Cross-linking of proteins catalyzed by tissue transglutaminase has been suggested to play key roles in a variety of cellular events, including cell apoptosis and human pathogenesis (e.g. polyglutamine and Alzheimer diseases). It has often been suggested that tissue transglutaminase enhances aggregation and precipitation of damaged or pathogenic proteins. To ascertain whether this is accurate, we investigated the effects of tissue transglutaminase-catalyzed modulation on the aggregation of structurally damaged and unfolded proteins. Our results indicated that the aggregation and precipitation of some unfolded proteins were inhibited by transglutaminase-catalyzed reaction, although the effect was strongly dependent upon the target protein species. To elucidate the molecular events underlying the inhibitory effect, extensive analysis was performed with regard to reduced β-lactoglobulin using a number of techniques, including chromatography and spectroscopy. The results indicated that cross-linking yields high molecular weight soluble polymers but inhibits the growth of insoluble aggregates. The cross-linked β-lactoglobulin retained stable secondary structures with a hydrophobic core. We concluded that the transglutaminase-catalyzed intermolecular cross-linking did not necessarily enhance protein aggregation but could sometimes have a suppressive effect. The results of the present study suggested that tissue transglutaminase modifies aggregation and deposition of damaged or pathogenic proteins in vivo in a wide variety of manners depending on the target protein species and solution conditions.

Protein clustering occurs in the highly concentrated intra- and extracellular solutions of the living body (1, 2). Fine balancing of the intermolecular forces of proteins in biological fluids may realize a variety of physical states, such as dispersed, equilibrium clustered, glass-like, or crystal states, as in pure solutions (3–7), because biological environments are highly diverse. Investigation of the factors that affect the intermolecular interactions of proteins are of primary importance from a biological viewpoint. Such information is also essential for clinical control of abnormal protein aggregation, such as amyloid formation in patients with neurodegenerative diseases (8–12).

Biological protein assembly is mediated not only by non-covalent interactions but also by covalent cross-linking catalyzed by enzymes. The most important group of cross-linking enzymes is the transglutaminase (TG) family (13–15). TG are thiol- and Ca²⁺-dependent acyl transferases that catalyze formation of amide bonds between the γ-carboxamide groups of peptide-bound glutamine residues and the primary amino groups of various compounds, including the ε-amino group of lysine in proteins. It is commonly held that covalent cross-linking of protein molecules always enhances protein aggregation and precipitation. In fact, cross-linking by TG stabilizes blood clots and skin keratin under physiological conditions (13, 15). Tissue-type transglutaminase (tTG) is also a candidate for the factor that induces formation of neuronal inclusions in the brains of patients with human diseases, such as the polyglutamine and Alzheimer diseases (16–19).

However, we have recently demonstrated that the cross-linking reaction catalyzed by tTG does not always enhance protein aggregation; the cross-linking reaction in the intramolecular mode was shown to strongly suppress the aggregation of disease-related proteins (20). This led to the question of whether the intermolecular mode of cross-linking would also suppress protein aggregation. In fact, Lai et al. (21) recently reported that tTG-catalyzed intermolecular cross-linking of a polyglutamine-containing protein formed high molecular weight soluble polymers, but inhibited precipitation. An accurate answer to this question must be pursued, as the TG-catalyzed cross-linking has been suggested to play key roles in a variety of cellular events, including regulation of cellular growth, differentiation, apoptosis, and human pathogenesis (14, 22).

In the present study, we examined whether the intermolecular cross-linking of damaged and structurally unfolded proteins enhances or inhibits their aggregation and precipitation. To assess this problem from a general viewpoint, we examined four structurally unrelated proteins as substrates of TG: bovine β-lactoglobulin A (βLG; β-sheet-type), bovine serum albumin (BSA; α-helix-type), human α-lactalbumin (αLA; α/β-type), and bovine α-casein (natively unfolded type). βLG, αLA, and BSA were unfolded by reducing the disulfide bonds, which might be the simplest model for biological protein damage. First, we performed simple aggregation and cross-linking analysis of all the proteins. This was followed by extensive analysis for βLG as a typical case using biochemical and biophysical techniques.

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1 The abbreviations used are: TG, transglutaminase; tTG, a tissue-type transglutaminase; βLG, bovine β-lactoglobulin A; αLA, human α-lactalbumin; BSA, bovine serum albumin; DTT, dithiothreitol; SEC, size-exclusion gel chromatography.
**EXPERIMENTAL PROCEDURES**

**Materials**—Guinea pig liver transglutaminase was purified on an immunoabsorbent column as described previously (23). βLG, αLA, BSA, bovine α-casein, and bovine α-chymotrypsin were purchased from Sigma-Aldrich and were used without further purification. Other chemicals of reagent grade were purchased from Nacalai Tesque Co. (Kyoto, Japan).

**Aggregation and Cross-linking Reactions**—All of the sample solutions were prepared in 10 mM Tris (pH 7.5), and the pH was adjusted carefully with small amounts of HCl. Non-covalent aggregation of βLG, BSA, and αLA was initiated by adding 10 mM dithiothreitol (DTT) and 5 mM CaCl2 at 37 °C. The ITG-catalyzed cross-linking reaction was initiated by adding 130, 260, or 650 mM ITG, 10 mM DTT, and 5 mM CaCl2 at 37 °C. Irreversibly inactivated ITG used for control experiments was prepared by heat treatment of tTG at 70 °C for 5 min. A reference experiment under reducing conditions without aggregation and cross-linking of βLG was performed in a solution containing 10 mM DTT and 1 mM EDTA. SDS-PAGE analysis was performed by the standard method of Laemmli (24) with densitometric measurement of the Coomassie Brilliant Blue-stained gel. Solution turbidity was measured by absorbance at 320 nm using a U-3000 spectrometer (Hitachi, Tokyo).

In the experiments using α-casein, the protein was dissolved in 10 mM Tris (pH 7.5), 5 mM CaCl2, and 10 mM DTT and incubated at 37 °C for 24 h. At this time point, tTG was added to the solution, and then the turbidity was monitored.

**Size-exclusion Gel Chromatography and Microfiltration**—Analytical size-exclusion gel chromatography (SEC) was performed using the Class M10A HPLC system (Shimadzu, Kyoto, Japan) equipped with a G3000SW column ( Tosoh, Tokyo). The equilibration and elution buffers were 0.1 mM sodium phosphate buffer (pH 7.5), and the flow rate was 1 ml/min. Absorbance at 280 nm was monitored. The samples loaded onto SEC were pretreated by microfiltration with an Ultrafree-MC filter ( pore size, 0.22 μm; Millipore Co., Bedford, MA).

**Spectroscopic Measurements—Circular dichroism (CD) spectra** were measured with a Jasco J-820 spectropolarimeter (Jasco, Tokyo) using a quartz cell with a path length of 0.5 or 1 mm. The protein concentrations were 6 and 150 μM for far- and near-UV measurements, respectively. The temperature of the cell was controlled using a Peltier-type FTC-423S apparatus (Jasco).

**Preparation of βLG Solutions in the Presence of CaCl2**—The cross-linked samples were prepared in advance by adding 650 mM ITG and 5 mM CaCl2 to the reduced reference sample followed by incubation at 37 °C for 24 h. The reduced reference and cross-linked samples containing 100 μM βLG and various concentrations of CaCl2 were incubated at 37 °C for 24 h. The samples after incubation were subjected to microfiltration ( pore size, 0.22 μm), and the protein concentration of the supernatant was determined by averaging estimates obtained from absorbance at 280 nm and the BCA method. The concentrations of CaCl2 in the presence of 1 mM EDTA were determined by the method of Goldstein (25).

**Proteolytic Digestion Experiments**—The reaction mixtures containing 270 μM βLG and 10 mM DTT were used for the digestion experiments monitored by SDS-PAGE. Depending on the sample species, 650 mM ITG, 5 mM CaCl2, or 1 mM EDTA was added to the solutions. The mixtures were incubated at 37 °C for 24 h. Then, α-chymotrypsin was added at the final concentration of 10 μM/μl, and the mixtures were incubated at 25 °C with stirring.

**RESULTS**

**tTG-catalyzed Suppression of Non-covalent Protein Aggregation and Precipitation**—Structurally damaged and unfolded protein molecules have a strong tendency to form aggregates because exposure of hydrophobic parts of the protein interior to the aqueous environment enhances the intermolecular hydrophobic interactions that overcome electrostatic or other types of intermolecular repulsion. Accordingly, we expected that reduction of disulfide bonds and the subsequent unfolding of proteins would also induce their non-covalent aggregation in a medium of relatively high ionic strength that partially shields electrostatic repulsion. This type of aggregation and precipitation was actually observed for βLG, αLA, and BSA at neutral pH at 37 °C in the presence of DTT and Ca2+ (Fig. 1. A–C, star symbols). Aggregation of the reduced βLG was suppressed completely by chelation of trace ions by EDTA (Fig. 1A, filled circles). This sample containing highly reduced βLG without any aggregates in the presence of 1 mM EDTA is referred to as the “reduced reference” sample throughout this report. Addition of an excess amount of CaCl2 (e.g., 5 mM) to this reduced reference solution strongly induced non-covalent aggregation and precipitation of βLG (Fig. 1D, star symbols).

The addition of tTG to the aggregating solutions described above inhibited the increase in turbidity of the proteins depending on the concentration of tTG (Fig. 1). Notably, the effect of tTG was much weaker for αLA than for βLG and BSA. Heat-inactivated tTG did not show this inhibitory effect (data not shown). Ca2+-induced aggregation of the reduced reference βLG shown in Fig. 1D could also be suppressed very efficiently by tTG (Fig. 1D, open symbols). Furthermore, disassembly of preformed aggregates by tTG was observed for α-casein. A solution of α-casein became turbid in the presence of CaCl2, and the turbidity decreased sharply by adding tTG at 37 °C (Fig. 2). These results indicated that the tTG-catalyzed reaction inhibited the non-covalent aggregation of unfolded proteins. The solubility of tTG-modified βLG as a function of ionic strength was compared with that of the reduced reference βLG by changing the concentration of CaCl2 and measuring the residual protein concentration in the solution phase at 37 °C. The tTG-modified βLG exhibited phase separation and precipitation at [CaCl2] ≥ 20 mM, whereas the reduced reference βLG was precipitated at [CaCl2] ≥ 0.5 mM (Fig. 3). These results confirmed that tTG-catalyzed modification of the unfolded protein increased its solubility.

The proteins used here were cross-linked very efficiently by tTG in the presence of Ca2+ and DTT (Fig. 4 for βLG; Refs. 26 and 27). Therefore, the cross-linking reaction was the most plausible mechanism for the inhibition of precipitation and increase in protein solubility. To determine the detailed molecular events underlying this paradoxical phenomenon, further analyses were performed for the case of βLG shown in Fig. 1A. Note that cross-linking of βLG, αLA, and BSA did not occur without reduction of the disulfide-bonds (data not shown; Refs. 26, 27), probably because exposure of lysine and glutamine...
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FIG. 2. Turbidity changes of α-casein solution. α-Casein was dissolved in 10 mM Tris (pH 7.5), 5 mM CaCl₂, and 10 mM DTT and incubated at 37 °C for 24 h. At this time point, tTG was added at a concentration of 0 (●) or 260 nM (○). The solution turbidity was monitored by determining the absorbance at 320 nm.

FIG. 3. Solubility of reduced βLGs as a function of [CaCl₂]. Concentrations of the reduced reference (●) and cross-linked βLGs (○) in the solution phase were determined after incubation at 37 °C for 24 h. The horizontal axis is represented on a logarithmic scale. See “Experimental Procedures” for details.

FIG. 4. tTG-catalyzed cross-linking of βLG monitored by SDS-PAGE. The βLG solution at a concentration of 270 μM contained 10 mM Tris (pH 7.5), 10 mM DTT, 5 mM CaCl₂, and 650 nM tTG and was incubated at 37 °C for 24 h. Arrows 1 and 3 indicate monomeric and highly polymerized βLGs, respectively. Arrow 2 indicates the position of tTG.

residues located on flexible protein chains is necessary for efficient cross-linking.

Size-exclusion Gel Chromatography Combined with Microfiltration—Microfiltration (pore size, 0.22 μm) and SEC were combined to monitor the aggregation and cross-linking processes of βLG (Fig. 5). This method separated three different populations of βLG, although some errors caused by dissociation of rapidly dissociating oligomers during the time of chromatographic measurement could not be excluded. The three groups were large aggregates of >0.22 μm (group L; star symbols in Fig. 5, B and C), small aggregates of <0.22 μm (group S; filled symbols in Fig. 5, B and C), and non-aggregated βLG (group N; open symbols). Fractional changes in each population were monitored by measuring peak areas on the chromatograms (Fig. 5, B and C). The peak labeled “N” in Fig. 5A corresponds to the N group. The area of the S group was estimated by integrating the chromatogram at the elution time of 5 to 8.7 min including the void fraction (labeled “S” in Fig. 5A). The fractional values for the N and the S groups were calculated by dividing the areas by that of the N group at zero reaction time. The L fraction was estimated by assuming that the sum of the N, S, and L fractions should be unity.

In the case of the reduced reference sample containing 1 mM EDTA, neither the L nor S group appeared even after incubation for 24 h (data not shown). In the sample containing CaCl₂ without tTG, the large aggregates of the L group appeared soon after the beginning of incubation, but the S fraction did not increase significantly (Fig. 5B). All of the molecules were in the L group after incubation for 24 h.

In contrast, in the presence of tTG and CaCl₂, the L fraction did not increase substantially, whereas the S fraction increased rapidly (Fig. 5C). In this case, essentially all the molecules were trapped in the S group after incubation for 24 h (Fig. 6, open symbols). These results indicated that reduced βLG modified by tTG could not grow to form large aggregates. The half-decay time constant (τ) of the N fraction of the tTG-containing sample was 4.2 ± 0.2 h (Figs. 5C and 6, open symbols). These decay kinetics were similar to those observed for the decay of the monomer fraction determined by SDS-PAGE analysis (Fig. 6, filled symbols). Both decays advanced at the same rate in the initial 4 h of incubation, indicating that growth of the S fraction in the presence of tTG could be explained completely by the cross-linking reaction at the early stages. The cross-linked high molecular weight βLG was trapped in the solution phase. At later stages of incubation, the decay of the N fraction of SEC was slightly faster than that of the monomer fraction on SDS-PAGE (Fig. 6), suggesting a minor contribution of non-covalent mechanisms for the later assembly process of βLG.

Notably, most of the βLG molecules in the S group under cross-linking conditions were eluted in the void fraction at all incubation times (Fig. 5A, bottom panel). Oligomers with lower molecular weights were not present to a large extent. These observations were consistent with the results of SDS-PAGE analysis (Fig. 4), which did not exhibit a clear ladder-type pattern of polymerization. These results suggested that the rate-limiting step in polymerization was not the cross-linking step itself. The details of the cascade of events leading to cross-linking will be discussed later.

Conformational Changes of βLG Accompanied by Cross-linking—The conformational changes of βLG accompanying reduction and cross-linking were analyzed by CD spectroscopy. The spectrum in the near-UV region of the reduced reference βLG showed a total loss of tertiary structures by incubation at 37 °C for 24 h (Fig. 7A, thick solid line). The decrease in ellipticity at ~206 nm of the far-UV CD spectrum also demonstrated unfolding of secondary structures by reduction (Fig. 7A, thick
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Kinetic changes in the monomer components in the presence of 650 nM tTG. The fraction of the N group monitored by SEC (○) and the monomer fraction monitored by SDS-PAGE (●) of cross-linked βLG. The solution contained 270 μM βLG, 650 nM tTG, 10 mM DTT, and 5 mM CaCl₂ and was incubated at 37 °C.

The fraction of the N group monitored by SEC (○) and the monomer fraction monitored by SDS-PAGE (●) of cross-linked βLG. The solution contained 270 μM βLG, 650 nM tTG, 10 mM DTT, and 5 mM CaCl₂ and was incubated at 37 °C.

Kinetic changes in the [θ]_295 and [θ]_222 of βLG. A, far-UV CD spectra of reduced βLG with and without tTG-catalyzed cross-linking. Thick solid line, without cross-linking. Broken line, cross-linked. B, far-UV CD spectra. The sample species were the same as those in A. C, far-UV CD spectra. The sample species were the same as those in A. D, far-UV CD spectra. The sample species were the same as those in A.

Fig. 7CD spectra of βLGs. A, near-UV CD spectra of the samples incubated at 37 °C for 24 h in the presence of 10 mM DTT. Thick solid lines, 1 mM EDTA. Broken lines, 5 mM CaCl₂ and 650 nM tTG. The spectrum of native βLG without reduction is also presented for comparison (thin solid line). B, far-UV CD spectra. The sample species were the same as those in A. C, far-UV CD spectra of reduced βLG with and without tTG-catalyzed cross-linking. Thick solid line, without cross-linking. Broken line, cross-linked. In this case, the reduced samples were prepared in advance by incubation of βLG (100 μM) in the presence of 10 mM Tris (pH 7.5), 10 mM DTT, and 1 mM EDTA at 37 °C for 24 h. Then, the cross-linked sample, prepared by adding 5 mM CaCl₂ and 650 nM tTG to this highly reduced sample, was further incubated at 37 °C for 12 h.

Kinetic details of the conformational changes of βLGs were monitored by ellipticities at [θ]_222 and [θ]_295. Tertiary structures of βLG represented by [θ]_293 were lost in a single-phase manner for both the reduced reference and the cross-linked βLGs (Fig. 8A). The decay time constants of the two were similar to each other (τ = 4.2 ± 0.3 and 5.0 ± 0.4 h, respectively), indicating that the cross-linking reaction did not substantially perturb the unfolding process of the tertiary structures. In contrast, the cross-linking reaction significantly altered the kinetic trace of [θ]_293. The trace for the reduced reference sample decreased in a biphasic manner with τ₁ = 1.8 ± 0.2 and τ₂ = 20.7 ± 3.2 h (Fig. 8B, filled symbols). The trace for the cross-linking sample also decreased monotonically until 5 h of incubation (Fig. 8B, open symbols). The decay constant of this part was 1.8 ± 0.3 h, exactly the same as that of the faster phase of the reduced reference sample. However, the change in [θ]_293 of the slower phase was blocked completely by the tTG-catalyzed reaction. The trace exhibited a relatively sharp corner at 4–6 h of incubation, and the ellipticity even increased marginally at longer incubation times. These results indicated that the cross-linking reaction inhibited unfolding of the secondary structures at later stages. Note that CD analysis was not informative for the non-covalently aggregated sample without tTG because of the strong light scattering of the turbid solution.

Stability of Cross-linked βLG—The stability of the residual secondary structure of cross-linked βLG was compared with that of the reduced reference βLG by far-UV CD spectroscopy. By decreasing the temperature from 25 to −3 °C, the residual structure of the reduced reference βLG was unfolded as indicated by a strong decrease in ellipticity at −200 nm (Fig. 9A, broken line). This change was completely reversible, indicating that the residual structure still contained a cooperative hydrophobic core (28, 29). The spectrum of the cross-linked βLG also changed reversibly in the direction of unfolding by cooling, but the magnitude of the change was much smaller (Fig. 9B, broken line). The results indicated that the residual secondary structure with a hydrophobic core of the cross-linked βLG was formed more tightly than that of the reduced reference βLG. We also compared the stability of the residual secondary structures by guanidine hydrochloride (GdnHCl)-induced denaturation. The denaturation curve monitored by [θ]_222 was composed of two phases for both the βLG species (Fig. 9C). The molecular origin of this biphasic behavior was not pursued in the present study. The results shown in Fig. 9C indicate that the residual secondary structure of the cross-linked sample was more stable than that of the reduced reference against the denaturant.

Finally, stability against α-chymotrypsin digestion was tested for the three βLG species. The reduced reference βLG was digested easily by the protease (Fig. 10, Reference), whereas digestion of the non-covalently formed aggregates was negligible (Fig. 10, Non-covalent). The cross-linked sample was also degraded at a significant rate (Fig. 10, Cross-linked). These results indicated that the susceptibility of cross-linked βLG to the proteolysis was much greater than that of the non-covalent aggregates and was even comparable with that of the reduced βLG monomer.

DISCUSSION

Inhibition of Protein Aggregation by tTG-catalyzed Cross-linking—Clustering behaviors of unfolded protein molecules in solution are finely influenced by changes in the attractive and repulsive intermolecular forces. Here, we studied a covalent mechanism that could shift the balance of the association-dissociation equilibrium. It is natural to suppose that the covalent cross-linking of unfolded protein molecules would always enhance their aggregation and precipitation (Fig. 1A, Case 1). However, we found that the tTG-catalyzed cross-linking of some unfolded proteins inhibited their non-covalent aggregation and precipitation by producing soluble multimers (Fig. 1A, Case 2). Notably, the efficiency of the tTG effect was strongly dependent upon the species of substrate proteins (Fig. 1, A–C), indicating that the inhibitory effect is active only for some specific protein groups or conformations. It is known that 1 g of guinea pig liver contains ~5.8 nmol of tTG (23), which gives a rough estimate of 1–3 μM for the intracellular concentration of tTG. The concentrations of tTG used in the present study were comparable with or lower than this estimate.
The detailed molecular events underlying the paradoxical inhibition of protein aggregation by tTG were analyzed for βLG. Based on all of the results, we propose the scheme shown in Fig. 11B. Three different denatured states of reducing βLG (D1, D2, and D3) were identified using the kinetic data of [θ]293 and [θ]206 of the reduced reference sample (Fig. 8, filled circles). The unfolding of secondary structures of the reduced reference βLG monitored by [θ]206 progressed in a biphasic manner with τ1 = 1.8 ± 0.2 and τ2 = 20.7 ± 3.2 h, whereas that of tertiary structures monitored by [θ]206 advanced with a single decay of τ = 4.2 ± 0.3 h. The kinetic results indicated that βLG under reducing conditions initially unfolded to a mildly denatured state (D1), which lacked some secondary structure but still contained tertiary structure. Then, the unfolding advanced to the D2 state in which the tertiary structures of βLG disappeared completely. After a longer incubation time, βLG in the D2 state further lost its residual secondary structure and fell into the more extensively unfolded state, D3.

Cross-linking did not affect the decay of [θ]293 (Fig. 8A) but inhibited the slower phase of the change in [θ]206 (Fig. 8B). This observation indicated that cross-linking blocked the transition from the D2 state to D3 and trapped the molecules as soluble multimers (Fig. 11B). The decay constant of the non-aggregating fraction by SEC for the cross-linked sample was 4.2 ± 0.2 h (Figs. 5C and 6), which was the same as that observed for the decay of the tertiary structure (τ = 4.2 ± 0.3 h). This result indicated that the cross-linking reaction occurred rapidly in the D2 state of βLG. The rate-limiting step of the cross-linking reaction is probably the conformational change of βLG. The extensive unfolding from D2 to D3 could also be reverted by the action of tTG (Fig. 7C). The decay time constant of the N fraction of the non-covalent aggregation processes (4.4 ± 0.4 h; Fig. 5B) was also identical to that of [θ]293 of the reduced reference βLG, suggesting that the non-covalent aggregation was also initiated in the D2 state (Fig. 11B).

Properties of Cross-linked βLG Molecules—The cross-linked βLG could not form precipitates under low ionic strength. Higher concentrations of CaCl2 were required for its phase separation than for the reduced βLG monomer (Fig. 3). This indicated that cross-linking weakened the intermolecular attractive forces or, alternatively, that it enhanced the electrostatic repulsion. The observation that the effect of tTG on the aggregation of αLA was much weaker than that of βLG and BSA (Fig. 1, A–C) can also be explained by variations in force balancing; the attractive force enhanced by unfolding of αLA was probably too strong to be overcome by the cross-linking effect.

The change in the force balance caused by the tTG activity could have originated from the conformational shift of the unfolded βLG by cross-linking because cross-linking substantially suppressed unfolding of the secondary structure (Fig. 8B). Furthermore, the residual structures with a hydrophobic core were more stable for the cross-linked βLG than for the reduced reference (Fig. 9). It is plausible that cross-linking trapped βLG in the conformation with less exposure of the hydrophobic core, which would reduce the hydrophobic attractive force among the molecules and increase the solubility of...
It should also be noted that tTG and N\footnote{Ref} linking occurs with the substrate proteins in an extremely high aggregation process was strongly dependent on the species of the and precipitation. We found that the effect of tTG on the aggregation does not necessarily inhibit protein aggregation and could be real pathogens for degenerative disease (12). Increased their solubility and inhibited their fibril formation. The cross-linking of polyglutamine-containing proteins including both enhancement and inhibition of aggregation deamyloid-type aggregation. In fact, Lai et al. (21) showed that the cross-linking of polyglutamine-containing proteins increased their solubility and inhibited their fibril formation. Soluble oligomers of pathogenic proteins are not always benign and could be real pathogens for degenerative disease (12).

However, it should also be emphasized that tTG-catalyzed cross-linking does not necessarily inhibit protein aggregation and precipitation. We found that the effect of tTG on the aggregation process was strongly dependent on the species of the substrate proteins (Fig. 1, A–C). Furthermore, when cross-linking occurs with the substrate proteins in an extremely high concentration, it will induce gelation of the proteins. This is probably the case for blood clotting and skin keratin formation. It should also be noted that tTG and N\footnote{Ref}-(γ-L-glutamyl)-L-lysine linkage are often co-localized with the intracellular inclusions in patients with the neurodegenerative diseases (36–39). Cultured cell models over-expressing tTGase and/or amyloidogenic proteins, such as α-synuclein, and huntingtin, have also been shown to exhibit tTGase-induced intracellular cross-linking and deposit formation (35, 38, 39). These observations suggested that tTG-catalyzed cross-linking enhances the aggregation and deposition of some pathogenic proteins \textit{in vivo}. It is possible that tTG-catalyzed cross-linking modifies protein aggregation and deposition \textit{in vivo} in a variety of fashions, including both enhancement and inhibition of aggregation depending on the target protein species and solution conditions. Further studies will be required to examine larger numbers of protein species under a variety of solution conditions, to establish the biological significance of the present findings.

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