Thiol-dependent Gelation of Conalbumin in the Presence of a Denaturant

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The effects of urea concentration on the dithiothreitol-dependent gelation of conalbumin (ovotransferrin) were investigated. In the presence of a high concentration of urea, conalbumin gelation was induced at lower molar ratios of dithiothreitol to protein than in the absence of the denaturant. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that in the presence, but not in the absence of urea, intermolecular disulfide bonds were mainly involved in the gelation process. Under a scanning electron microscope, the gel formed in the presence of urea was composed of more fibrous networks than that formed in the absence of the denaturant.

Egg white¹⁾ and its components including conalbumin (ovotransferrin)^{2~4)} and lysozyme⁵⁾ have been shown to form gels by incubation with thiols under mild temperature and pH conditions. We have shown that the first step in the gelation of conalbumin involves conformational changes in the protein molecule which are caused by the cleavage of intramolecular disulfide bonds.²⁾ These changes in protein conformation are accompanied by the exposure of hydrophobic residues and subsequent aggregation through intermolecular hydrophobic interactions.²⁾ Little interchain covalent interaction through disulfide bonds is involved in the conalbumin aggregation.²⁾

During the course of examining the conditions for improving gel texture, we have found that conalbumin gel can be induced by dithiothreitol in the presence of a high concentration of urea. The question has arisen as to whether the thiol-dependent conalbumin gelation proceeds *via* different processes in the presence of urea, which is known to weaken protein hydrophobic interactions. In the pres-

ent report, we have investigated the effective conditions and possible mechanisms for thiol-dependent gelation in the presence of a high concentration of urea. By scanning electron microscopy, the network structures were compared between the gels formed in the presence and absence of urea.

MATERIALS AND METHODS

Conalbumin was purified as described previously.¹⁾ Sodium iodoacetate, dithiothreitol, 5,5'-dithiobis(2-nitrobenzoic acid) and other chemicals were obtained from Nakarai Chemicals Ltd.

Gelation was induced by incubating a 2% conalbumin solution at 37°C for 20 hr with thiol reagents in 0.1 M sodium phosphate buffer (pH 8.0) or 0.1 M Tris-HCl buffer (pH 8.0) containing indicated concentrations of urea. The gel hardness was determined with a rheometer (Yamaden Co., Rheoner type RE-3305) using a plunger of 8 mm in diameter and an aluminum cup (24 mm in diameter and 7 mm in depth). The clearance between the plunger and plate was 3 mm.

The appearance of turbidity in conalbumin solutions was measured at 600 nm with a spectrophotometer (JASCO Co., type UNIVAC-1MS). The solution containing 2% conalbumin was incubated in 0.1 M sodium phos-

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phate buffer (pH 8.0) or in 0.1 M Tris-HCl buffer (pH 8.0) at 37°C for different times in the presence or absence of 6 M urea.

The contents of free sulfhydryl groups in the protein was assayed spectrophotometrically using 5,5'-dithiobis(2-nitrobenzoic acid) as described previously.²⁾

The quantification of the protein was carried out by the method of Lowry *et al.*⁶⁾ using conalbumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with Atto SJ-1139 using a slab gel (1.0 mm × 13 cm × 13 cm) in the presence of the standard buffer system.⁷⁾ The concentrations of acrylamide were 10% in separating gels and 3% in stacking gels. Electrophoresis was performed at a constant voltage of 40 V for 16 hr, and proteins were stained with Coomassie blue.

Scanning electron microscopy was carried out as described previously.³⁾ The conditions for gelation were 2% conalbumin in 0.1 M Tris-HCl buffer (pH 8.0) in the presence or absence of 6 M urea. The conalbumin solution was incubated at 37°C for 20 hr at dithiothreitol to conalbumin molar ratios of 15:1 in the absence of urea, and in the ratio of 2:1 in the presence of 6 M urea.

RESULTS

Correlation between the cleaved number of disulfide bonds and the gel hardness of conalbumin

In a previous report, we have shown that the hardness of conalbumin gel was a maximum at 70 mM 2-mercaptoethanol.³⁾ Conalbumin contains 15 intramolecular disulfide bonds.⁸⁾ At 70 mM of the thiol, about nine of the intramolecular disulfide bonds are cleaved.²⁾ However, the correlation between the gel hardness and the cleaved number of disulfide bonds has remained to be investigated in more detail.

Upon the cleavage of protein disulfide bonds by a mono-thiol including 2-mercaptoethanol, mixed disulfides are known to be formed.⁹⁾ The presence of mixed disulfides disturbs the quantification of the cleaved number of protein disulfide bonds. In contrast, few stable mixed disulfides are formed by dithiols, since the formation of an intramolecular disulfide bond of the reagent is favored.¹⁰⁾ Therefore, the correlation between the gel hardness and the cleaved number of disulfide bonds was examined using dithiothreitol.

As shown in Fig. 1, with increasing molar ratio of the thiol to the protein, the number of

free sulfhydryl groups was increased, and the amount of free proteins in the gel sap decreased. The gel hardness showed a maximum value at the molar ratio of 16. Under these conditions, about ten disulfide bonds were cleaved. About 20% of conalbumin was detected in the gel sap, showing that about 80% of the protein was involved in the gel networks. A further increase in the molar ratio of dithiothreitol to protein resulted in an increase in the cleaved number of disulfide bonds and in the amount of protein involved in the gel networks, but in a decrease of the gel hardness.

Effects of urea concentration on conalbumin gelation

The effects of urea concentration on conalbumin gelation were examined at the 2-mercaptoethanol concentration (70 mM) that induced the maximum gel hardness.²⁾ As

FIG. 1. Gelation by Dithiothreitol in the Absence of Urea.

Three milliliters of conalbumin solution (20 mg/ml) were incubated at 37°C in 0.1 M Na-phosphate buffer (pH 8.0) with different concentrations of dithiothreitol. After a 20 hr reaction, the gel hardness (—○—), the number of cleaved disulfide bonds (---●---), and free proteins in the gel sap (—■—) were determined. For determining the concentrations of free proteins in the gel sap, 0.5 ml of a conalbumin solution was incubated under the same conditions. The formed gels were extensively crushed with a glass rod, and the samples were centrifuged at 2,000 × *g* for 5 min. The supernatant (50 μl) was diluted with 40 volumes of 0.1 M Na-phosphate buffer containing 8 M urea and 1.0 mM EDYA, and then incubated with 50 mM iodoacetate at 37°C for 15 min. A part of the sample was analyzed for protein concentration.

shown in Fig. 2, the gelation by this thiol was strongly inhibited by the presence of urea, no gelation being observed at urea concentrations higher than 4M. The same was also true with the conalbumin gelation, which was induced at the dithiothreitol to protein ratio of 16:1.

However, in the presence of a lower molar ratio of dithiothreitol to conalbumin, gelation was induced at urea concentrations higher than 4M (Fig. 2). These data show that the denaturant differently affected the gelation under the two different molar ratios of dithio-

threitol to conalbumin.

Effects of anion on the gelation in the presence of urea

As shown in a previous report, the hardness of conalbumin gel induced in the absence of urea was several times greater in phosphate buffer than in Tris-HCl buffer.³⁾ The hardness of the gel induced in the presence of a high concentration of urea was compared between the two buffers.

FIG. 2. Effects of Urea Concentration on the Gel Hardness.

A conalbumin solution (20 mg/ml) was incubated in 0.1 M Na-phosphate buffer (pH 8.0) with 70 mM 2-mercaptoethanol (---▲---) or dithiothreitol at the molar ratios of 1:1 (—●—) or 16:1 (—○—) in the presence of different concentrations of urea. The incubation was continued at 37°C for 20 hr, and the gel hardness was determined.

FIG. 3. Gelation in the Presence of a High Concentration of Urea.

Conalbumin (20 mg/ml) was incubated in 6 M urea containing 0.1 M Tris-HCl buffer, pH 8.0 (—●—) or 0.1 M Na-phosphate buffer, pH 8.0 (---○---), with different concentrations of dithiothreitol at 37°C for 20 hr, before the gel hardness was determined.

FIG. 4. Time Course for Turbidity Changes in Tris Buffer.

A: A conalbumin solution (20 mg/ml) was incubated with dithiothreitol in Tris-HCl buffer, pH 8.0, at 37°C for different times in the absence of urea, and the absorbance at 600 nm was measured.

B: The conditions were the same as those in A, except that the solution contained 6 M urea.

The molar ratios of dithiothreitol to protein were 1:1 (●), 2:1 (⊗), 4:1 (□), 6:1 (■), 8:1 (△), 10:1 (●), 12:1 (▲) and 16:1 (○).

When conalbumin was incubated in the presence of 6 M urea with dithiothreitol in Na-phosphate buffer (pH 8), the gel hardness was a maximum at an equimolar ratio of the thiol to the protein (Fig. 3). With an increasing thiol to protein ratio, the hardness was decreased, and at molar ratios higher than four, no gelation occurred. In Tris-HCl buffer (pH 8.0), the gel hardness was a maximum at the molar ratio of two. With an increasing dithiothreitol to conalbumin ratio, the gel hardness was decreased, no gelation being observed at molar ratios greater than eight. The data in Fig. 3 represent that, unlike the gel formed in the absence of urea, in the presence of urea the maximum gel hardness was similar under the two different buffer conditions.

In the absence of urea, 2-mercaptoethanol-dependent gelation of conalbumin is closely correlated with the appearance of turbidity.³⁾ The time course for the appearance of turbidity was compared in the presence and absence of urea under the two different buffer conditions. When conalbumin was incubated with dithiothreitol in Tris-HCl buffer, the rate of appearance of turbidity was increased with the increasing molar ratio of thiol to protein in the absence of urea (Fig. 4A). In the presence of 6 M urea, however, with increasing molar ratio, the initial increase was followed by a decrease in the rate (Fig. 4B). The appearance of tur-

bidity showed a maximum rate at the molar ratio of four (Fig. 4B). Similar profiles for the time course of the appearance of turbidity were observed in the phosphate buffer (Fig. 5). The major difference between the two buffer systems was that the rate of turbidity appearance was much greater in the Tris buffer than in the phosphate buffer, both in the presence and absence of urea. In the phosphate buffer, the rate of turbidity appearance was a maximum at the molar ratio of 1:1 in the presence of 6 M urea.

Analyses by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The data in Figs. 2~5 show that the conditions for thiol-dependent gelation of conalbumin were quite different in the presence and absence of urea, suggesting that the gelation proceeded *via* different mechanisms under the different urea conditions.

In the absence of urea, intermolecular disulfide bonds are not involved in the 2-mercaptoethanol-dependent gelation of conalbumin.²⁾ With respect to dithiothreitol-induced gels, the possibility for the formation of intermolecular disulfide bonds was tested by gel electrophoresis. Conalbumin was incubated in the phosphate buffer with dithiothreitol in the presence or absence of 6 M urea for different times. The molar ratios of thiol to

FIG. 5. Time Course for Turbidity Changes in Na-Phosphate Buffer.

The changes in turbidity were measured in 0.1 M Na-phosphate buffer, pH 8.0, in the presence (B) and absence (A) of 6 M urea. The other conditions and symbols used for the molar ratios of dithiothreitol to the protein are the same as those in Fig. 4.

FIG. 6. Analyses by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.

The conalbumin solution (20 mg/ml) was incubated with dithiothreitol in Na-phosphate buffer, pH 8.0, at 37°C for the indicated time in the presence (A and C) or absence (B and D) of 6 M urea. The molar ratios of dithiothreitol to conalbumin were 1:1 in the presence of 6 M urea (A and C) and 16:1 in the absence of the denaturant (B and D). The samples were diluted with 19 volumes of 0.1 M Tris-HCl buffer containing 8 M urea, 1 mM EDTA, 1% sodium dodecyl sulfate and 0.1 M iodoacetamide. After 1 min of incubation at 37°C, the samples were immediately analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with A and B. With C and D, the samples were incubated with 0.2 M 2-mercaptoethanol in a boiling-water bath for 2 min prior to gel electrophoresis.

The arrows a, b and c represent the mobilities of standard proteins of catalase (M_r , 240,000), aldolase (M_r , 158,000), and ovalbumin (M_r , 45,000), respectively.

conalbumin were 1:1 in the presence of urea and 16:1 in the absence of the denaturant. Under these conditions, the conalbumin gel showed its maximum hardness, and the pro-

files for the time course of turbidity appearance were almost exactly the same (see Figs. 1, 3 and 5). The reaction was terminated by diluting the samples and by blocking the

FIG. 7. Scanning Electron Micrographs.

Gels were formed by incubating 20 mg/ml of conalbumin solution at 37°C for 20 hr in 0.1 M Tris-HCl buffer, pH 8.0, in the presence (A) or absence (B) of 6 M urea. The molar ratios of dithiothreitol to protein were 2:1 and 16:1 in A and B, respectively.

generated free sulfhydryl groups with iodoacetamide. The samples with or without the 2-mercaptoethanol treatment were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Figure 6A shows that in the presence of urea, conalbumin took highly polymerized forms in a short incubation time. In contrast, in the absence of urea, no polymerized form was detected in the same incubation time (Fig. 6B). A few bands with slightly lower mobility that appeared during the incubation in the absence of urea were not ascribed to polymerized forms, but to different species with altered numbers of cleaved disulfide bonds, since their apparent molecular weights were markedly lower than the dimer form (M_r , 156,000). As shown in Fig. 6C and D, when the samples were incubated with 2-mercaptoethanol prior to electrophoresis, most, if not all, of the proteins migrated as a monomeric form. These data show that in the presence, but not in the absence of urea, conalbumin was highly polymerized through interchain disulfide bonds in a short incubation time with dithiothreitol.

Scanning electron micrographs of the gel

To see if the difference in the involvement of intermolecular disulfide bonds was reflected in the gel network structures, the gels formed in the presence or absence of urea were analyzed by scanning electron microscopy. As shown in Fig. 7, the network structures were quite different between the two gels that were formed in the presence and absence of a high concentration of urea. More fibrous network structures were observed with the gel formed in the presence of urea than that formed in the absence of the denaturant.

DISCUSSION

The data in the present study show that the conditions required for the thiol-dependent conalbumin gelation were quite different in the presence and absence of a high concentration of urea. At a high molar ratio of dithiothreitol to conalbumin, which induced gels with a maximum hardness in the absence of a denaturant, urea strongly inhibited the gelation. This is consistent with the previous data showing that intermolecular hydrophobic interactions were mainly involved in the 2-

mercaptoethanol-induced gelation processes.²⁾

In the presence of urea concentrations higher than 4 M, however, much lower molar ratios of the thiol to the protein were required for the formation of the gel. This could be explained as follows: It is known that iron-free conalbumin denatures in urea concentrations higher than 4 M.^{11,12)} This probably renders the protein more accessible to a thiol reagent in the presence of a high concentration of urea.¹³⁾ Consequently, lower molar ratios of dithiothreitol to conalbumin would be sufficient to cleave the disulfide bonds in the protein. Intermolecular non-covalent interactions through hydrophobic and hydrogen bonds are known to be strongly inhibited by the presence of a high concentration of urea. However, during the incubation under these conditions, conalbumin would be polymerized through intermolecular disulfide bonds, as can be seen in Fig. 6. At higher molar ratios of the thiol to the protein, making more reduced conditions, the formation of intermolecular disulfide bonds would not be favored. Therefore, in the presence of a high concentration of urea, the gelation of conalbumin may be induced at lower molar ratios of dithiothreitol to protein than in the absence of the denaturant.

This gelation profile of conalbumin is very similar to the previous data by van Kleef *et al.*, who have observed that gels formed by heating an ovalbumin solution in 6 M urea are solubilized by incubation with a thiol reagent.¹⁴⁾ Some of the four intrinsic sulfhydryl groups in ovalbumin¹⁵⁾ may be involved in the intermolecular disulfide bond formation in the presence of urea. Likewise, the process of conalbumin gelation in urea may be correlated with the rheological properties of wheat flour dough, which are maintained by the sulfhydryl and disulfides in glutenin.^{16,17)}

Scanning electron microscopy has been widely used to analyze the structure of food protein gels.¹⁸⁾ We tested whether the difference in the urea conditions caused alterations in the structure of the gel networks. As can be seen in the scanning electron micrographs

(Fig. 7), the gel formed in the presence of a high concentration of urea was composed of a more fibrous network than that formed in the absence of the denaturant. In the absence of urea, hydrophobic interactions are mainly involved in the gelation of conalbumin.²⁾ By these interactions, conalbumin tends to form branched or less-ordered aggregates.²⁾ This is probably due to the occurrence of a great number of hydrophobic regions in a denatured conalbumin molecule.²⁾ However, in the presence of 6 M urea, the gelation was induced at a low molar ratio of dithiothreitol to conalbumin. Consequently, only a limited number of the sulfhydryl groups employed for intermolecular interactions was probably generated. Therefore, it is very likely that the protein formed less branched aggregates. The difference in the processes of conalbumin gelation may be reflected in the gel network structures formed under the two urea conditions.

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