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Self-Assembly of Tissue Transglutaminase into Amyloid-Like Fibrils Using Physiological Concentration of Ca^{2+}

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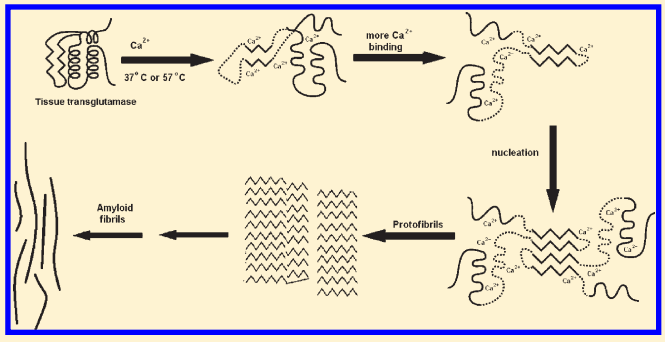
Supporting Information

ABSTRACT: Tissue transglutaminase (tTG or TG2) is a member of the transglutaminase family that catalyzes calcium dependent formation of isopeptide bonds. It has been shown that the expression of TG2 is elevated in neurodegenerative diseases such as Parkinson's, Huntington's, and Alzheimer's. We have investigated the self-assembly of TG2 *in vitro*. First, using software, hot spots, which are prone for aggregation, were identified in domain 2 of the enzyme. Next we expressed and purified recombinant TG2 and its truncated version that contains only the catalytic domain, and examined their amyloidogenic behavior in various conditions including different temperatures and pHs, in the presence of metal ions and Guanosine triphosphate (GTP).



To analyze various stages leading to TG2 fibrillation, we employed various techniques including Thioflavin T (ThT) binding assay, Congo-Red, birefringence, Circular Dichroism (CD), 8-anilino-1-naphthalene sulfonic acid (ANS) binding, Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM). Our results indicated that using low concentrations of Ca^{2+}, TG2 self-assembled into amyloid-like fibrils; this self-assembly occurred at the physiological temperature (37 °C) and at a higher temperature (57 °C). The truncated version of TG2 (domain 2) also forms amyloid-like fibrils only in the presence of Ca^{2+}. Because amyloid formation has occurred with domain 2 alone where no enzymatic activity was shown, self-cross-linking by the enzyme was ruled out as a mechanism of amyloid induction. The self-assembly of TG2 was not significant with magnesium and zinc ions, indicating specificity of the self-assembly for calcium ions. The calcium role in self-assembly of TG2 into amyloid may be extended to other proteins with similar biophysical properties to produce novel biomaterials.

INTRODUCTION

Amyloidosis relates to a group of diseases with amyloid fibrils deposition and protein aggregation. Among the well-known amyloid diseases are Alzheimer's, Parkinson's, Huntington's, and Prion disorders.[1,2] Alzheimer's is characterized by extracellular deposition of aggregated amyloid-beta (Aβ) as senile plaques and neurofibrillary tangles.[3,4] However, in diseases such as Prions and Parkinson's intercellular aggregations have been observed.[5] Amyloid formation has been seen in both folded proteins and natively disordered ones. In folded proteins, a partial denaturation can lead to misfolding and amyloid formation, whereas in a natively disordered one, a folding intermediate can be formed that could lead to formation of amyloid fibrils.[6,7] A protein that undergoes amyloidosis displays a number of physiochemical characteristics that discern the amyloid formation from amorphous aggregation; these include an increase in β-sheet content, binding to amyloid specific dyes (Thioflavin T and Congo Red), birefringence, specific β-cross fibril formation, and toxicity of both intermediates (oligomers) and the fibril.[4,8,9]

Understanding the self-assembly of proteins into fibrils has been crucial in finding inhibitors for amyloid formation.[10] Another usage of amyloid fibrils have been in nanotechnology where fibrillar proteins, because of their unique biophysical characteristics, have been utilized to produce novel biomaterials such as nanowires and nanoscalfolds.[11,12] Therefore, there is a surge in finding novel ways to produce protein self-assembled biomaterials.

Transglutaminase 2 belongs to a widespread enzyme family of transglutaminases that catalyze Ca^{2+}-dependent acyl-transfer

Received: February 25, 2011
Revised: July 5, 2011
Published: July 26, 2011

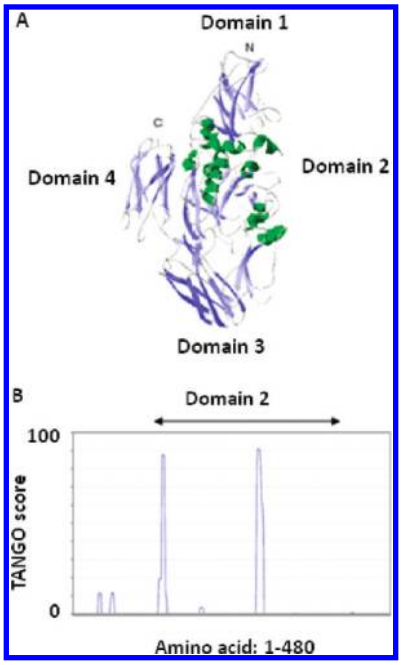


Figure 1. Schematic structure of TG2. (A) 3D structure of TG2 (1FAU). N- and C-terminals are shown by N and C. TG2 domains include an N-terminal β-sandwich domain, a catalytic core, and two C-terminal barrels. (B) Predicting amylogenic regions of tissue transglutaminase using *TANGO* software. Most of the amylogenic regions are located in the catalytic domain.

reactions, and the enzyme produces intra- and intermolecular isopeptide bonds in proteins.[3,13] The products of enzyme activity are stable, rigid, and insoluble protein complexes that show resistance to 2% sodium dodecyl sulfate (SDS) and 8 M urea or enzymatic degradations. These complexes are similar to the protein aggregates that are present in neurodegenerative diseases.[14] Moreover, elevated expressions and activity of TG2 (tissue transglutaminase) have been found in most neurodegenerative diseases;[3] Aβ, α-synuclein, tau, and huntingtin are among TG2 substrates.[15]

Recent studies have shown that isopeptidase activity increases along with increased TG2 activity in Alzheimer's disease.[3] Indeed, colocalization of TG2 occurs with aggregated Aβ in senile plaques and tau in neurofibrillary tangles.[14] TG2 employs stringent specificity in distinguishing an acyl donor, and poor specificity for the acyl-acceptor amine group.[15] The substrates for TG2 have been found, as a growing list, in a number of biological processes; they encompass cytoskeleton network organization proteins such as actin, myosin, and filamin 1,[15,16] factors involved in protein folding including heat shock proteins,[16] proteins in transport processes like clathrin heavy chain, and importin β1 subunit,[16] and matrix-associated proteins, exemplified by collagen, fibronectin, and laminin.[15,16]

TG2 has four structural domains (Figure 1A), with an N-terminal β-sandwich, catalytic core, and two β-barrels in its C-terminal. Several binding sites have been identified for TG2. Binding site for fibronectin and integrin in domain 1, interaction site for phospholipase Cδ_1 in domain 4, and Ca^{2+} and guanosine triphosphate (GTP)-binding sites in domains 2 and 3, respectively.[13] TG2 catalyzes several biological reactions in the cell at different cellular localization; in addition to its isopeptidase activity, TG2 can modulate proteins via incorporating amine and also carrying out protein deamidation. TG2 is also known to possess GTP hydrolyzing activity by which it is involved in cell signaling.[13,17] Recently, TG2 has been found to show kinase activity and disulfide isomerase activity as well.[18,19] TG2 isopeptidase catalytic activity requires millimolar concentrations of Ca^{2+} and is inhibited by guanine nucleotides. Therefore, the intracellular TG2 does not perform enzymatic activity, but rather it functions as a G-protein in the phospholipase C mediated signal transduction.[20] However, in the extracellular matrix, it performs its Ca^{2+}-dependent activity and binds tightly to both fibronectin in the extracellular matrix and integrin as a co-receptor on the cell surface; as a result, TG2 could affect cell adhesion, signaling, motility, and differentiation without dependence on its catalytic activity.[20,21]

In the present study, we have examined the amyloidogenic properties of TG2. Initially, we became more interested in studying TG2 amyloid formation because online aggregation software predicted a number of regions as "hot spots" for aggregation. Additionally, as mentioned previously, TG2 has been found to colocalize with aggregated Aβ in senile plaques and tau in neurofibrillary tangles.

In order to study TG2 in vitro, bacterially expressed TG2- and its truncated version (139−480 a.a.) were purified; the truncated version encompassed the core of the enzyme, the catalytic domain. Furthermore, various conditions were applied to study the behavior of TG2. The results indicate that TG2 self-assembled to form amyloid fibrils in the presence of a physiological concentration of Ca^{2+} at 37 °C and at an elevated temperature (57 °C). The aggregations that are formed have a number of physiochemical characteristics of amyloid and are toxic to human cells. The truncated version also forms amyloid in the presence of Ca^{2+}; this result eliminates self-cross-linking by the enzyme as a cause of amyloid formation since the truncated version lacks the isopeptidase activity and still forms amyloid fibrils in the presence of Ca^{2+}. Using metal ions to self-assemble non-amyloidogenic biomolecules into amyloids can be exploited to produce novel biomaterials for various applications in nanotechnology.

RESULTS

TG2 Contains Aggregation-Prone Regions. Although many proteins can, in some in vitro conditions, form aggregates, there are some local regions in the polypeptide sequence that contain "hot spots" for aggregation.[22] Indeed, with a great accuracy, using algorithms that consider physiochemical properties of proteins, one can predict these sensitive areas.[22−25] Therefore, the first approach was to examine the TG2 sequence for "hot spot" regions, using various software such as *TANGO*, *PASTA*, and *Waltz*. As shown in Figure 1B, using *TANGO*, TG2 appears to possess a few aggregation-prone regions belonging to domain 2, the domain responsible for catalysis. Other software such as *Amylpered* and *Waltz* also predicted "hot spots" that are mostly localized to the catalytic domain of the polypeptide (data not shown).

Ca^{2+} Self-Assembles TG2, Showing Amyloid-Specific Dye Binding. To study TG2 in vitro, the TG2 cDNA was cloned into the bacterial expression vector and the recombinant protein was purified using affinity chromatography (Supporting Information Figure S1) and described in detail in the Materials and Methods section. The activity of purified TG2 was examined and it showed activity in the presence of 10 mM Ca^{2+} as described previously.[26,27]

In order to study the aggregation properties of TG2, various in vitro conditions were examined including acidic and alkaline

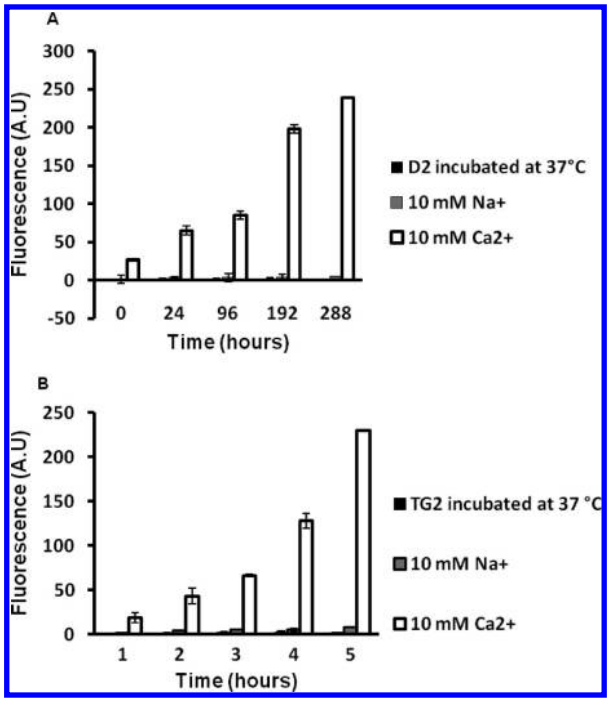


Figure 2. TG2 Thioflavin T binding assay in the presence of metal ions. The purified TG2 (0.2 mg/μL) was incubated at pH 8 (glycine 50 mM) in the presence of metal ions for several days at two different temperatures. At each time, 30 μL aliquots of samples were taken to measure for ThT binding assay. The standard deviation represents the average of three independent repeats. (A) TG2 samples were incubated at 57 °C. (B) TG2 samples were incubated at 37 °C.

environments, changes in temperature, GTP addition, and different metal ion concentrations (Figure 2A,B). The last two factors, namely, GTP and metals, were examined in addition to the generic factors such as temperature and pH change in lieu of TG2 interaction with both nucleotides and Ca^{2+}. To our surprise, only in the presence of Ca^{2+} was aggregation seen with TG2 in pH ranging from 7.0 to 8; indeed, the aggregation was visible at an elevated temperature (57 °C) by the naked eye after about four hours. As shown in Supporting Information Figure S2, the aggregation measured at 350 nm as turbidity[28] was increasing as the time of incubation increased, in the presence of Ca^{2+}. The aggregation was not detected in the presence of Na^+ or at the high temperature alone (Figure 2A,B). Moreover, in extreme pH, very acidic or very alkaline, no aggregation was detected, and in the presence of GTP (100 μM), no aggregation was detected with TG2 either (data not shown).

To better study the nature of TG2 aggregation in the presence of calcium ions, Thioflavin T (ThT) binding assay was conducted. Indeed, one of the main methods to detect amyloid formation is ThT binding assay.[29] TG2 was incubated at physiological temperature (37 °C) and elevated temperature 57 °C in the presence or the absence of metal ions, namely, Ca^{2+} and Na^+. As shown in Figure 2A,B, TG2 showed ThT binding only in the presence of calcium regardless of temperature. TG2 in the presence of Na^+ did not show any ThT binding. This result was quite interesting to us, and therefore, we decided to examine various concentrations of the metals in amyloid formation of TG2 in vitro. As shown in Supporting Information Figure S3A, various physiological concentrations of Ca^{2+} did show ThT binding, but the maximum binding was at 10 mM of Ca^{2+}.

TG2 Displays β-Sheet Enrichment and Exposure of Hydrophobic Region in the Presence of Ca^{2+}. In order to study the conformational changes that take place in the presence of Ca^{2+} ions, circular dichroism (CD) spectroscopy was performed. The enrichment of β-sheet took place only when Ca^{2+} was present; at high heat alone or in the presence of Na^+, the conversion to β-sheet secondary structure did not occur (Figure 3A,B). As shown in Figure 3C, only in the presence of Ca^{2+} there was a great increase in absorbance near 218 nm, corresponding to the β-sheet secondary structure. The enrichment of β-sheet structure is one of the hallmarks of amyloid formation that is seen with many amyloid forming proteins.[30,31]

To further investigate the mechanism of TG2 amyloid formation, 8-anilino-1-naphthalene sulfonic acid (ANS) binding was measured during TG2 amyloid formation in the presence or absence of metal ions. In the presence of various concentrations of Na^+, ANS binding did not change much (data not shown). However, in the presence of Ca^{2+}, ANS binding increased, indicating that structural changes had occurred in the presence of the divalent ion (Figure 4A). Generally, ANS binding increases as more of the hydrophobic region in protein is exposed to the solvent. Indeed, the increased ANS binding is seen when amyloid formation takes place due to exposure of proteins as it undergoes protein fibrillation.[30]

TG2 Aggregates Display Amyloid-Like Morphology and Display Birefringence. In order to visualize the morphology of TG2 aggregates, TG2 samples in the presence of Ca^{2+} were examined using transmission electron microscopy (TEM). As shown in Figure 4B and Supporting Information Figure S3B, TEM negative stains indicated that the TG2 aggregates in the presence of Ca^{2+} displayed amyloid-like fibrils. However, as a number of samples were examined, we did not find an abundance of fibrils but rather aggregates of proteins that look more like amorphous aggregations (data not shown). To obtain better results, we performed atomic force microscopy (AFM) to detect fibrils. As shown in Figure 4C, the AFM analysis confirmed the presence of amyloid-like fibrils in TG2 samples that were incubated in the presence of calcium ions. The height distribution of a good fraction of fibrils turned out to be 9 nm (Figure 4D).

One of the ultimate tests for confirming amyloid fibrils is birefringence.[32] The amyloid fibril, stained with Congo Red, when viewed under cross-polarized light generates green−blue light. As shown in Figure 5, TG2 fibrils were examined for birefringence. Under cross-polarized light, TG2 fibrils in the presence of Ca^{2+} at both 37 °C and higher temperature (57 °C) displayed the blue−green birefringence. However, TG2 that was incubated in the presence of Na^+ did not demonstrate any birefringence (Figure 5C).

Domain 2 Alone Can Self-Assemble in the Presence of Ca^{2+}; Aggregates of TG2 and Its Truncated Version are Toxic to Human Cells. As our data have indicated, TG2 is able to form amyloid in the presence of Ca^{2+}. However, TG2 has several domains (Figure 1A) that could be involved in amyloid formation. The catalytic domain of the enzyme is located in domain 2 (D2) where all the known calcium binding sites are also located. As described in Materials and Method, D2 region, spanning residues 139 to 480, was cloned and expressed in bacteria. Although the domain is much smaller than TG2, it formed inclusion bodies much greater than TG2 itself. Nevertheless, after refolding and purification of the truncated domain (Supporting Information Figure S4), D2 was used in the

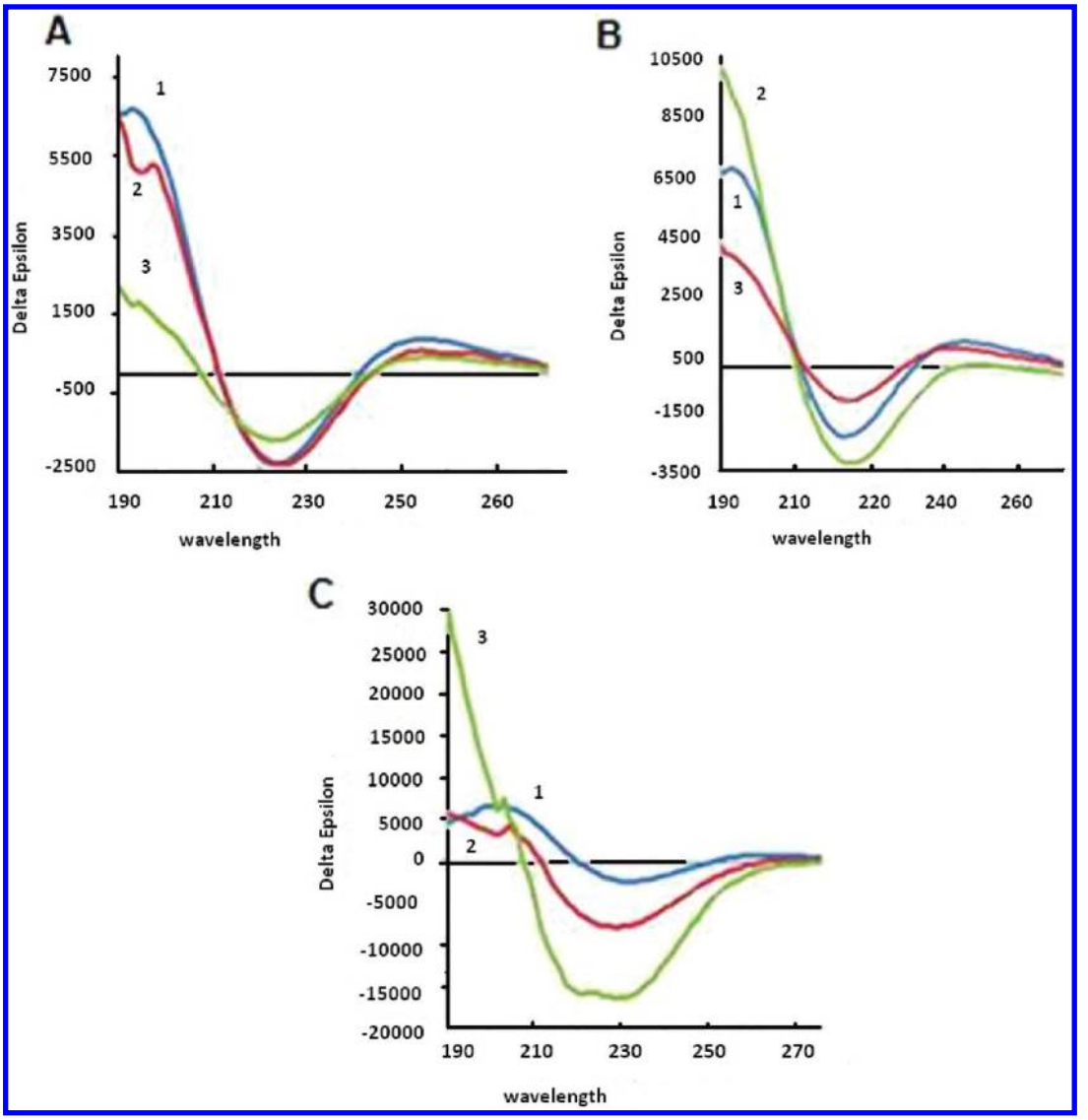


Figure 3. Near-UV CD spectra of TG2 incubated at 57 °C. Aggregation assay was done as described in the legend of Figure 2. Aliquots (0.2 mg/mL) were taken for CD analysis. (A) 1: Native TG2. 2: TG2 after 24 h. 3: TG2 after 288 h. (B) 1: Native TG2. 2: TG2 in the presence of 10 mM Na^+ after 24 h. 3: TG2 in the presence of 10 mM Na^+ after 288 h. (C) 1: Native TG2. 2: TG2 in the presence of 10 mM Ca^{2+} after 24 h. 3: TG2 in the presence of 10 mM Ca^{2+} after 288 h.

aggregation assay in the presence of Ca^{2+}. As shown in Figure 6A, D2 alone was able to form amyloid in the presence of Ca^{2+} alone. To further confirm the amyloid formation, birefringence assay was performed, and it also proved positive for the presence of amyloid (Figure 6A). Since D2, which lacks any enzymatic activity on its own, was able to form amyloids, the self-cross-linking activity of enzyme is ruled out as a primary cause of amyloid formation. As shown in Figure 6C, the TEM negative stain of D2 in the presence of Ca^{2+}, showed amyloid-like fibrils.

One of the important characteristics of amyloids relates to their toxicity. To examine the amyloid formed by TG2 and its truncated version (D2), RBC hemolysis was performed as previously reported.[33] As shown in Figure 7, the amyloids formed in the presence of Ca^{2+} from both TG2 and D2 are cytotoxic to human RBC. The cytotoxicity was at a maximum from the protein samples that were exposed to 10 mM Ca^{2+} during aggregation assay.

DISCUSSION

Tissue transglutaminase, known as "natural glue", has been shown to be involved in number of important biological reactions.[15] The enzyme through its cross-linking activity shapes ECM and regulates apoptosis, for example, cross-linking caspase 3.[34] It also regulates several proteins that are involved in apoptosis through its GTPase activity .When calcium concentration is elevated, TG2 undergoes a large conformational change to become an isopeptidase otherwise it acts as a GTPase.[35] In a few neurodegenerative diseases, the expression and the activity of the enzyme is upregulated.[3,14,15] More interestingly, TG2 has been found to be colocalized with amyloidogenic proteins including tau and Aβ.[3] In addition, it has been somewhat controversial whether the proteins that are cross-linked by TG2 are of amyloid type or simply amorphous aggregations.[36]

In this study, we have examined the self-assembly of TG2 and its catalytical domain into amyloids. Our results clearly indicate that TG2 at physiological concentrations of Ca^{2+} can form

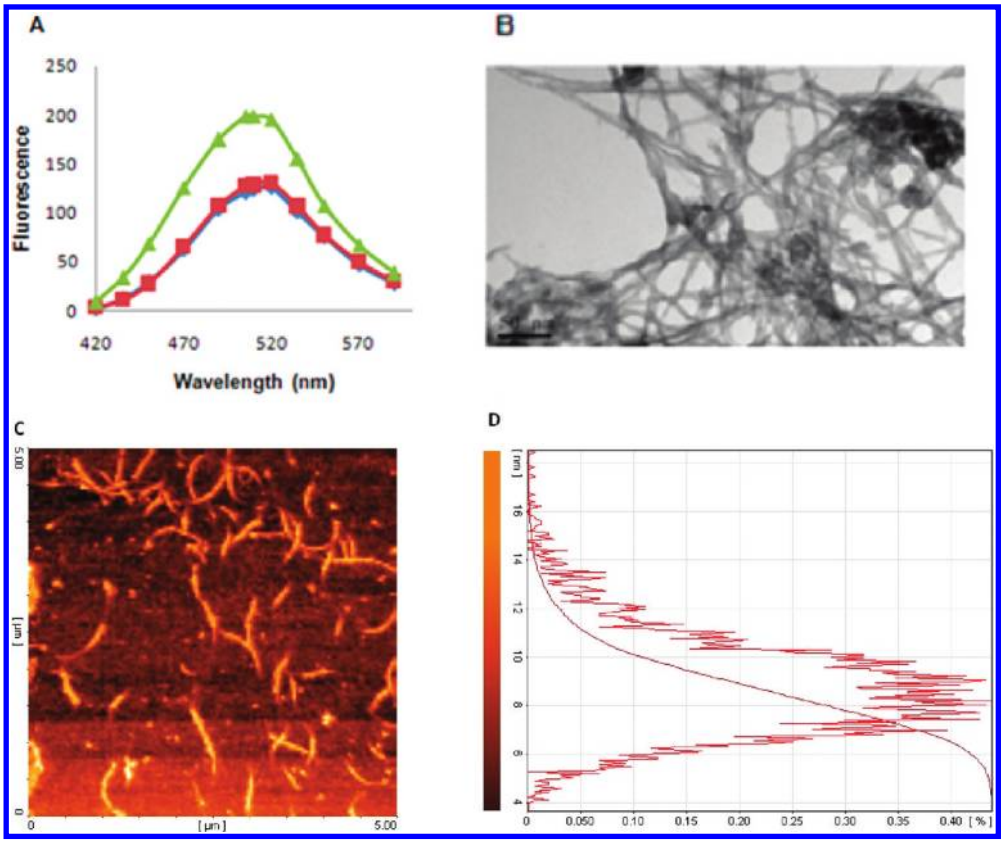


Figure 4. TG2 ANS binding assay and Morphology of TG2 aggregates using TEM and AFM. TG2 fibrillation was done as explained in the legend of Figure 2. At each time point, aliquots were removed for the reaction for ANS binding. For TEM and AFM, the samples from the last time point were used. (A) ANS binding assay of TG2 in the presence of metal ions. (♦) TG2 Native, (■) TG2 in the presence of 10 mM Na^+ after 288 h, (▲) TG2 in the presence of 10 mM Ca^{2+} after 288 h. (B) TEM of TG2 aggregates generated at 57 °C in the presence of calcium ions. The fibrillation was done as explained in the legend of Figure 2. The samples were taken at the end of fibrillation reaction (288 h). (C) AFM phase image of TG2 aggregates at 57 °C in the presence of calcium ions. The fibrillation reaction was performed as explained in the legend of Figure 4B. (D) Height distribution of fibrils detected by AFM.

protein fibrils. The catalytical domain (D2) on its own was also shown to form amyloid at physiological concentration using Ca^{2+}. Since domain 2 of TG2 did not show enzymatic activity, the cross-linking of the enzyme was ruled out as the mechanism of the amyloid formation.

The significance of our work relates to the initiation of TG2 amyloid formation by calcium ions. There have been a handful of amyloidogenic proteins where the rate of their amyloid formation is affected upon metal ion treatment: to name a few, α-synuclein in the presence of Hg^+,[31] Aβ when exposed to Ca^{2+} and Cu^{2+},[37,38] and islet amyloid poly peptide (IAPP) in the presence of Zn^{2+}.[39] However, TG2 is not considered an amyloidogenic protein, and Ca^{2+} actually initiates TG2 amyloid formation under our conditions. In other words, the Ca^{2+} interaction with TG2 makes up a unique case in which metal ions can convert a non-amylogenic protein into amyloid. Calcium ions have been used to promote the self-assembly of α-lactalbumin to nanotubes when the protein was hydrolyzed[42] but not when the protein was in its native form as in our study; other divalent metal ions could have also formed the self-assembled α-lactalbumin,[40] whereas in our study, only Ca^{2+} among the divalent ions examined could produce any significant amyloid fibrils (Figure 8).

How could Ca^{2+} bring about TG2 amyloid formation? As aforementioned, TG2 is a calcium binding protein that undergoes a conformational change upon binding to calcium.[35] The metal interaction can break the interaction between domain 2 (the catalytic domain) and domains 3 and 4, leading to movement of protein domains.[15] The exact calcium binding sites have not been identified for TG2, but up to 6 sites have been predicted to be the calcium binding sites. In recent studies, five noncanonical Ca^{2+} for TG2 have been characterized;[41] indeed, because TG2 has several negatively charged amino acids which display high surface potential, three might be more Ca^{2+} binding sites than have been characterized.[42] Interestingly, an active site mutant (C277S) of TG2, although shown to lack enzyme activity, was found to bind 6 Ca^{2+}.[41] In other words, calcium binding is not dependent on TG2 enzymatic activity.[41,43] In fact, all of the calcium binding sites are located in domain 2, the catalytic domain.

The mechanism that we propose for the amyloid formation of TG2 relates to the roles of these extra calcium sites that we like to call "cryptic" sites. In the normal situation when TG2 is performing its catalytic reaction, the cryptic sites are not calcium bound as much. However, in situations where the enzyme is exposed to a high concentration of the ions, the cryptic sites are calcium bound, and as a result, the protein undergoes a huge conformational change in the direction of β-sheet enrichment, as evidenced by the CD and ANS analyses. Once the protein is forced to unfold, misfolding could take place and amyloid formation would be the next step.

Could TG2 self-cross-linking initiate amyloid formation? Since TG2 was shown to cross-link itself,[44] there is a possibility that self-cross-linking by the enzyme could occur, leading to amyloid formation. However, as shown in Supporting Information Figure S5, the activity was sharply reduced as the incubation time increased, whereas the amyloid formation increased (Figure 2A). More importantly, domain 2 which should not have any enzyme activity (data not shown) due to the omission of several substrate recognizing domains, still gave rise to amyloid formation in the presence of Ca^{2+} (Figure 6). Therefore, the self-cross-linking of TG2 under our conditions is not the primary reason for the amyloid formation.

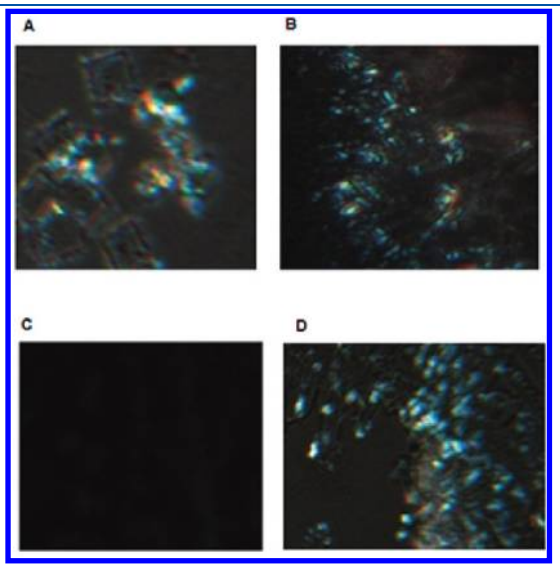


Figure 5. Detection of TG2 amyloid using Congo Red birefringence assay. Green birefringence was done as described in Materials and Methods. TG2 aggregation was set up as described in the legend of Figure 2. (A) TG2 aggregate in the presence of Ca^{2+} after 288 h incubation at 57 °C. (B) TG2 aggregate in the presence of Ca^{2+} after 288 h incubation at 37 °C. (C) TG2 in the presence of Na^{+} after 288 h incubation at 57 °C. (D) Lysozyme fibrils as positive control. Magnification 250×.

Another important question is whether the self-assembly seen by calcium could be seen with other metal ions? To answer this question, we examined two other divalent metal ions, namely, zinc and magnesium. None of the ions examined showed any significant self-assembly with TG2, indicating the specificity of calcium ions in the self-assembly (Figure 8). There is also one more line of evidence in the literature that supports our findings. Factor XIII which is actually a member of the transglutaminase family has been predicted to have an amyloidogenic region, and the protein was shown to form amyloid at 37 °C.[45]

What would be the consequences of TG2 amyloid induced formation in extra cellular matrix (ECM)? There exist many calcium binding proteins in ECM such as cadherin, BM-40, and fibrillin that contain several epidermal growth factor (EGF) modules.[42,45−47] For those proteins, Ca^{2+} has been proposed to help in structure stabilization, active site optimization, and formation of supramolecular assembly.[47] Since the concentration of Ca^{2+} in ECM at some sites reaches up to 26 mM,[48] there is a possibility that the calcium binding proteins of ECM undergo the same amyloid formation as TG2 does under our assay conditions. Indeed, in this sense the calcium binding proteins can behave more like functional amyloids that have been seen with a growing number of proteins and peptides.[48] Therefore, it could be

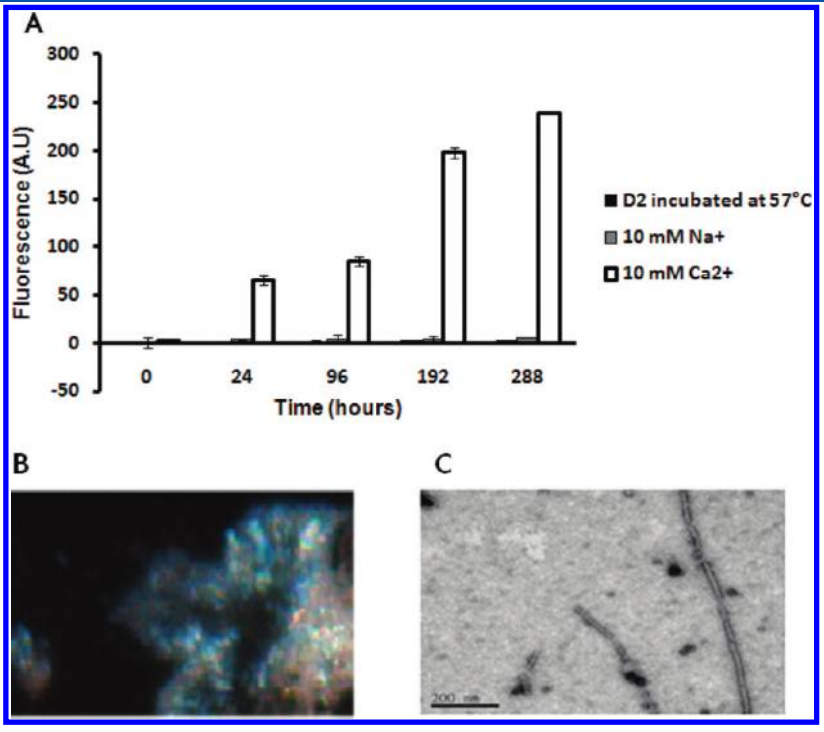


Figure 6. ThT binding assay, birefringence assay, and TEM for domain 2 (D2) at 57 °C. The aggregation assay was set up as described in the legend of Figure 2 except that Domain 2 (D2) was used as the source of the protein. (A) ThT binding assay for D2 aggregate. The reaction was performed as described. (B) Congo Red birefringence assay for D2 sample that was incubated in the presence of Ca^{2+} after 288 h. (C) Electron micrographs obtained of in the presence of Ca^{2+} after 288 h incubation at 57 °C.

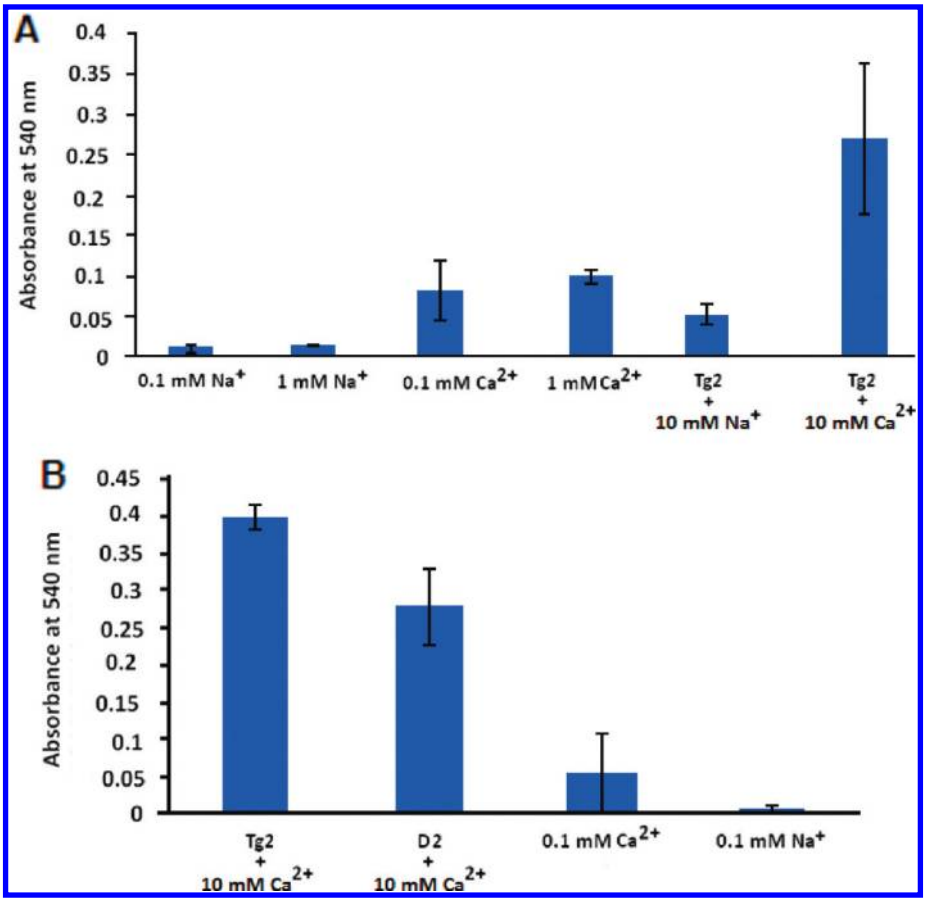


Figure 7. Cytotoxicity assay for TG2 and D2 aggregates. One milliliter of isolated RBC was incubated 40 min at 37 °C in the presence of (A) various concentrations of metal ions alone or TG2 samples generated in the presence of 10 mM Ca^{+2} or Na^{+} after 288 h. (B) first column (from left to right), TG2 incubated at 37 °C in the presence of Ca^{2+} after 288 h; second column, D2 incubated at 57 °C in the presence of Ca^{2+} after 288 h; third column, 0.1 mM Ca^{2+}; fourth column, 0.1 mM Na^{+} and after that the absorbance of supernatant were measured at 540 nm.

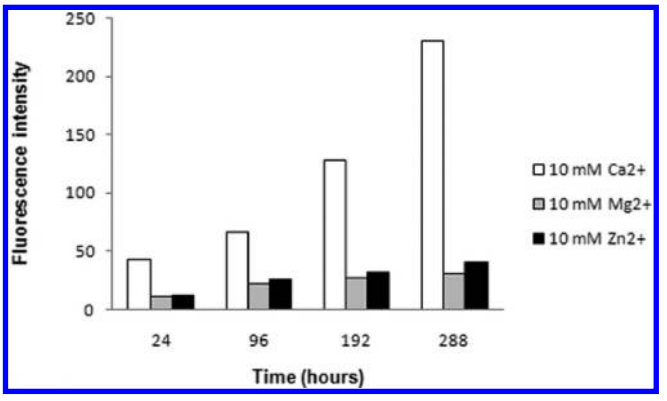


Figure 8. ThT binding assay for TG2 in the presence of different metal ions. The purified TG2 (0.2 mg/μL) was incubated at pH 8 (Glycine 50 mM) in the presence of different divalent ions for several days. All samples were incubated at 57 °C. Aliquots were taken at various time points of ThT binding assay as described in the Materials and Methods.

interesting to check other calcium binding proteins that reside in ECM for the amyloid formation.

Another important issue is whether the TG2 amyloid formation can be seen in vivo. Indeed, the above question should be investigated in mammalian organisms or tissue cultures expressing TG2. As we were preparing the present manuscript for submission, a very recent paper reported that the maize transglutaminase can form amyloid-like aggregates in plant cells.[49] Therefore, there is a good possibility that TG2 can form amyloids in a mammalian system.

At the moment, we are examining the effects of other divalent metal ions in the amyloid formation of TG2. In addition, we are planning to better localize the aggregation sites by changing a few calcium binding sites in domain 2 by site-directed mutagenesis, and also investigate the TG2 amyloid formation in the mammalian expression system.

CONCLUSION

In our present studies, we showed that TG2 self-assembled in the presence of a physiological concentration of calcium ions. Using a number of approaches, we show that the self-assembled TG2, induced by Ca^{2+}, was indeed amyloid. Domain 2 of TG2 alone was shown to form amyloid as well, indicating that the TG2 amyloid formation was not due to self-cross-linking of TG2 by its enzymatic activity. The self-assembly, induced by Ca^{2+}, occurred at both low and relatively high temperature, revealing the sole role of calcium in the amyloid formation. Our results can be important on several fronts; this is the first time that a non-amyloidogenic TG2 has been shown to form amyloid in the presence of metal ions. Furthermore, our finding that calcium ions specifically can induce self-assembly of TG2 into fibrils may

suggest the existence of other proteins with similar chemicophysical properties as TG2 that can be self-assembled into novel biomaterials using the metal ions.

MATERIALS AND METHODS

Prediction of Amylogenic Regions for Aggregation. To predict the propensity of TG2 for aggregation, we used online software such as *Tango*, *Waltz*, and *Amylpered*.[50] These programs are specialized for the detection of regions within a protein which are prone to protein aggregation.

Cloning, Expression, and Refolding Purification of TG2 and Its Domain2 (D2). The following primers were used to subclone the TG2 cDNA (a gift from Prof. Daniel Aeschlimann) into pET28a bacterial expression vector: 5′AACCTCGAGTTAGGCGGGGCCAATGATGACATTCC 3′ (*Eco*R I cloning site, underlined) and 5′CCGGAATTCATGGCCGAGGAGCTGGTCTTAGAGAGG3′ (*Xho* I cloning site, underlined). For cloning Domain2, spanning nucleotides 420−1440, corresponding to 139−480 amino acids of TG2, the following primers were used: 5′ATTCCATATGAACGCCTGGTGCCCAG3′ (*Nde* I cloning site, underlined) and 5′ACGCGTCGACTTAGCCCACACGGATC3′ (*Sal*I cloning site, underlined). The PCR reactions were performed as recommended by Sambrook and Russell using LA-Taq (Takara, Japan).The amplified products were purified and digested using the restriction enzymes and subsequently ligated and transformed into bacteria. The correct clones were chosen for DNA sequencing (Macrogen, Korea). The best condition for expression was at 1 mM Isopropyl β-D-1-thiogalactopyranoside IPTG (Fermentase, Lithuania), 4 mM lactose (Merck, Germany), and the cells were incubated at 22 °C for 14 h as previously reported.[51,52] 10−20% of TG2 expressed protein was found in the soluble fraction, whereas domain 2 (D2) was found entirely in inclusion bodies. The proteins that were found in inclusion bodies were refolded using urea gradient as described elsewhere.[52] Purifications of the recombinant proteins were done using Ni-sepharose (Sigma, Germany). The purified proteins were dialyzed overnight in 50 mM glycine buffer, pH 8, and 1 mM 2-mercaptoethanol (Merck, Germany). The protein solution was concentrated using stirred ultrafiltration cell (Amicon, USA), and protein concentrations were determined using TCA-Lowery assay. For refolded TG2, the enzymatic activity, and for refolded domain 2, Native PAGE, was performed to substantiate their folding.

Aggregation Assay. To set up the aggregation assay, purified TG2 or D2 (0.2 mg/mL) was used in 50 mM glycine buffer pH 2−12, at 37 and 57 °C In order to investigate the effect of metal ions, we used calcium chloride and sodium chloride (total concentrations were 2 and 10 mM for NaCl and 1, 5, and 10 mM for $CaCl_2$). To investigate the effect of crowding agent on TG2 aggregation, polyethylene glycols (Merck, Germany) (PEG) 4000 and 6000 (150 mg/mL) were utilized. Sodium azide 0.05% was added to the samples and incubated at 37 °C.

Turbidity and Thioflavin T Binding Assay. To measure turbidity, 30 μL aliquots of each sample were diluted up to 500 μL with 50 mM glycine buffer pH 8 and absorbance was measured at 350 nm.

Thioflavin T (Sigma, Germany) stock solution was prepared by dissolving ThT in sodium phosphate (NaH_2PO_4 50 mM, Na_2HPO_4 50 mM, pH 8) and filtered through a 0.2 μm filter. ThT (20 μM) was added to 30 μL of the sample and ThT fluorescence intensity was measured at an excitation at 440 nm and emission at 490 nm (excitation and emission bandwidths were kept within 5 and 10 nm).

Hemolytic Assay for Cytotoxicity. The hemolytic assay was described elsewhere.[33] Briefly, red blood cells were isolated from fresh blood and washed three times with phosphate buffered saline (PBS) (pH 7.4). Red blood cells (RBCs) were incubated with samples at 37 °C for 40 min and then centrifuged at 1000 *g* for 10 min. Absorbance of supernatant measured at 540 nm is an indicator of the extent of hemolysis.

Circular Dichroism (CD). CD spectra of samples were obtained in the far-UV region (190−260 nm) using a circular dichroism spectrometer model 215. The sample concentrations were 0.2 mg/mL and 0.1 cm; quartz cell was utilized.

ANS Fluorescence Assay. Stocks of ANS (Sigma, Germany) fluorescence assay were prepared by dissolving ANS in absolute ethanol and filtered using a 0.2 μm filter. Final concentrations of the samples were 0.05 μM mixed with ANS final concentration 50 μM. ANS was excited at 390 nm and emission was measured from 420 to 600 nm. Bandwidths of excitation and emission were 5 and 10 nm, respectively.

Congo Red Birefringence Assay. A staining solution (5 mL) was made using ethanol/distilled de-ionized (DDI) water in ratio 4:1 and to the staining solution a saturating amount of sodium chloride was added. The solution was stirred to dissolve sodium chloride and filtered away the excess sodium chloride. A saturating amount of Congo red (Merck, Germany) was added to the solution, stirred, and filtered to get the working solution. The protein samples (10 μL) were added to a microslide and allowed to dry. An excess amount of staining solution was added to the dried samples. After removing extra dye, the samples were visualized under a polarized microscope.

Transmission Electronic Microscopy (TEM). Samples (0.2 mg/mL) were diluted 20 times and stained with 1% (w/v) uranyl acetate. The grids were subsequently washed with DDI, examined, and photographed at an accelerating voltage of 80 kV.

Atomic Force Microscopy (AFM). Ten microliter samples were removed from fibrillation reaction, deposited onto very smooth glass, and air-dried for 5 min. All the images were obtained using a DualScope DS95 AFM Scanner (DME - Danish Micro Engineering A/S, Copenhagen, Denmark) under ambient conditions. Images were obtained in ac mode. Each image was taken with 256 data sample points in each sweep line. The ac probe had a rectangular cantilever with a very sharp silicon conical tip with a typical resonance frequency of 200 kHz, a force constant of 40 N/m, and a normal tip radius of less than 10 nm. Several images were obtained from separate locations to ensure reproducibility. All the images were analyzed using the accompanied software of the AFM microscope to calculate height distribution.

ASSOCIATED CONTENT

Supporting Information. TG2 Protein purification and its activity, TG2 turbidity assay, ThT binding assay for TG2 and TEM image of TG2, D2 protein purification, cross-linking activity of TG2 during aggregation assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

We would like to thank Mohsen Janmaleki for his assistance in AFM imaging and analysis.

REFERENCES

(1) Stefani, M; Dobson, C. M. *J. Mol. Med.* **2003**, *81*, 678–99.
(2) Spie, J. D.; Cohen, A. S. *J. Struct. Biol.* **2000**, *130*, 88–98.
(3) Wilhelmus, M. M. M.; van Dam, A.-M.; Drukarch, B. *Eur. J. Pharmacol.* **2008**, *585*, 467–472.
(4) Williams, T. L; Day, I. J.; Serpell, L. C. *Langmuir* **2010**, *26*, 17260–8.
(5) Cookson, M. R. *PLoS Biol.* **2004**, *2*, e401.

(6) Vendruscolo, M.; Zurdo, J.; MacPhee, C. E.; Dobson, C. M. *Philos. Trans. R. Soc. London, Ser. A: Math. Phys. Eng. Sci.* **2003**, *361*, 1205–22.
(7) Dobson, C. M. *Philos. Trans. R. Soc. London, Ser. B: Biol. Sci.* **2001**, *356*, 133–45.
(8) Biancalana, M.; Makabe, K.; Koide, A.; Koide, S. *J. Mol. Biol.* **2009**, *385*, 1052–63.
(9) Li, H., Rahimi, F., Sinha, S., Maiti, P., Bitan, G., and Murakami, K. In *Encyclopedia of Analytical Chemistry*, Myers, R. A., Ed.; John Wiley & Sons Ltd: New York, 2009; p 1.
(10) Gazit, E. *Prion* **2007**, *1*, 32–5.
(11) Reches, M.; Gazit, E. *Science* **2003**, *300*, 625–7.
(12) Pilkington, S. M.; Roberts, S. J.; Meade, S. J.; Gerrard, J. *A. Biotechnol. Prog.* **2010**, *26*, 93–100.
(13) Fesus, L.; Piacentini, M. *Trends Biochem. Sci.* **2002**, *27*, 534–9.
(14) Wang, D. S.; Dickson, D. W.; Malter, J. S. *Int. J. Clin. Exp. Pathol.* **2008**, *1*, 5–18.
(15) Griffin, M.; Casadio, R.; Bergamini, C. M. *Biochem. J.* **2002**, *368*, 377–96.
(16) Orrù, S.; Caputo, I.; D'Amato, A.; Ruoppolo, M.; Esposito, C. *J. Biol. Chem.* **2003**, *278*, 31766–73.
(17) Hwang, K. C.; Gray, C. D.; Sivasubramanian, N.; Im, M. J. *J. Biol. Chem.* **1995**, *270*, 27058–62.
(18) Mishra, S.; Murphy, L. J. *J. Biol. Chem.* **2004**, *279*, 23863–8.
(19) Hasegawa, G.; Suwa, M.; Ichikawa, Y.; Ohtsuka, T.; Kumagai, S.; et al. *Biochem. J.* **2003**, *373*, 793–803.
(20) Nakaoka, H.; Perez, D. M.; Baek, K. J.; Das, T.; Husain, A.; Misono, K.; et al. *Science* **1994**, *264*, 1593–6.
(21) Zemskov, E. A.; Janiak, A.; Hang, J.; Waghray, A.; Belkin, A. *M. Front. Biosci.* **2006**, *11*, 1057–76.
(22) Sánchez de Groot, N; Pallarés, I.; Avilés, F. X.; Vendrell, J.; Ventura, S. *BMC Struct Biol.* **2005**, *5*, 18.
(23) Oliveberg, M. *Nat. Methods* **2010**, *7*, 187–8.
(24) Galzitskaya, O. V.; Garbuzynskiy, S. O.; Lobanov, M. Y. *PLoS Comput. Biol.* **2006**, *2*, e177.
(25) Pawar, A. P.; Dubay, K. F.; Zurdo, J.; Chiti, F.; Vendruscolo, M.; et al. *J. Mol. Biol.* **2005**, *350*, 379–92.
(26) Jeitner, T. M.; Fuchsbauer, H. L.; Blass, J. P.; Cooper, A. *J. Anal. Biochem.* **2001**, *292*, 198–206.
(27) Wu, Y. W.; Tsai, Y. H. *J. Biomol. Screen.* **2006**, *11*, 836–43.
(28) Hall, D.; Minton, A. P. *Anal. Biochem.* **2005**, *345*, 198–213.
(29) Nilsson, M. R. *Methods* **2004**, *34*, 151–60.
(30) Kalhor, H. R.; Kamizi, M.; Akbari, J.; Heydari, A. *Biomacromolecules* **2009**, *10*, 2468–75.
(31) Bartolini, M.; Bertucci, C.; Bolognesi, M. L.; Cavalli, A.; Melchiorre, C.; et al. *ChemBioChem* **2007**, *8*, 2152–61.
(32) Westermark, P.; Benson, M. D.; Buxbaum, J. N.; Cohen, A. S.; Frangione, B.; et al. *Amyloid* **2005**, *12*, 1–4.
(33) He, J.; Xing, Y. F.; Huang, B.; Zhang, Y. Z.; Zeng, C. M. *J. Agric. Food Chem.* **2009**, *57*, 11391–6.
(34) Tucholski, J.; Johnson, G. V. *J. Neurochem.* **2002**, *81*, 780–91.
(35) Pinkas, D. M.; Strop, P.; Brunger, A. T.; Khosla, C. *PLoS Biol.* **2007**, *5*, e327.
(36) Karpuj, M. V.; Garren, H.; Slunt, H.; Price, D. L.; Gusella, J. et al. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 7388–93.
(37) Isaacs, A. M.; Senn, D. B.; Yuan, M.; Shine, J. P.; Yankner, B. A. *J. Biol. Chem.* **2006**, *281*, 27916–23.
(38) Kowalik-Jankowska, T.; Ruta-Dolejsz, M.; Wisniewska, K.; Lankiewicz, L.; Kozlowski, H. *Environ. Health Perspect.* **2002**, *110*, 869–70.
(39) Brender, J. R.; Hartman, K.; Nanga, R. P.; Popovych, N.; de la Salud Bea, R.; et al. *J. Am. Chem. Soc.* **2010**, *132*, 8973–83.
(40) Graveland-Bikker, J. F.; Ipsen, R.; Otte, J.; de Kruif, C. *G. Langmuir* **2004**, *20*, 6841–6.
(41) Király, R.; Csosz, E.; Kurtán, T.; Antus, S.; Szigeti, K.; et al. *FEBS J.* **2009**, *276*, 7083–96.
(42) Hynes, R. O. *Science* **2009**, *326*, 1216–9.
(43) Ahvazi, B.; Boeshans, K. M.; Idler, W.; Baxa, U.; Steinert, P. M. *J. Biol. Chem.* **2003**, *278*, 23834–41.
(44) Barsigian, C.; Stern, A. M.; Martinez, J. *J. Biol. Chem.* **1991**, *266*, 22501–9.
(45) Kallberg, Y.; Gustafsson, M.; Persson, B.; Thyberg, J.; Johansson, J. *J. Biol. Chem.* **2001**, *276*, 12945–50.
(46) Maurer, P.; Hohenester, E.; Engel, J. *Curr. Opin. Cell. Biol.* **1996**, *8*, 609–17.
(47) Stenflo, J.; Stenberg, Y.; Muranyi, A. *Biochim. Biophys. Acta* **2000**, *1477*, 51–63.
(48) Fowler, D. M.; Koulov, A. V.; Alory-Jost, C.; Marks, M. S.; Balch, W. E.; et al. *PLoS Biol.* **2006**, *4*, 6.
(49) Villar-Piqué, A.; Sabaté, R.; Lopera, O.; Gibert, J.; Torne, J. M.; Santos, M.; Ventura, S. *PLoS One* **2010**, *10*, 13625.
(50) TANGO (http://tango.crg.es/)/ Waltz (http://waltz.vub.ac.be/)/ Amylpered (http://biophysics.biol.uoa.gr/AMYLPRED/input.html).
(51) Ambrus, A.; Fésüs, L. *Prep. Biochem. Biotechnol.* **2001**, *31*, 59–70.
(52) Liu, X. Q.; Yang, X. Q.; Xie, F. H.; Song, L. Y.; Zhang, G. Q.; et al. *Protein Expr. Purif.* **2007**, *51*, 179–86.