Covalent Blocking of Fibril Formation and Aggregation of Intracellular Amyloidgenic Proteins by Transglutaminase-Catalyzed Intramolecular Cross-Linking†

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Received October 26, 2004; Revised Manuscript Received November 25, 2004

ABSTRACT: Two different types of physical bonding have been proposed to involve in the formation of neuronal inclusions of patients with neurodegenerative diseases such as Alzheimer's, Parkinson's, and polyglutamine diseases. One is the noncovalent bonding that stabilizes the amyloid-type fibrous aggregates, and the other is the covalent cross-linking catalyzed by tissue transglutaminase. The cross-linking is subdivided into the inter- and intramolecular cross-linking. Little attention has been paid to the pathological roles of the intramolecular cross-linking. To elucidate the possible interplay between the intramolecular cross-linking and the amyloid-type fibril formation, we performed an in vitro aggregation analysis of three intracellular amyloidgenic proteins (a domain of τ protein, α-synuclein, and truncated yeast prion Sup35) in the presence of tissue transglutaminase. The analysis was performed in low concentrations of the proteins using techniques including thioflavin T binding and mass spectrometry. The results demonstrated that the amyloid-type fibril formation was strongly inhibited by the transglutaminase-catalyzed intramolecular cross-linking, which blocked both the nucleation and the fiber extension steps of the amyloid formation. Far-UV CD spectroscopy indicated that the cross-linking slightly altered the backbone conformation of the proteins. It is likely that conformational restriction imposed by the intramolecular cross-links has impaired the ordered assembly of the amyloidgenic proteins. Nonamyloid type aggregation was also suppressed by the intramolecular cross-links. On the basis of the results, we proposed that tissue transglutaminase is a modulator for the protein aggregation and can act defensively against the fibril deposition in neurons.

The formation of neuronal inclusions is a hallmark pathology of neurodegenerative diseases (*1*−*4*). The inclusions are principally composed of proteins such as τ protein for Alzheimer's disease (AD),[1] α-synuclein for Parkinson's disease (PD), and huntingtin for Huntington's disease (HD) (*5*−*7*). One of the essential problems left unresolved is the physical nature of the bonding that stabilizes the highly insoluble inclusions. Two different types of bonding have been proposed. First, the proteins are deposited in the form of aggregates with specific unbranched fibrous shape, known as amyloid (*4*, *8*, *9*). Note that we use the term amyloid in a wider sense than its traditional one that strictly indicates the fibrillation of extracellular proteins such as Alzheimer's Aβ protein. In vitro studies have elucidated the β-sheet-rich structure of the amyloid that is stabilized by the hydrogen-bonding network (*9*, *10*). In addition to the noncovalent mechanism, covalent cross-linking catalyzed by tissue transglutaminase (tTGase) has been proposed to enhance or stabilize the neuronal inclusions (*11*−*13*). Interplay of the two different bonding types potentially modulates the physical properties of the neuronal inclusion and its toxicity.

tTGase is ubiquitous thiol- and Ca^{2+}-dependent acyl transferase implicated in a number of cellular processes, located both in the intra- and in the extra-cellular spaces (*14*, *15*). It catalyzes the cross-linking reactions that form an amide bond between the γ-carboxamide groups of peptide-bound glutamine residues and the primary amino groups in various compounds, including the ϵ-amino group of lysine residues. The tTGase-catalyzed cross-linking is subdivided into the inter- and the intramolecular cross-linking. Involvement of tTGase in the neurodegenerative diseases including AD, PD, and polyglutamine diseases has been supported by several lines of experimental indications. The tTGase and its mRNA contents in the brain of the patients were

† This work is supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan (15659050) and CREST of Japan Science and Technology Corporation.

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[1] Abbreviations: AD, Alzheimer's disease; PD, Parkinson's disease; HD, Huntington's disease; tTGase, tissue-type transglutaminase; τ4RD, four-repeat domain of human τ protein; SupNM, the N-terminal region of Sup35; βLG, β-lactoglobulin A; ThT, thioflavin T; DTT, dithiothreitol; SEC, size-exclusion gel chromatography; TEM, transmission electron microscopy; CD, circular dichroism.

10.1021/bi047722d CCC: $30.25
Published on Web 01/15/2005

substantially higher than those of normal controls (*16*−*22*). tTGase and the N^{ϵ}-(γ-L-glutamyl)-L-lysine linkage were often colocalized with the intracellular inclusions of the patients (*20*−*23*). The cultured cell models overexpressing tTGase and/or amyloidgenic proteins such as τ, α-synuclein, and huntingtin have exhibited tTGase-induced intracellular cross-linking and deposit formation (*22*−*24*). In vitro experiments have also demonstrated the tTGase-catalyzed intermolecular cross-linking of the amyloidgenic proteins (*16*, *23*−*27*).

However, several essential problems are left untouched. Especially, potential roles of the intramolecular cross-linking in the neurodegenerative diseases have been rarely commented on while much attention has been devoted to the intermolecular one as a potential enhancer of the protein aggregation. The lack of the studies focused on the intramolecular cross-linking should be made up because of the following three reasons. First, the tTGase-catalyzed intramolecular cross-linking has actually been found in vivo for τ protein and a fragment of huntingtin (*20*, *22*, *28*, *29*). Second, the intramolecular cross-linking may be more dominant than the intermolecular one in the biologically relevant low concentration range of substrate proteins. Third, it is a reasonable expectation that the intramolecular cross-linking would impose conformational restrictions upon amyloidgenic proteins and would therefore affect their amyloid formation significantly.

In the present study, to elucidate the possible interplay between the tTGase-catalyzed intramolecular cross-linking and the amyloid formation, we constructed an in vitro experimental system using a four-repeat domain of human τ protein (τ4RD), human α-synuclein, and the N-terminal region of yeast prion Sup35 (SupNM). τ4RD comprises the amyloid-forming part of τ protein that is accumulated in neurofibrillary tangles of patients with AD (*5*, *30*, *31*). α-Synuclein is the principal component of Lewy bodies deposited in the brain of patients with PD (*6*, *32*). SupNM is the prion-forming part of the translation-termination factor Sup35 that is responsible for the prionic [PSI+] phenotype of *Saccharomyces cerevisiae* (*33*, *34*). SupNM is very rich in glutamine and asparagine residues and might share common physical properties with the glutamine-rich proteins such as huntingtin of HD (*7*). The three are natively unfolded intracellular amyloidgenic proteins (*35*−*37*), which were chosen as representatives since they are related to the pathological cases where the involvement of tTGase has been indicated as described previously. The analysis was performed in the low protein concentrations where the intramolecular cross-linking was preferentially formed. Several techniques including mass spectrometry and thioflavin T (ThT) dye binding have been combined to monitor the cross-linking and the amyloid formation processes. In addition to these amyloidgenic cases, the effect of tTGase on the amorphous aggregation process was tested for bovine β-lactoglobulin A (βLG) in a reducing condition. The results presented next demonstrate a novel action of the tTGase-catalyzed reaction that inhibits the amyloid formation and protein aggregation.

MATERIALS AND METHODS

Materials. Guinea pig liver transglutaminase was purified on an immunoadsorbent column as described previously (*38*). Bovine βLG was purchased from Sigma Chemicals Co. (St. Louis, MO). Other chemicals of reagent grade were purchased from Nacalai Tesque Co. (Kyoto, Japan).

Cloning, Expression, and Purification of Recombinant Proteins. cDNAs encoding τ4RD, α-synuclein, and SupNM were cloned by polymerase chain reaction amplification from an adult human brain cDNA library (for τ4RD and α-synuclein) or genomic DNA library of *S. cerevisiae* (for SupNM) using the primers GCCAAGAGCCATATGCAGACAGCCCCCGTG and TTTGGCGTTCTCGAGGAAGGTCAGCTTGTG for τ4RD, CTCTCGGAGTGGCCATTCGACGACAGTGTG and TGAGTGGGGGCAGGATCCGATACTTCAATC for α-synuclein, and CCCACTAGCAAGCATGCCGGATTCAAAC and GAAACGTGATCTTGTCGACCAAACTAATCG for SupNM. The PCR-amplified fragment of τ4RD was digested with NdeI and BamHI and was ligated to the NdeI−BamHI fragment of the pET-22b vector (Novagen, EMD, Darmstadt, Germany). The PCR-amplified fragment of α-synuclein was digested with NcoI and BglII and was ligated to the NcoI−BamHI fragment of the pET-3d vector (Novagen, EMD, Darmstadt, Germany). The PCR-amplified fragment for SupNM was digested with SphI and SalI and was ligated to the SphI−SalI fragment of the bacterial vector pQE82L (Qiagen, CA). The correct DNA sequences were confirmed by DNA sequencing. The expression products of τ4RD and SupNM contain His-tags in the N- and the C-terminal ends, respectively. Formula weights of τ4RD, α-synuclein, and SupNM were 15 607, 14 460, and 30 109, respectively. Recombinant α-synuclein was purified by the methods of Conway et al. (*39*). Recombinant τ4RD and SupNM were purified using a standard protocol for His-tagged proteins by means of the Ni-bound chelating sepharose column (Amersham, Piscataway, NJ) followed by cationic or anionic ion-exchange chromatography for τ4RD or SupNM, respectively.

Aggregation Experiments. All the sample solutions were prepared by lyophilized protein powders being dissolved to the buffer solution containing 10 mM Tris (pH 7.5) 10 mM dithiothreitol (DTT) and 5 mM $CaCl_2$ (or 2 mM EDTA), the pH being adjusted carefully with small amounts of HCl. Irreversibly inactivated tTGase used for the control experiments was prepared by heat treatment at 70 °C for 5 min. Intact tTGase or the heat-inactivated tTGase was added to the sample solutions and was incubated at 37 °C. The samples containing α-synuclein and SupNM were stirred during the incubation using a revolution mixer RVM-101 (Iwaki, Japan) at the rotation rate of 0.1 Hz. Some more details for the aggregation experiments are described in legends to Figures 1, 4, and 8.

Analysis of Aggregation Processes. ThT binding analysis of α-synuclein and SupNM was performed by diluting 40 times an aliquot of the sample solutions at each incubation time with a buffer solution containing 10 mM Tris (pH 7.5) and 10 μM ThT. ThT fluorescence was measured by a F2500 spectrofluorimeter (Hitachi, Japan). The excitation and emission wavelengths were 440 and 480 nm, respectively, and bandwidths of excitation/emission lights were 5:5 nm. For τ4RD, the sample solution containing 10 μM ThT was prepared in the 5 × 5 mm quartz cell and incubated for the amyloid formation at 37 °C in a cell holder equipped with a water bath. ThT fluorescence for τ4RD was measured without dilution. Turbidity of the sample solutions was

measured by absorbance at 340 nm without dilution using a 2 μL quartz cell and a Genespec spectrometer (Naka Instrument, Ibaraki, Japan). The SDS−PAGE analysis was done by the standard method of Laemmli (*40*) using 12 or 15% polyacrylamide gel. The samples for the electrophoresis were prepared by the TCA precipitation method and were dissolved in 20 μL of the loading dye solution.

Spectroscopic Analysis of Cross-Linked Proteins. The samples for the mass spectrometric measurement were desalted using Micro Bio-Spin 6 chromatography column (Bio-rad Laboratories, CA). The mass spectra were measured using a MALDI-TOF MS spectrometer Voyager DE-STR (Applied Biosystems, CA). Circular dichroism (CD) spectra were measured with a Jasco J-820 spectropolarimeter (JASCO Co., Tokyo, Japan) using a quartz cell with a path length of 0.5 mm at 37 °C. The protein concentration was adjusted to 6 μM for the CD measurements.

Characterization of Aggregates. Transmission electron microscopy (TEM) of aggregates was taken with a H-7000 electron microscope (Hitachi, Japan) operated at 75 kV, at a magnification of 40 000. The samples were applied to carbon grids and stained with 2% uranyl acetate. The images were recorded on FG electron-microscopic films (Fuji Film, Japan) and developed in a D-19 developer (Kodak, Rochester, NY) for 3 min. Analytical size-exclusion gel chromatography (SEC) was performed using the Class-M10A HPLC system (Shimadzu, Kyoto, Japan) equipped with a G3000SW$_{XL}$ column (7.8 × 300 mm; TOSO, Tokyo, Japan). Equilibrium and elution buffers were the 0.1 M sodium phosphate buffer (pH 7.5), and the flow rate was 1 mL/min. The sample of 20 μL was loaded to SEC, and the chromatogram was monitored by absorbance at 220 nm.

RESULTS

tTGase-Catalyzed Inhibition of Amyloid-Type Aggregation. All the three proteins tested here had highly unfolded conformations in the monomeric form at the initial points of the incubation, which was confirmed by the CD spectroscopy and the size exclusion gel chromatography (data not shown). At the beginning of this study, we searched for the reaction condition where tTGase preferentially catalyzed the intramolecular cross-linking of τ4RD, α-synuclein, and SupNM. It could be achieved by employing relatively low concentrations of the proteins (typically 1.3 μM for τ4RD, 50 μM for α-synuclein, and 2.5 μM for SupNM) in a low salt solution as confirmed in the next section. These sample conditions were used in the following experiments. Note that, in the higher protein concentrations, we observed the occurrence of a mixture of the intra- and intermolecular cross-linking reactions (data not shown).

Amyloid-type aggregation in the reaction mixtures was monitored by the binding of an amyloid-specific fluorescence dye ThT and solution turbidity (Figure 1). The results of the ThT binding showed that intact tTGase strongly inhibited the amyloid formation of all three proteins (filled triangles of Figure 1A−C) as compared to the solutions containing heat-inactivated tTGase (filled circles of Figure 1A−C). Turbidity of the sample solutions also changed in parallel with the ThT binding (open symbols of Figure 1A−C). Even after sufficient incubation times, the tTGase-treated samples were still transparent, indicating that the tTGase-catalyzed

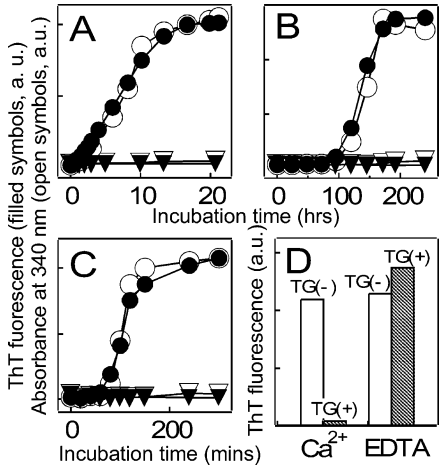

FIGURE 1: Effects of tTGase on the amyloid formation processes. Amyloid formation of τ4RD (A), α-synuclein (B), and SupNM (C) was monitored by ThT binding (filled symbols) and turbidity (open symbols) in the presence of heat-inactivated tTGase (circles) or intact tTGase (down triangles). The solution contained 10 mM Tris (pH 7.5), 10 mM DTT, and 5 mM $CaCl_2$ and was incubated at 37 °C. The protein concentration was 1.3 μM for τ4RD, 50 μM for α-synuclein, and 2.5 μM for SupNM. The concentration of tTGase was 0.13 μM for panel A, 1.3 μM for panel B, and 0.06 μM for panel C. In the τ4RD solutions (A), 0.02 mg/mL heparin was additionally included since the efficient amyloid formation of τ and its fragments requires cofactors such as heparin and arachidonic acids (*30*, *31*). (D) The amount of the amyloid of SupNM measured by ThT binding after incubation for 24 h at 37 °C. All the samples contained 2.5 μM SupNM in 10 mM Tris (pH 7.5), 10 mM DTT. They additionally contained 5 mM $CaCl_2$ or 2 mM EDTA and also heat-inactivated (TG (−)) or intact tTGase (TG (+)) in 0.06 μM.

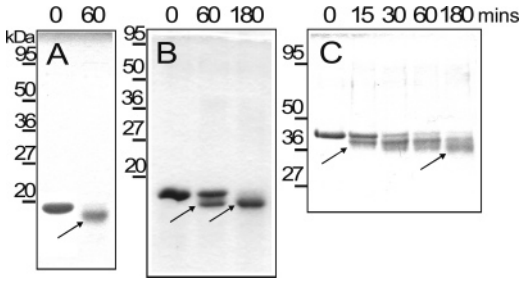

FIGURE 2: SDS−PAGE analysis of the tTGase-catalyzed reaction. SDS−PAGE gels of τ4RD (A), α-synuclein (B), and SupNM (C) incubated at 37 °C in the presence of intact tTGase. The solution compositions were the same as those for Figure 1. Arrows in the figure indicate the populations with intracellular cross-links.

reaction suppressed not only the amyloid formation but also the formation of large aggregates of any type. By chelating Ca^{2+} by EDTA, the inhibitory effect of tTGase was totally abolished for all three proteins (Figure 1D for SupNM).

Intramolecular Cross-Linking Inhibits the Amyloid Formation. The samples containing intact tTGase used for Figure 1A−C were analyzed by the SDS−PAGE method (Figure 2). Covalently linked multimers were not formed significantly in all the protein species. Instead, some bands migrating faster than the authentic monomers appeared in the early incubation times (arrows in Figure 2). To exclude the possibility of proteolytic degradation and to get further structural information, mass-spectrometric analysis was performed for the tTGase-treated samples. The mass peaks corresponding to the monomer of τ4RD and α-synuclein were split into several peaks by the tTGase treatment (Figure 3). The molecular weight of each split peak decreased regularly by 16∼17 mass

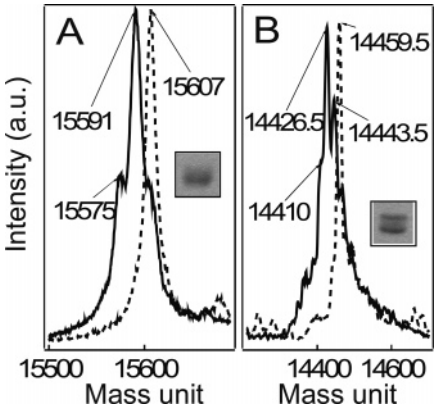

FIGURE 3: MALDI-TOF mass spectrometric analysis of the tTGase-treated samples. Spectra of the intramolecular cross-linked proteins for τ4RD (A) and α-synuclein (B) are presented (solid lines). The sample was taken from the preparation used in Figure 1A at 60 min for τ4RD or Figure 1B at 180 min for α-synuclein. The SDS−PAGE pattern of the samples used for the mass spectrometric measurements are shown in the insets. Dashed lines are control spectra for the proteins without the tTGase treatment.

units, which exactly corresponds to the value expected for formation of an intramolecular cross-link. No significant fraction of mass peaks for the degradation products was observed. Unfortunately, the high-resolution mass spectrum for SupNM could not be obtained, but the spectrum lacked any signs for degradation (data not shown). The SDS−PAGE bands migrating faster than the unmodified monomer for the three proteins should be assigned to the molecular species with compact conformations imposed by the intramolecular cross-links.

To examine the correlation between the intrachain cross-linking and the ability to form an amyloid, τ4RD and α-synuclein with various degrees of the cross-linking were prepared, and the amyloid formation was induced for each preparation (see the legend to Figure 4 for the details). The samples used here essentially lack the SDS−PAGE bands for the intermolecular cross-linked populations (not shown in Figure 4). The results demonstrated an inverse correlation between the degree of the cross-linking and the amount of amyloid for both τ4RD and α-synuclein (Figure 4). The results of Figures 1−4 indicate that the Ca^{2+}-dependent tTGase activity inhibits the aggregation of the amyloidgenic proteins via the intramolecular cross-linking.

Structure of the Intramolecular Cross-Linked Proteins. CD spectra were measured to examine the effects of the intramolecular cross-linking on the protein conformation in the solution phase. τ4RD and α-synuclein without the cross-link showed a typical spectral pattern of a highly unstructured conformation (broken lines of Figure 5). After the incubation with intact tTGase, a slight but significant change in the ellipticity was observed at around 200 nm. The similar result was also obtained for SupNM (data not shown). The results indicate that the intramolecular cross-linking substantially altered the backbone conformation of the proteins although the modified proteins still remained in the highly randomized states.

Aggregation States of the tTGase-Treated Samples. Negatively stained TEM images were taken for the samples in the final plateau phases of Figure 1A−C. In the absence of the tTGase activity, the aggregates of the amyloidgenic proteins exhibited fibrous shape (Figure 6A,C,E). These

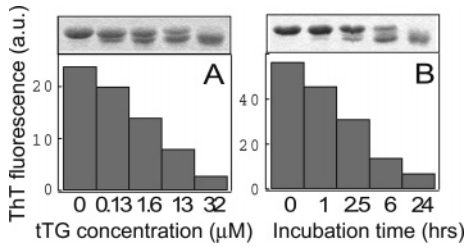

FIGURE 4: Correlation between the degree of the intramolecular cross-linking and the amount of amyloid. The lower panels demonstrate the amount of amyloid formed using the samples with various degrees of the intramolecular cross-links shown in the upper panels. The τ4RD (A) and α-synuclein (B) solutions were prepared in 10 mM Tris (pH 7.5), 10 mM DTT, and 5 mM $CaCl_2$ as follows. τ4RD samples were prepared by varying the tTGase concentrations. The concentration of τ4RD was 32 μM. τ4RD in this condition did not form the intermolecular cross-linking significantly (data not shown). The tTGase concentration was 0, 0.13, 1.6, 13, or 32 μM. The samples were incubated for 12 h at 37 °C. tTGase became inactive within 3 h probably because of the cross-linking between τ4RD and tTGase. To these preparations, heparin was added in the final concentration of 0.3 mg/mL. The samples were further incubated at 37 °C for 8 h, and the amyloid content was measured by the ThT binding method. α-Synuclein samples were prepared by the incubation time being varied. The sample containing 50 μM α-synuclein and 0.4 μM tTGase was incubated at 37 °C. At each incubation time (0, 1, 2.5, 6, and 24 h), the tTGase was inactivated by being heated at 70 °C for 5 min. After that, heparin was added in the final concentration of 0.3 mg/mL to enhance and accelerate the amyloid formation of α-synuclein (*51*) and incubated at 37 °C under stirring for 3 days. The amyloid content of the samples was determined by the ThT binding method.

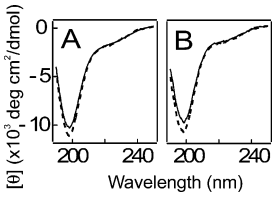

FIGURE 5: Conformation of the cross-linked proteins monitored by the far-UV CD spectroscopy. Spectra of the intramolecular cross-linked proteins (solid lines) for τ4RD (A) and α-synuclein (B) were obtained for the samples in the final plateau phase of Figure 1A,B. Dashed lines are control spectra for the samples without the tTGase treatment.

fibers could bind an amyloid specific dye Congo Red and also contained large amounts of β-sheet structures as measured by FTIR (data not shown), indicating that the fibrous aggregates are of typical amyloid. On the other hand, in the presence of intact tTGase, the fibrous component was absent (Figure 6B,D,F). The TEM images for the tTGase-treated τ4RD and α-synuclein showed only a few fine granular components (Figure 6B,D), while relatively large granules were observed for SupNM (Figure 6F).

The aggregation state of the tTGase-treated samples was further examined by SEC. The largest fraction of the tTGase-treated α-synuclein eluted at the monomer position of the chromatogram, and no aggregated fraction was detected significantly (Figure 7A). Note that the tTGase-treated α-synuclein eluted slower than the untreated monomer, which is consistent with the faster migration of the SDS−PAGE band for the tTGase-treated sample (Figure 2B). On the other hand, for the tTGase-treated SupNM, a fraction of 63% was eluted at the monomer position, while the rest appeared in the void position of the SEC chromatogram. Note also that the monomer peak of unmodified SupNM (∗ in Figure 7B)

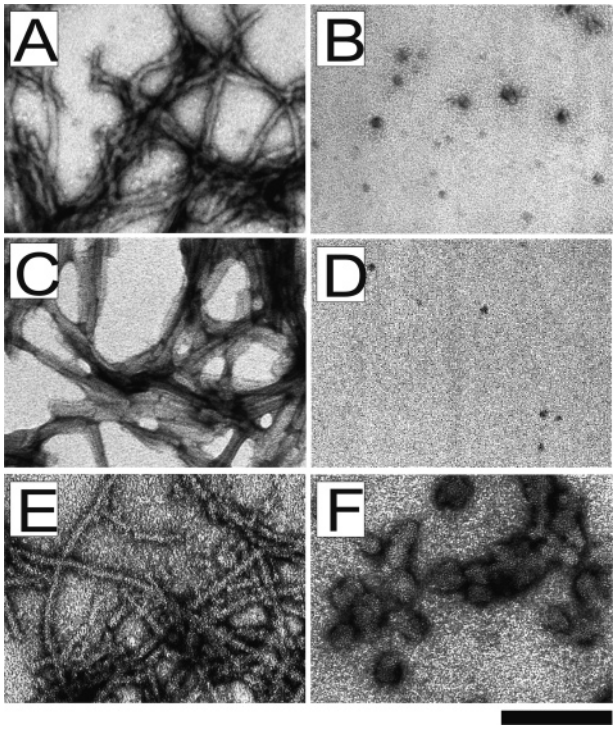

FIGURE 6: TEM analysis of the tTGase-treated samples. The TEM images were taken for the samples in the final plateau phases of Figure 1A−C. (A) τ4RD and tTGase (−). (B) τ4RD and tTGase (+). (C) α-synuclein and tTGase (−). (D) α-synuclein and tTGase (+). (E) SupNM and tTGase (−). (F) SupNM and tTGase (+). Scale bar: 200 nm.

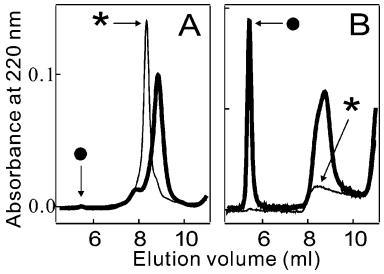

FIGURE 7: SEC analysis of the tTGase-treated samples. The chromatograms were taken for α-synuclein (A) and SupNM (B). Thick lines: tTGase-treated. Thin lines: untreated. Peaks for the untreated monomers are indicated by an asterisk. The void fraction was indicated by (●). Note that the asterisk region of SupNM is small because the molecules were partly adsorbed to the SEC column. The data for τ4RD are lacking because of the similar adsorption problem.

is substantially small because the molecules were partly adsorbed to the SEC column. The results of TEM and SEC indicated that the transparent tTGase-treated samples sometimes contained nonamyloid type aggregates depending on the protein species. The aggregates were, however, small in size and in the soluble state.

Cross-Linking Inhibits Both Nucleation and Extension Steps of Amyloid Formation. The amyloid formation proceeds in a nucleation-dependent manner (*41*). The rate-limiting step is the nucleus formation, followed by a faster phase of the fiber extension. This type of kinetics was well-established for τ protein, α-synuclein, and SupNM (*37*, *42*, *43*). A question arises as to whether the tTGase-catalyzed intrachain cross-linking has affected either the nucleation or the extension step. This question could be addressed by monitoring the aggregation of the samples seeded by the matured amyloid or the tTGase-treated proteins. The results for SupNM are summarized in Figure 8. The amyloid formation of the intact SupNM seeded by the intact amyloid fibers proceeded without an initial lag phase, exhibiting a typical seeding effect (asterisks in Figure 8). On the other hand, addition of the aged tTGase-treated sample did not accelerate the amyloid formation of the intact SupNM (open circles in Figure 8), indicating that the tTGase-treated SupNM even after a long incubation time did not contain a significant amount of amyloid seeds. Furthermore, the tTGase-treated sample did not exhibit growth of amyloid fibers even after seeding of the intact SupNM amyloid (solid triangles in Figure 8). The results for the seeding tests showed that the tTGase-treated SupNM could neither form a nucleus or participate in the extension reaction (the scheme of Figure 8). Essentially, the same results were obtained for α-synuclein, although the data quality was worse because EDTA used in the experiments substantially weakened the amyloid formation propensity of the protein (data not shown).

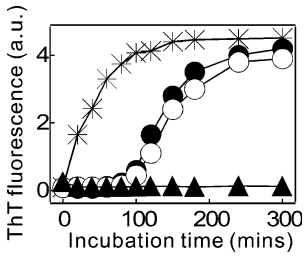

FIGURE 8: Seeding effects of intact or intramolecular cross-linked SupNM. The amyloid formation process was monitored by the ThT binding for intact SupNM (2.5 μM) without seeds (●), intact SupNM seeded by intact SupNM amyloid (*), intact SupNM seeded by the tTGase-treated SupNM (○), and tTGase-treated SupNM seeded by intact SupNM amyloid (▲). All the samples were prepared in 10 mM Tris (pH 7.5), 10 mM DTT, and 2 mM EDTA and incubated at 37 °C under stirring. The intact amyloid seed was prepared by incubating intact SupNM (2.5 μM) in 10 mM Tris (pH 7.5), 10 mM DTT, and 5 mM $CaCl_2$ for 12 h at 37 °C. The tTGase-treated SupNM used for (○) and (▲) were prepared by incubating 2.5 μM intact SupNM in 10 mM Tris (pH 7.5), 10 mM DTT, 5 mM $CaCl_2$, and 60 nM tTGase for 12 h at 37 °C. Final concentrations of the used seeds for (*), (○), and (▲) were 50 nM in the monomer level.

tTGase-Catalyzed Inhibition of Nonamyloid-Type Aggregation and Precipitation. The results presented previously were concerned with the amyloid-type aggregation. We additionally provide here the results for the nonamyloid-type aggregation for generalization of the present conclusion. Reduction of disulfide bonds of folded proteins often damages their conformation and induces their noncovalent aggregation. This might provide a convenient in vitro model for intracellular protein damages. The reduction-induced aggregation and precipitation was observed for many protein species including β-lactoglobulin A (βLG) in the presence of DTT (solid circles in Figure 9A). TEM analysis indicated that the βLG aggregation is of amorphous type (data not shown). Addition of intact tTGase to the reduced βLG solution drastically suppressed their turbidity increase (Figure 9A). tTGase predominantly catalyzed intramolecular cross-linking in the present solution condition as demonstrated by SDS−PAGE (Figure 9B) and mass spectrometric analyses (Figure 9C), although some faint bands of covalently linked multimers could also be observed in the SDS−PAGE gel

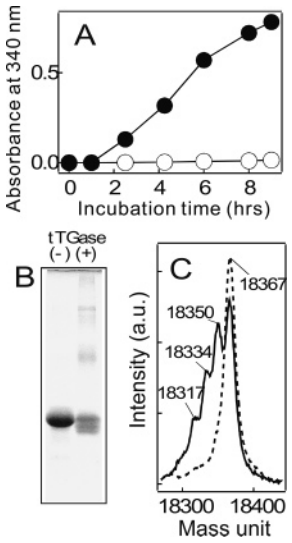

FIGURE 9: Effects of the tTGase-catalyzed intramolecular cross-linking on the amorphous aggregation process of reduced βLG. (A) Turbidity change of βLG solutions in the presence of intact tTGase (○) or heat-inactivated tTGase (●). The concentrations of βLG and tTGase were 27 μM and 260 nM, respectively. The solutions contain 10 mM Tris (pH 7.5), 10 mM DTT, and 5 mM $CaCl_2$ and were incubated at 37 °C. (B) SDS−PAGE analysis of the samples with the intact and the heat-inactivated tTGase in the turbidity experiment above after 3 h incubation. (C) MALDI-TOF mass spectrometric analysis of the samples used in panel B. The solid line is the spectrum for the tTGase-treated sample, and the dashed line is the control spectrum for the sample without the tTGase treatment.

(Figure 9B). The similar tTGase-catalyzed suppression of the reduction-induced aggregation was observed for α-lactalbumin and bovine serum albumin (data not shown). The results indicated that the amorphous-type aggregation was efficiently suppressed by the tTGase-catalyzed cross-linking.

DISCUSSION

Inhibition of Amyloid Formation by the Intramolecular Cross-Linking. In this study, the ThT binding and TEM analyses have clearly demonstrated the inhibition of the amyloid formation by the Ca^{2+}-dependent tTGase activity (Figures 1A and 6). The formation of both the nucleus and the matured amyloid was blocked efficiently (Figure 8). The mechanism of the inhibition should be assigned to the intramolecular cross-linking (Figures 2−4) since the intermolecular cross-linking was not detected significantly in the present solution conditions (Figure 2). The intramolecular covalent link must restrict the conformational freedom of the protein and alter its backbone conformation as indicated by the far-UV CD spectrum (Figure 5), which probably distorts the specific protein conformation required for the tightly ordered amyloid-type assembly.

The inhibition of the amyloid formation by the intramolecular cross-linking was demonstrated here for the three different protein species. Their natively unfolded conformation (*35*−*37*) must be the structural factor that enhances the susceptibility to the tTGase-catalyzed reaction because the flexible protein chain in the unfolded state can easily coordinate its Lys and Gln residues on the active site of tTGase. Natively folded amyloidgenic proteins such as transthyretin and β2-microglobulin also unfold when they fall into the amyloid formation pathway (*8*, *9*), which suggests that a wide range of amyloidgenic proteins can be modulated by the tTGase-catalyzed cross-linking. This study employed the intracellular protein species as representatives since those are tightly linked to the cases where the pathological involvement of tTGase has been proposed (i.e., tau in AD, α-synuclein in PD, and huntingtin in HD). However, tTGase is located not only in the intracellular but also the extracellular spaces of the living organisms and therefore might modulate the extracellular amyloidgenic proteins such as Alzheimer's Aβ peptides in situ.

The tTGase-catalyzed intramolecular cross-linking suppressed not only the amyloid formation but also the turbidity increase of the sample solutions (Figure 1A−C). Although the tTGase-treated SupNM formed a substantial amount of amorphous aggregates (37% of total molecules; Figures 6F and 7B), they are relatively small in size and soluble (Figure 1C). The observation suggests that the intramolecular cross-linking by tTGase suppresses not only the amyloid-type but also the amorphous-type protein aggregation. This is confirmed by the results obtained for reduced βLG in Figure 9, which found that the amorphous aggregation of the protein was strongly inhibited by the tTGase activity. It is likely that the cross-link formation increases the solubility of unfolded proteins by reducing the exposure of their hydrophobic cores.

Biological Implications. Many previous studies have proposed the pathological relevance of the tTGase-catalyzed intermolecular cross-linking that forms insoluble multimers of amyloidgenic proteins or that attaches other molecules such as polyamines to the proteins (*16*−*29*). Those studies have often argued that the tTGase-catalyzed cross-linking reaction could enhance the in situ protein aggregation. Most of them were, however, concerned with the intermolecular mode of the cross-linking reaction, which could naturally be dominant in the solution containing high concentrations of the substrate proteins in vitro or under the overexpression of the substrate proteins in the cell. Furthermore, the amyloid type properties (e.g., dye binding or morphology) of the tTGase-induced aggregates have rarely been examined.

We would complement these previous studies by arguing that the tTGase-catalyzed intramolecular cross-linking may be an in situ modulator upon the amyloid formation process. At the beginning of this study, we could easily find the reaction condition where tTGase preferentially catalyzes the intramolecular cross-linking in the relatively low concentrations of the amyloidgenic proteins. This suggests that the intramolecular cross-linking may be dominant in the low concentration range of the proteins in the living bodies. In fact, several studies have found a large degree of the tTGase-catalyzed intramolecular cross-linking of τ protein in the brain of patients with AD and progressive supranuclear palsy (*20*, *28*, *29*) and of a fragment of huntingtin in a cultured cell model (*22*), although the authors of the reports have not clearly discriminated between the pathological implications of the inter- and the intra-modes of the cross-linking and proposed that both the cross-linking modes could similarly enhance the protein aggregation.

The tTGase-catalyzed intramolecular cross-linking may lead to dysfunction of the amyloidgenic proteins by altering their conformation. However, we would rather emphasize its potentially protective action against the amyloid deposition. The present results have shown that the intramolecular

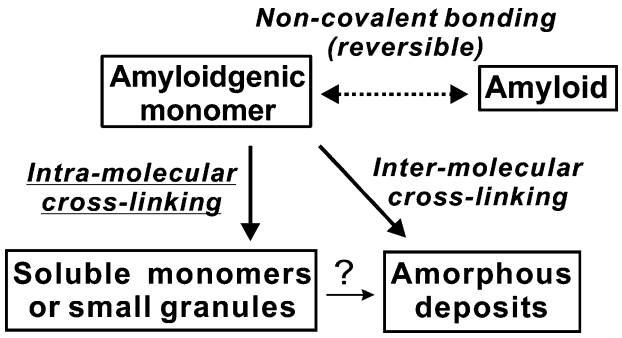

FIGURE 10: Scheme for the action of the tTGase-catalyzed cross-linking that inhibits the amyloid formation pathway.

cross-linking takes the amyloidgenic monomer away from the amyloid formation pathway (Figure 10), which inhibits the formation of amyloid and its potentially toxic precursors (*44*). Furthermore, it makes the protein molecules remain in the monomeric state or the soluble granular state (Figure 10). The protein fraction preserved in the solution phase can be more easily removed by a fluid flow or simple proteolytic systems than the phase-separated deposits. Even when it grows in size to precipitate (a thin arrow labeled with "?" in Figure 10), the deposit of amorphous type might be benign as compared to the amyloid or its precursors. The small amorphous deposit with looser packing density may be treated efficiently by the chaperone and the ubiquitin−proteasome degradation systems (*45*−*47*). The preferential degradation of the cross-linked products is consistent with the observation that the N^{ϵ}-(γ-L-glutamyl)-L -lysine linkage content of the cerebrospinal fluid is higher for patients with neurodegenerative diseases than for normal controls (*48*, *49*). At present, however, we cannot exclude the possibility that the small aggregates trapped by the intramolecular cross-linking could be toxic. Inhibition of the amyloid formation by the intramolecular cross-linking may not always be protective against the disease progression but sometimes be an enhancer of the diseases.

It is known that the aggregates of huntingtin formed by tTGase in vitro were of nonamyloid type (*17*). It has also been reported recently that the intermolecular cross-linking of a polyglutamine-containing protein inhibits its insoluble aggregation and forms high-molecular weight soluble polymers (*50*). It is likely that the intermolecular cross-linking stabilizes amorphous aggregates or the soluble high-molecular weight polymers rather than the amyloid because of the conformational restriction of the amyloidgenic proteins forced by the intermolecular covalent linkages. This presumption, together with the present results, suggests that the intra- and the intermolecular cross-linking may cooperate with each other in blocking the in situ formation of amyloid (Figure 10). Both types of the cross-linking may even dissociate the matured amyloid by reducing the effective concentration of the amyloidgenic monomer in equilibrium with the amyloid (the arrow with a broken line in Figure 10). The tTGase-catalyzed cross-linking that alters the physical properties of the protein aggregates may act defensively against the diseases whose primary pathogen is amyloid deposit.

Concluding Remarks. The present in vitro study cast a new light on the action of the intramolecular cross-linking catalyzed by tTGase as a defensive modulator against the amyloid formation and protein aggregation. One of the critical questions to be answered is whether the action is really effective in situ or not. Another question to be addressed is whether the intramolecular cross-linked population of the amyloidgenic protein is benign or toxic. Furthermore, we did not determine the positions of the cross-linking sites of the proteins, which should be done in future to elucidate molecular details underlying the events demonstrated here. It is also interesting to extend the present study to the cases of extracellular amyloidgenic proteins such as Alzheimer's Aβ and β2-microglobulin. Better knowledge of the tTGase-mediated modulation upon protein aggregation and cell apoptotic events would disclose novel therapeutic strategies against human degenerative diseases.

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BI047722D