formation and sulfhydryl content. In the absence of DTT, a strong gel was only formed after 100 °C heat treatment. Measurement of sulfhydryl (SH) group content using Ellman's reagent indicated a trace amount of SH groups even in the control unheated solution. Although the lysozyme molecule reportedly contains four disulfide (S–S) groups and no SH groups, the detection of trace SH groups due possibly to external contamination has been reported previously (Li-Chan and Nakai, 1989; Hayakawa and Nakamura,

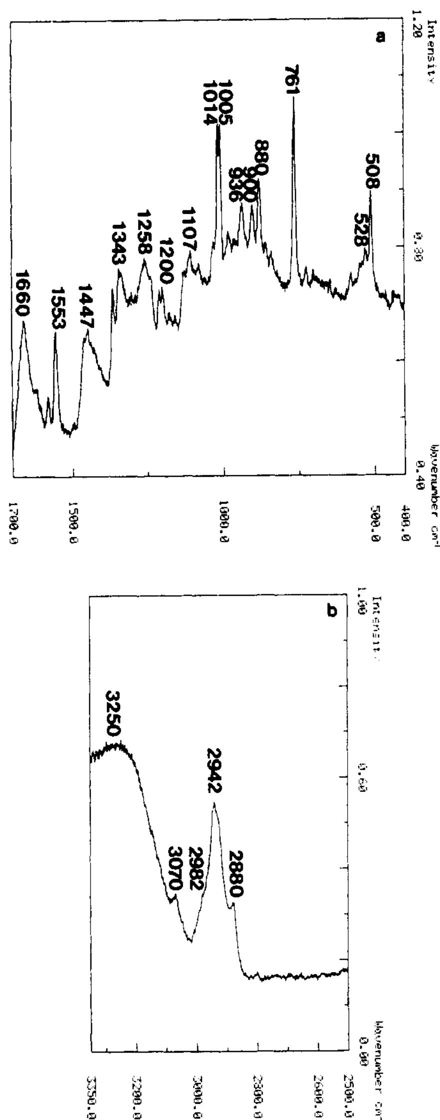

Figure 1. Raman spectrum of 10% lysozyme solution (0.05 M NaCl, pH 7.2) in the wavenumber shift regions (a) 400–1700 and (b) 2500–3350 cm^{-1}.

1986). No significant change in total SH group content resulted from any of the heat treatments of lysozyme in the absence of DTT (Table I), but the occurrence of disulfide interchange resulting in intermolecular cross-links during 100 °C heating was suggested by the formation of an opaque white gel which could not be solubilized by 8 M urea.

In the presence of DTT, weak or moderately strong gels were formed after incubation at 37 or 60 °C, while strong and very strong gels were formed at 80 and 100 °C, respectively. A total of 0.19–1.3 mol of SH/mol of protein was detected after heat treatment in the presence of 10 mM DTT, while up to 6.0 mol of SH/mol of protein resulted from heating in the presence of 100 mM DTT. The involvement of covalent intermolecular disulfide bonds in the gels formed by heating at 80 or 100 °C in the presence of 10 mM DTT was suggested by the insolubility of these gels in 8 M urea, whereas gels formed in the presence of 100 mM DTT were soluble even in 0.05 M NaCl with the aid of brief sonication.

Parts a and b of Figure 1 show typical Raman spectra obtained for lysozyme in 0.05 M NaCl solution at pH 7.2, in the wavenumber regions from 400 to 1700 cm^{-1} and from 2500 to 3350 cm^{-1}, respectively. The assignment of some of the major bands to vibrational modes of amino acid side chains or peptide backbone is shown in Table II. Figures 2 and 3 show the spectra in these two wavenumber regions for lysozyme after various heat treatments, in the absence (Figures 2 and 3a–e) or presence (Figures 2

Table II. Tentative Assignment of Some Major Bands in the Raman Spectrum of Lysozyme Solution (0.05 M NaCl, pH 7)

wavenumber,[a] cm^{-1}	tentative assignment[b]
508	ν S–S, g–g–g
528	ν S–S, g–g–t
538, 543, 754	Trp
626	Phe
644	Tyr
699	ν C–S, Met
761	Trp
839, 859	Tyr
879	Trp
900, 936	ν C–C (α-helix)
980	ν C–C
1005	Phe
1014	Trp
1030	Phe
1080, 1107, 1130 (sh)	ν C–N
1178	Tyr
1200, 1210	Tyr, Phe
1238 (sh), 1258, 1278 (sh)	amide III (ν C–N, δ N–H, ν CH_3–C)
1343, 1365	Trp and δ C–H
1447, 1460 (sh)	δ CH_2
1553	Trp
1582	Trp
1620 (sh)	Tyr, Trp, Phe
1660	amide I (ν C=O, δ N–H), δ H–O–H
2880, 2942, 2982 (sh)	aliphatic ν C–H
3070	aromatic ν C–H
3250	ν O–H and ν N–H

[a] (sh) refers to shoulder. [b] ν and δ are stretching and bending vibrations, respectively; g–g–g and g–g–t refer to the gauche (g) or trans (t) conformations of the C–S–S–C bonds of the disulfide grouping.

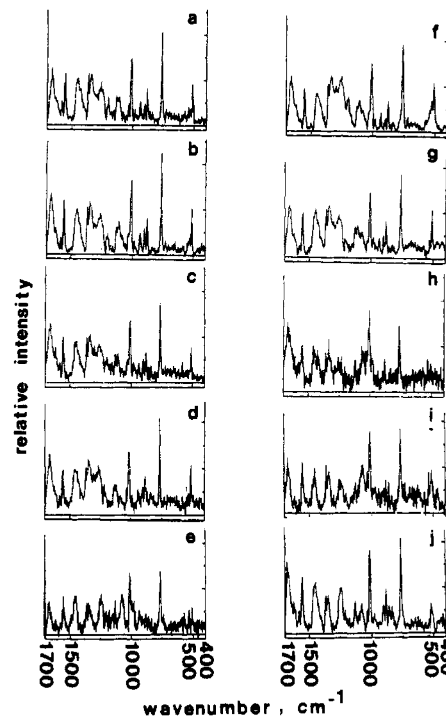

Figure 2. Raman spectra from 400 to 1700 cm^{-1} of 10% lysozyme in the absence (a–e) or presence of 10 mM DTT (f–j), with the following heat treatments: a and f, control (no heating); b and g, 37 °C for 30 min; c and h, 60 °C for 15 min; d and i, 80 °C for 12 min; e and j, 100 °C for 12 min. Spectra were normalized to the intensity of the H–C–H deformation mode at 1445–1450 cm^{-1} after baseline correction.

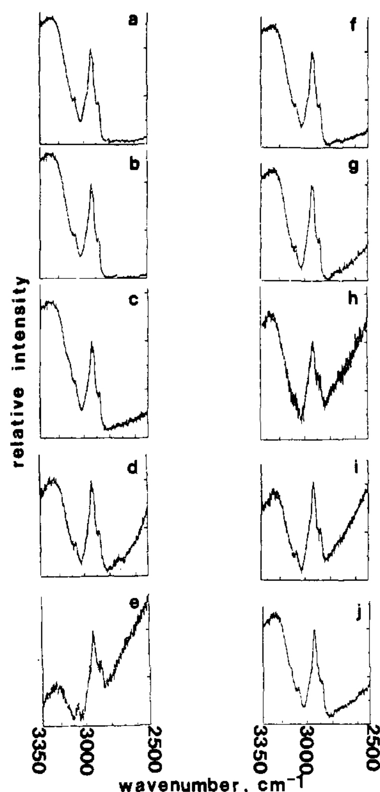

Figure 3. Raman spectra from 2500 to 3350 cm^{-1} of 10% lysozyme in the absence or presence of 10 mM DTT and various heat treatments, as described in the caption to Figure 2. Spectra were normalized to the intensity of the C–H stretching band at 2940 cm^{-1}, without baseline correction.

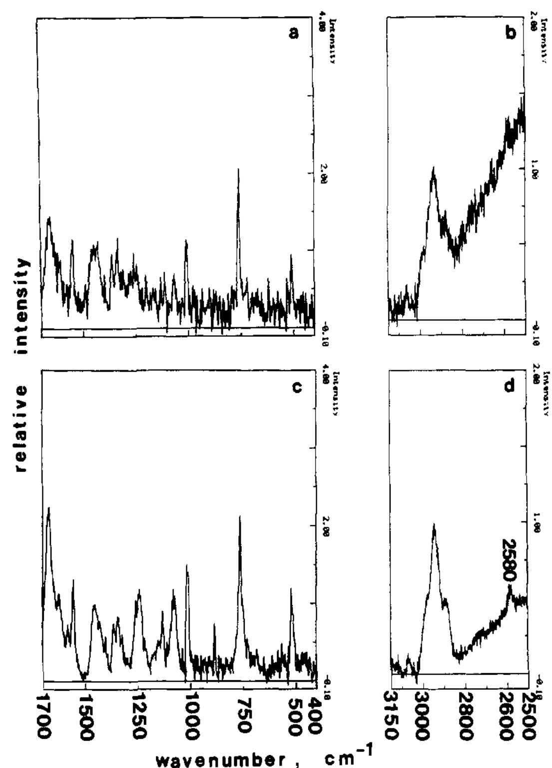

Figure 4. Raman spectra of 10% lysozyme solution containing 100 mM DTT: a and b, control (without heating); c and d, after heat treatment at 80 °C for 12 min. Spectra in the 400–1700-cm^{-1} region were normalized to the intensity at 1445–1450 cm^{-1} after baseline correction, while spectra in the 2500–3150-cm^{-1} region were normalized to the intensity at 2940 cm^{-1} after subtraction of the solvent (100 mM DTT, 0.05 M NaCl, pH 7.2) spectrum.

and 3f–j) of 10 mM DTT. The results obtained by heating in the absence of DTT are similar to those reported previously by other workers [e.g., Chen et al. (1973)]. Little or no change in the spectra were observed for lysozyme after heating at 37, 60, or 80 °C (Figures 2 and 3b–d), except for somewhat poorer signal to noise ratios of the 60 and 80 °C heated samples, probably due to their turbidity. After 100 °C heat treatment (Figures 2e and 3e), the most evident changes include the following: (1) a change in the relative intensity of the peaks at 508 and 528 cm^{-1}, corresponding to disulfide stretching vibrations, from about 3:1 to 1:1 ratio, which may suggest change of one of the four disulfide groups from an all gauche to a gauche–gauche–trans conformation after heating; (2) a decrease in the intensity of the peaks at 760, 880, and 1360 cm^{-1}, which indicates increased exposure of tryptophan residues to the aqueous environment; (3) shifts in the center of the amide III region from about 1258 to 1245 cm^{-1} and in the amide I region from 1660 cm^{-1} to a doublet at 1660 and 1674 cm^{-1}, which suggest a decrease in α-helical structure and increases in β-sheet and random coil structure; (4) an increase in intensity of backbone C–C and C–N stretching vibrations in the 980- and 1080-cm^{-1} regions; (5) an increase in the intensity of the CH stretching vibration at 2938–2942 cm^{-1} relative to the broad water line at 3250 cm^{-1}, which suggests increased exposure of aliphatic side chains to the aqueous environment (Verma and Wallach, 1977). A decrease in the relative intensity at 3250 cm^{-1} after gelation may be related to changes in strength of hydrogen bonding or structure of water molecules or to protein–water interactions, as reported by others who observed dependence of relative scattering intensity attributed to O–H stretching of water molecules as a function of temperature and of concentration of protein in solutions (Brooker et al., 1989; Cavatorta et al., 1976; Samanta and Walrafren, 1978).

The Raman spectrum of unheated lysozyme solution treated with 10 mM DTT (Figures 2f and 3f) did not differ appreciably from that of untreated lysozyme (Figures 2a and 3a), which is consistent with the insignificant changes in hydrophobicity as well as sulfhydryl group content reported previously (Li-Chan and Nakai, 1989). Many of the changes observed only after 100 °C heat treatment in the absence of DTT start to appear in the spectrum obtained after heating in the presence of 10 mM DTT at 37 °C (Figures 2g and 3g), with more marked changes after 60 and 80 °C heat treatment (Figures 2 and 3h,i, respectively). Interestingly, heating at 100 °C in the presence of 10 mM DTT (Figures 2j and 3j) resulted in less marked changes than heating at 60 or 80 °C.

The Raman spectra of lysozyme heated in the presence of 100 mM DTT were generally similar to those heated with 10 mM DTT, with one important exception, that reduction of some of the disulfide bonds to sulfhydryl groups was suggested by the appearance of a peak at about 2580 cm^{-1} (Figure 4d). The S–H stretching vibration has been reported to appear in the vicinity of 2570–2580 cm^{-1} for many organic compounds (Tu, 1986). This result is in agreement with the measurement of sulfhydryl groups using Ellman's reagent (Table I).

The spectra for unheated (Figure 4a,b) and 37 °C–30 min heated (not shown) samples with 100 mM DTT showed lower signal to noise ratios than other samples. Although the location of the laser excitation on the sample was changed by repositioning the capillary between each scan, in an attempt to avoid overheating of the sample solution, it was observed that these samples became increasingly turbid during collection of the Raman scattering data.

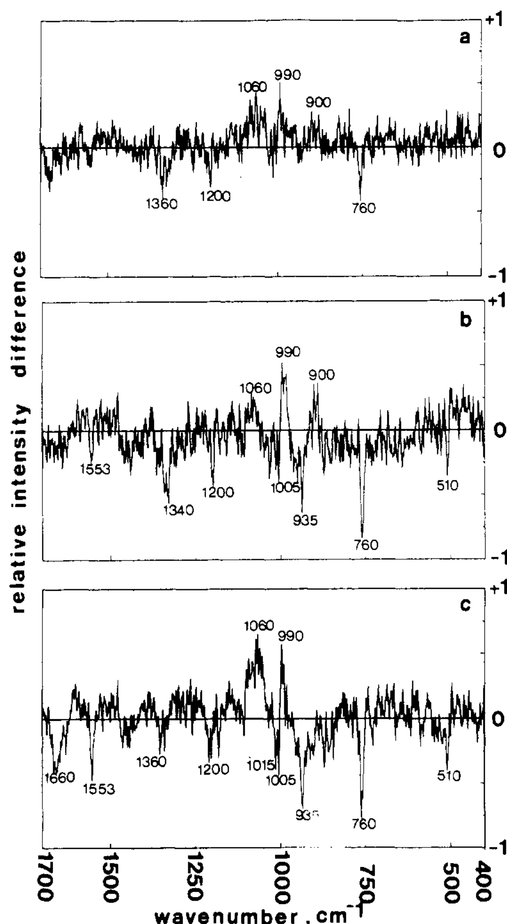

Figure 5. Raman difference spectra of 20% lysozyme solution in deuterium oxide, as follows: (a) [lysozyme, 10 mM DTT, 40 °C, 10 min] - [lysozyme, 10 mM DTT]; (b) [lysozyme, 10 mM DTT, 75 °C, 5 min] - [lysozyme, 10 mM DTT]; (c) [[lysozyme, 100 °C, 5 min] - [lysozyme]. Spectra were normalized to the intensity at the 1455-cm^{-1} band before computation of the difference spectrum.

Only slight turbidity resulted for samples with 10 mM DTT. It is likely that the higher concentration of DTT was conducive to reduction of the disulfide groups in lysozyme and subsequent conformational and structural changes being induced by the laser. On the other hand, samples that had already undergone more severe heat treatment (e.g., 80 °C) were stable during excitation with the laser source, yielding spectra with improved signal to noise ratios (e.g., Figure 4c,d, compared to Figure 4a,b).

Difference spectra of deuterated lysozyme that show the effect of heat treatment with or without 10 mM DTT are shown in Figure 5. The use of deuterium oxide rather than water as the solvating medium allows easier interpretation, particularly of any changes occurring in the amide I or amide III regions. A decrease in α-helical structure and an increase in antiparallel β-structure after heat treatment at 75 °C with 10 mM DTT (Figure 5b) or at 100 °C without DTT (Figure 5c) were suggested by the negative and positive peaks in the difference spectra at 935–950 and 980–995 cm^{-1}, respectively (Yager and Gaber, 1987). Other changes include the exposure of previously buried aromatic residues suggested by decreases in intensity at 760, 1005–1015, 1200, and 1340–1360 cm^{-1}, changes in the disulfide stretching conformations at 509–530 cm^{-1}, and increased C–C and C–N stretching vibrations at 900 and 1060–1080 cm^{-1}, respectively. It is interesting to note that some of these changes were apparent after heat treatment at 40 °C for only 10 min in the presence of 10 mM DTT (Figure 5a) but not in the absence of DTT (data not shown).

Secondary structure fractions were estimated from Raman spectral analysis of the amide I region (from 1500 to 1800 cm^{-1}). These results are summarized in Table III. Some discrepancies in the estimation of secondary structure by this RSAP approach when compared to X-ray analysis results have been reported, namely a consistent overestimation of antiparallel β-sheet content and an underestimation of ordered helix content. The percentages of total helix and total β-sheet contents were estimated as 25% and 31%, respectively, for lysozyme in solution at pH 7.2 in the current study (Table III), compared to 31% and 38%, respectively, calculated for lysozyme in lyophilized form by RSAP analysis and to 46% and 19%, respectively, calculated for lysozyme crystals by X-ray diffraction analysis (Przybycien and Bailey, 1989). Nevertheless, the RSAP analysis is suitable for assessing relative changes in bulk average secondary structure content (Przybycien and Bailey, 1989). As Table III shows, in the absence of DTT, little change in secondary structure fraction was observed by heat treatment at 37 or 60 °C; however, upon heat treatment at 80 °C, there was a decrease in helical structure accompanied by an increase in β-sheet structure. After heat treatment at 100 °C, the virtual loss of helical structure was accompanied by an increase in random structure. The position of the center of the amide I peak had shifted from 1660 cm^{-1} in the native lysozyme to 1668 cm^{-1} with shoulders at 1660 and 1675 cm^{-1} after heat treatment at 100 °C.

In the presence of 10 mM DTT, the decrease in helical structure and increase in β-sheet structure were observed after heat treatment at 80 °C, while in the presence of 100 mM DTT, these changes were observed after a 12-min heat treatment at either 80 or 100 °C or after prolonged heat treatment (24 h) at 37 °C. The broad amide I band observed for lysozyme with DTT at the lower temperatures was shifted to a higher wavenumber (1669–1672 cm^{-1}) and became sharper after heat treatment at 60 or 80 °C; however, broadening was observed again after heat treatment at 100 °C. The maximum formation of β-sheet structure appeared to be after heat treatment at 80 °C, whether in the absence of DTT or in its presence.

DISCUSSION

The mechanism of thiol-induced gelation of globular proteins such as conalbumin and lysozyme has not been clearly elucidated. Oe et al. (1986) suggested that the thiol-induced gelation of conalbumin was a result of cleavage of at least 9 of the 15 disulfide linkages, accompanied by an increase in hydrophobicity measured by fluorescence probes; the hardness of the resulting gels depended on specific anions and pH which could affect the noncovalent interactions, but intermolecular covalent interactions were postulated not to be involved in gel formation. Circular dichroism spectral analysis revealed that the thiol reagent may have induced some changes in tertiary structure of conalbumin, but no prominent changes in secondary structure were observed within the first 15 min of incubation; further analysis could not be carried out due to the onset of turbidity preceding gelation.

Hayakawa and Nakamura (1986) also hypothesized an ionic shielding effect by salt and the requirement for cleavage of disulfide bonds causing exposure of hydrophobic regions in the thiol-induced gelation of lysozyme, but they postulated that interchanges between sulfhydryl and disulfide groups were also involved in the resulting gels. However, these workers did not measure either sulf-

Table III. Secondary Structure Fractions Determined by Raman Spectral Analysis of Amide I Region of 10% Lysozyme Samples after Various Reducing Agent and Temperature-Time Treatments

		secondary structure fraction[b]				
				undefined		
treatment	amide I,[a] cm^{-1}	total α-helix	total β-sheet	turn	random	total
lysozyme control						
no heat treatment	1660	0.25	0.31	0.37	0.07	0.44
37 °C, 30 min	1660	0.25	0.31	0.36	0.08	0.44
37 °C, 24 h	1660	0.26	0.39	0.34	0.03	0.37
60 °C, 15 min	1659	0.32	0.31	0.32	0.05	0.37
80 °C, 12 min	1650, 1668	0.12	0.53	0.24	0.11	0.35
100 °C, 12 min	1660, 1668*, 1675	0.00	0.31	0.00	0.69	0.69
lysozyme + 10 mM DTT						
no heat treatment	1659B	0.46	0.26	0.12	0.17	0.28
37 °C, 30 min	1655*B, 1662, 1669	0.30	0.34	0.27	0.10	0.37
37 °C, 24 h	1658B	0.44	0.24	0.28	0.03	0.32
60 °C, 15 min	1662, 1669*	0.35	0.39	0.20	0.06	0.26
80 °C, 12 min	1656, 1665, 1672*	0.00	0.63	0.20	0.17	0.37
100 °C, 12 min	1671	0.35	0.48	0.03	0.14	0.17
lysozyme + 100 mM DTT						
no heat treatment[c]	1653, 1664B	0.22	0.46	0.22	0.09	0.31
37 °C, 30 min[c]	1661, 1663*B	0.60	0.02	0.07	0.31	0.38
37 °C, 24 h	1664B, 1677	0.08	0.73	0.05	0.14	0.19
60 °C, 15 min	1662, 1671*	0.31	0.46	0.02	0.23	0.25
80 °C, 12 min	1669	0.05	0.75	0.10	0.10	0.20
100 °C, 12 min	1668B	0.08	0.69	0.17	0.06	0.23

[a] B indicates a broad peak; * indicates the wavenumber with the highest relative peak intensity. [b] Secondary structure fractions were calculated by the Raman spectral analysis package reported by Przybycien and Bailey (1989). [c] Calculated secondary structure fractions of these samples may be less reliable than for other samples due to poor signal to noise ratio of their collected spectra (S/N of less than 10), as explained in the text.

hydryl or hydrophobic groups to support their hypothesis of the mechanisms of gelation of lysozyme under these conditions. The discrepancy regarding possible involvement of covalent disulfide bonds in the gel formation of conalbumin (Oe et al., 1986) compared to lysozyme (Hayakawa and Nakamura, 1986) may be explained by the different conditions for thiol-induced gelation—1.6% protein concentration, 70 mM 2-mercaptoethanol, and incubation at 35 °C for conalbumin, compared to 5% protein concentration, 7.0 mM dithiothreitol, and incubation at 80 °C for lysozyme.

In the present work, gelation of lysozyme has been investigated under conditions with no added reducing agent or in the presence of two concentrations of reducing agent. The concentrations of 10 and 100 mM DTT added with 10% lysozyme solution are equivalent to molar ratios of reducing thiol group in DTT/sulfhydryl groups in the lysozyme molecule of 0.36 and 3.6, respectively. It should also be noted that even in the absence of added DTT, trace amounts of free SH groups were measured in the lysozyme solution (Table I).

The results of this study suggest that the gelation of lysozyme requires a certain degree of molecular flexibility which allows unfolding and possible conformational rearrangement. This flexibility may be imparted by incorporating sufficient reducing agent in conjunction with mild heating conditions or by heating at high temperatures (e.g., 100 °C) in the absence of a reducing agent. Under these circumstances, disulfide bond reduction or sulfhydryl–disulfide or disulfide–disulfide interchange may occur, which can result in a "destabilized" molecular structure with less restriction on unfolding. Input of energy in the form of elevated temperature then allows exposure of previously buried hydrophobic groups, conformational rearrangement, and realignment of molecular segments, including perhaps the formation of intermolecular β-sheet structures, as proposed in the thermal denaturation and aggregation of ovalbumin (Painter and Koenig, 1976).

Figure 6 shows a hypothetical, schematic illustration of

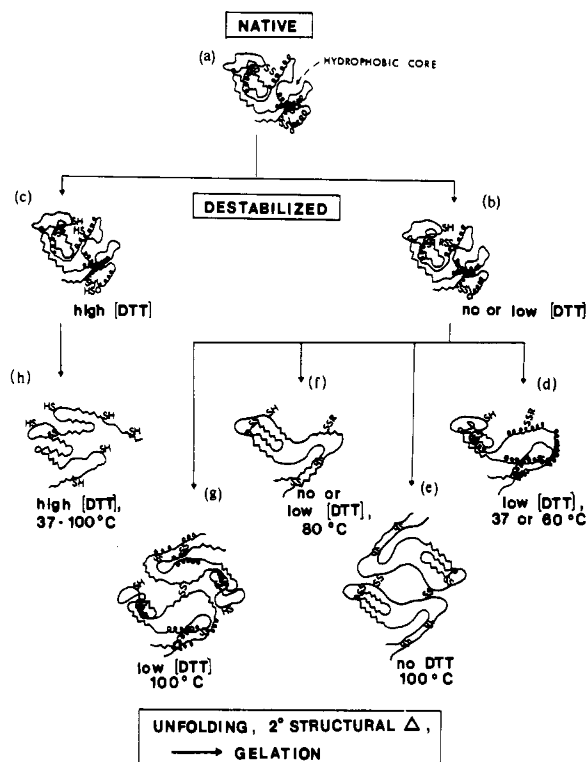

Figure 6. Schematic illustration of thiol- and heat-induced changes in lysozyme during various stages leading to gelation. The "native" structure was drawn on the basis of Blake et al. (1978), but proposed sites of changes shown in the figure, such as α-helix and β-sheet locations, reduction of disulfide bonds, or unfolding of the tertiary structure, are only hypothetical. (, α-helix; , β-sheet; SH, sulfhydryl group of cysteinyl residue; SS, disulfide bond between two cysteinyl residues; SSR, disulfide bond between cysteinyl residue of protein and thiol group of reducing agent).

the stages of destabilization or increased molecular flexibility, unfolding, and conformational rearrangement,

which may lead to gel formation. In the "native" lysozyme molecule, tertiary structure is stabilized by four intramolecular disulfide bonds (Figure 6a); many of the hydrophobic residues are located in the interior of a compact globular region, the so-called "hydrophobic box" or core (Blake et al., 1978). This type of structure is maintained under conditions such as the absence of reducing agents or after heat treatment at temperatures up to about 75 °C.

Cystine residues of proteins are susceptible to various types of reactions, depending on temperature and redox environment (Friedman, 1973). The first stage required for gelation of lysozyme involves a destabilization of the native structure by modification of the integrity of the intramolecular disulfide bonds. In the present investigation, the destabilization of the tertiary structure of lysozyme resulted from (1) SH–SS interchange (Figure 6b) induced by low concentration (10 mM) of DTT or by heat treatment at high temperatures (e.g., 100 °C) in the absence of added reducing agent or (2) reduction of SS bonds (Figure 6c) through incubation with a higher concentration (100 mM) of DTT. These destabilized structures have greater molecular flexibility, leading to unfolding and exposure of hydrophobic groups that were previously buried in the interior of the molecule.

The extent of unfolding and possibility of further conformational rearrangement leading to changes in secondary structures are dependent on the conditions. In the presence of 10 mM DTT and at temperatures of 37 and 60 °C (Figure 6d), the extent of unfolding appears to be sufficient to result in gel formation, possibly through hydrophobic interactions of exposed residues; however, little conformational rearrangement is seen in terms of the secondary structure fractions. Heating at 80 or 100 °C in the presence of 10 mM DTT or in its absence can result in the exposure of hydrophobic residues as well as changes in secondary structure (Figure 6e–g). Interestingly, gels formed at the higher temperature of 100 °C have less β-sheet structure than corresponding gels formed at 80 °C; in addition, little or no change in helical content is observed for gels formed at 100 °C in the presence of 10 mM DTT (Table III). The formation of intermolecular disulfide bonds favored at the higher temperature may have restricted the unfolding of helical segments (Figure 6g) and realignment of segments for β-sheet formation (Figure 6f,g). Although intermolecular disulfide cross-links were also suggested in the gel network formed by heat treatment at 80 °C in the presence of 10 mM DTT, the molecules in that gel show low helical and high β-sheet content. It is possible that the higher temperature of 100 °C may have promoted earlier formation of disulfide cross-links, before appreciable changes in secondary structure could take place, whereas at 80 °C the cross-links may be formed in a later stage of gel network stabilization. In the presence of 100 mM DTT, the reduction of three of the four disulfide intramolecular bonds results in little restriction in subsequent unfolding, and the changes in secondary structure as well as exposure of hydrophobic groups can be observed by incubation at 80 and 100 °C for short periods of time as well as more prolonged incubation (24 h) at 37 °C (Figure 6h).

The role of hydrophobic interactions, intermolecular disulfide cross-links, and β-sheet structures in the stabilization of the gel network can also be anticipated to depend on the conditions of temperature and reducing agent. Stabilization of the gel network formed in the presence of 10 mM DTT and incubation at temperatures of 37 or 60 °C appeared to be mainly through hydrophobic interactions. Noncovalent interactions consisting of both hydrophobic interactions and β-sheet structures were involved in gels formed with no or 10 mM DTT at 80 °C or for gels formed in the presence of 100 mM DTT either by short incubation at 80 or 100 °C or by longer incubation at 37 °C. Intermolecular disulfide cross-links were involved only in gels formed by heat treatment at 80 or 100 °C with 10 mM DTT or at 100 °C in the absence of DTT.

The importance of disulfide bonds in maintaining the secondary structure of lysozyme has been reported by White (1976, 1982), who, using circular dichroism to study the alteration of secondary structure through various stages of reduction, suggested a trend of decrease in helix and increase in β-structure for the reduced lysozyme. The continuous unfolding of helical structures, accompanied or followed by introduction or increase of β-structure by thermally induced denaturation of several proteins, has been reported by various workers [e.g., Sawyer et al. (1971), Lin and Koenig (1976), Painter and Koenig (1976), and Clark et al. (1981)]. In discussing secondary structures of globular proteins, Lim (1974) proposed that each separate helical region must have a hydrophobic side group which would permit the helix to attach itself to the hydrophobic core of the globule. This is in agreement with a destabilization or unfolding of helical structures which accompanies exposure of groups previously buried in the hydrophobic core of globular proteins such as lysozyme by denaturing treatment. Most workers agree on the subsequent formation of β-sheet structures, but whether or not this is an intermolecular process that contributes to the gel network or coagulum or an intramolecular process has been questioned by Clark and Lee-Tuffnell (1986). These workers suggested that interactions between exposed hydrophobic surfaces may be more important as the bonding mechanism.

The application of Raman spectroscopy in the present work enabled the study of structural changes at high concentrations of proteins, which is important in the investigation of the mechanism of gelation. The results indicated that molecular flexibility imparted by thiol- or heat-induced disulfide interchange or reduction of lysozyme is a necessary prerequisite for the formation of lysozyme gels. However, the relative importance of remaining helical structure, exposure of hydrophobic groups, increased proportion of β-sheet structure, or formation of new disulfide cross-links in intermolecular associations which stabilize the gel network depends on the conditions under which the gels were formed. The variations in secondary structure fractions, sulfhydryl group content, and solubility of the lysozyme gels formed by using low or high DTT concentrations and low or high temperature suggest that all of these changes can be involved in deciding the final gel structure but that their relative importance will depend on differences in the protein, composition of the solvating medium, and conditions such as heating temperature and time. Continued investigation of a wide variety of proteins by Raman spectroscopy can be expected to be valuable in the elucidation of the mechanisms of protein gelation in diverse food systems.

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