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The amyloid fibrils of the constant domain of immunoglobulin light chain

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ABSTRACT

Light chain-associated (AL) amyloidosis is characterized by dominant fibril deposition of the variable domain (VL) of an immunoglobulin light chain, and thus its constant domain (CL) has been considered not to be amyloidogenic. We examined the in vitro fibril formation of the isolated CL in comparison with β_2-microglobulin (β2-m), an immunoglobulin domain-like amyloidogenic protein responsible for dialysis-related amyloidosis. Two methods useful for β2-m at neutral pH also induced amyloid fibrils of CL, which were monitored by thioflavin-T binding and electron microscopy (EM). These results suggest that CL plays an important role, more than previously assumed, in the development of AL-amyloidosis.

1. Introduction

The deposition of amyloid fibrils in extra- and intracellular spaces is associated with various amyloidoses, including Alzheimer's disease (AD), Parkinson's, and Huntington's diseases and dialysis-related amyloidosis [1–4]. Among them, amyloidogenic light chain (AL) –amyloidosis is one of the most extensively studied diseases with respect to the molecular mechanism of the formation of amyloid fibrils [5–8]. The precursor protein of AL-amyloidosis is a monoclonal immunoglobulin light chain, which in its native form has a tertiary structure rich in β-sheets. AL-amyloidosis is produced by the neoplastic expansion of plasma B-cells that secrete large amounts of monoclonal light chain that aggregate as truncated proteins in extracellular amyloid deposits, causing organ dysfunction and death [9]. Mutation induced decrease in the stability of variable domain (VL) has been suspected to be the main factor in the increased amyloidogenicity [5,6].

The main component of the fibrils is VL and a varying part of the constant domain (CL) and, rarely, full-length light chains, suggesting that VL is amyloidogenic and that an impaired proteolytic cleavage occurs after deposition because of its structural flexibility. However, several cases have been reported that amyloid deposits dominantly comprised of CL [10,11]. It has been suggested that, while CL is usually susceptible to proteolysis after or during deposition, high susceptibility of VL and core fibril formation can lead to the dominant deposition of CL [10]. Although these results suggest that CL potentially contributes to the development of AL-amyloidosis, the roles of CL in the formation of amyloid fibrils and in AL-amyloidosis have not been clearly understood. In fact, no in vitro fibril formation of the isolated CL has been reported and instead, the isolated CL has been used as a model protein with no amyloidogenicity [12].

Here, we focus on a high similarity of CL with β_2-microglobulin (β2-m), a highly amyloidogenic protein responsible for dialysis-related amyloidosis [4]. Among various immunoglobulin domains, CL exhibits the highest homology to β2-m with 21% identity and 42% similarity in amino acid sequence on the basis of the sequence alignment generated by CulstalW2 [13] (Supplementary Fig. S1). In this study, we compared the in vitro fibril formation of CL of κ-type human light chain with that of β2-m. We used two methods established to be useful for inducing amyloid fibrils of β2-m at neutral pH: i.e., ultrasonication-induced fibril formation [14,15], and agitation-induced fibril formation [16,17]. Ultrasonication-induced fibrillation was performed in the presence of 0.5 mM sodium

Abbreviations: β2-m, β_2-microglobulin; CL, constant domain of the immunoglobulin light chain; EM, electron microscopy; Gdn-HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; TFE, trifluoroethanol; ThT, thioflavin-T

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dodecyl sulfate (SDS) [18], or 20% (v/v) trifluoroethanol (TFE) [19] at 37 °C. Previously, for agitation-induced fibril formation, we employed mild agitation at 37 °C in the presence of a high concentration of NaCl to induce aggregates followed by heating up the solution to 90 °C [17]. In this study, we found that only drastic agitation at 37 °C in the presence of NaCl or $NaClO_4$ is sufficient to induce fibrils. Under these conditions, CL formed fibrils prominently, suggesting that CL has a strong potential to form fibrils and to contribute markedly to the development of AL-amyloidosis.

2. Materials and methods

2.1. Materials

Recombinant human β2-m was expressed with an *Escherichia coli* expression system and purified as described previously [20]. Isolated DNA encoding human type κ CL domain was cloned into an *E. coli* expression vector, pAED4 [21] and expressed in BL21 (DE3) pLysS cells (Agilent Technologies, La Jolla, CA). The produced protein was accumulated in inclusion bodies as reported previously [22]. The expressed CL domain consists of 106 residues, the sequence of which is shown in Supplementary Fig. S1. Inclusion body from 1.6 L culture was resuspended in 3 ml of 100 mM Tris–HCl (pH 8.5) and solubilized by the addition of 0.6 g of solid guanidine hydrochloride (Gdn-HCl). The crude CL was purified over a Superdex-75 column (GE Healthcare, Waukesha, WI) pre-equilibrated with 6 M urea in 10 mM Tris–HCl (pH 8.5), and then reduced with 20 mM dithiothreitol (DTT), followed by dialysis against 10 mM Tris–HCl (pH 8.5) with 6 M urea at 4 °C for air oxidation for >48 h. The oxidized sample was dialyzed against 10 mM Na-acetate (pH 4.7) and purified using a Resource S cation-exchange column (GE Healthcare) equilibrated with 10 mM Na-acetate (pH 4.7). Concentrations of monomeric CL and β2-m were determined using a molar extinction coefficient of 10 950 M^{-1} cm^{-1} and 19 300 M^{-1} cm^{-1} at 280 nm, respectively, which were determined on the basis of amino acid composition [23].

2.2. Ultrasonication-induced formation of fibrils

A protein solution was ultrasonicated by using a water bath-type ultrasonic transmitter with temperature controller (ELESTEIN SP070-PG-M, Elekon, Tokyo, Japan) [14]. The frequency of the instrument was 17–20 kHz and the power output was set to deliver a maximum of 350 W. Reaction mixtures were ultrasonicated from three directions (i.e. from two sides and from the bottom) for 1 min and then incubated for 9 min without sonication, a process that was repeated during incubation at 37 °C. The protein concentration was 25 μM, and 0.5 mM SDS or 20% (v/v) TFE was added to 50 mM sodium phosphate buffer at pH 7.0 containing 100 mM NaCl.

2.3. Agitation-induced formation of fibrils

Thirty micromolar of CL or β2-m was incubated at 37 °C in a flat-bottomed insert-capped plastic tube after drastic stirring for 24 h using a Teflon-coated microstirring bar. NaCl, Na_2SO_4 or $NaClO_4$ was added to 50 mM sodium phosphate buffer (pH 7.0).

2.4. ThT assay and light scattering

The formation of amyloid fibrils was monitored by fluorescence analysis with thioflavin-T (ThT). An aliquot of 5 μl was taken from the reaction tube and mixed with 1.0 ml of 5 μM ThT in 50 mM sodium glycine buffer (pH 8.5). The wavelengths for excitation and emission were 445 nm and 485 nm, respectively [24]. For light scattering, the wavelengths were both set at 350 nm. ThT fluorescence and light scattering were measured using an F-4500 spectrofluorometer (Hitachi, Tokyo, Japan) at room temperature.

2.5. EM and CD measurements

Electron microscopy (EM) images were acquired using a JEOL 100CX transmission microscope (JEOL, Tokyo, Japan) with an acceleration voltage of 80 kV. The samples were diluted 10-fold by pure water or placed directly on carbon-coated copper grids for 1 min and negatively stained with 2% (w/v) uranyl acetate for 1 min as described previously [16,17,25]. All CD spectra were measured on a J-720 spectropolarimeter (Jasco, Tokyo, Japan) at 37 °C after 30 min of preincubation. The pathlength of the cell used for far-UV and near-UV CD measurements was 0.1 cm and 1.0 cm, respectively. The protein concentration for far-UV and near-UV CD measurements was 0.1 mg/ml and 0.5 mg/ml, respectively.

3. Results

3.1. Ultrasonication-induced formation of fibrils

Ultrasonication is usually used to induce breakage of preformed fibrils, thus producing seeds for the propagation of fibrils [26] or prion particles [27,28]. We previously established that ultrasonication is also useful for inducing fibrils in solution of β2-m monomers at acidic or neutral pH [14,15]. At neutral pH, native β2-m resists to fibril formation in vitro, although the fibrils are formed at neutral pH in patients [4]. Yamamoto et al. [18] established that the presence of 0.5 mM SDS induces fibril formation probably by facilitating partial denaturation and aggregate formation of β2-m. Alternatively, Yamamoto et al. [19] showed that 20% (v/v) TFE is useful for inducing amyloid fibrils of β2-m. Combining these promoting effects was shown to be effective for inducing amyloid fibrils [14].

In this study, we examined the ultrasonication-induced formation of fibrils of CL in comparison with that of β2-m at pH 7.0 and 37 °C in the presence of 0.5 mM SDS or 20% (v/v) TFE. As for β2-m, ThT fluorescence increased after 4–6 h of lag time in the presence of 0.5 mM SDS or 20% (v/v) TFE (Fig. 1B), and this result is consistent with previous studies [14]. In the case of CL, ThT fluorescence increased after 3–4 h of lag time in the presence of 0.5 mM SDS or 20% (v/v) TFE (Fig. 1A). In the case of β2-m, the final ThT fluorescence intensities were 3-fold higher. The formation of amyloid fibrils was confirmed by EM, revealing that the morphology varies depending on the solvent conditions. In the presence of 0.5 mM SDS, the short fibrils with diameters of 10–20 nm were formed from both CL and β2-m (Fig. 2A and B). The formation of short fibrils is expected because the ultrasonication tends to break the fibrils making relatively homogenous short fibrils [14,15]. In 20% (v/v) TFE, CL formed crumbs of fibrils with diameters of 20–40 nm and β2-m formed short rod-like fibrils with diameters of 10–25 nm (Fig. 2C and D).

To address the effects of SDS and TFE in promoting the formation of fibrils, secondary and tertiary structures of intact proteins under the respective conditions were monitored by far-UV and near-UV CD spectra, respectively. In the presence of 0.5 mM SDS, the far-UV CD spectra of both CL and β2-m were similar to those of the native conformations (Fig. 3A and C). On the other hand, the near-UV CD spectra were different between the two (Fig. 3B and D), indicating that, although 0.5 mM SDS did not change the secondary structure of CL and β2-m drastically, the effects on the tertiary structure of CL is most likely to be more extensive than that of β2-m. As for 20% (v/v) TFE, for both of CL and β2-m, the far-UV CD spectra showed destruction of the native structure and

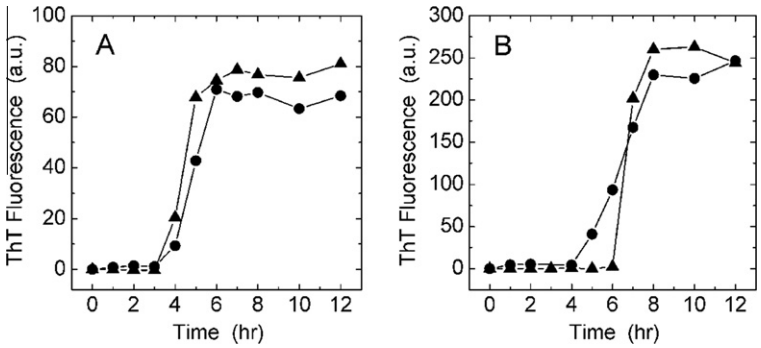

Fig. 1. Ultrasonication-induced formation of fibrils monitored by ThT fluorescence. Time course of the fibril extension of CL (A) or β2-m (B), in the presence of 0.5 mM SDS (●), or 20% (v/v) TFE (▲). CL or β2-m at 25 μM was ultrasonicated at pH 7.0 and 37 °C.

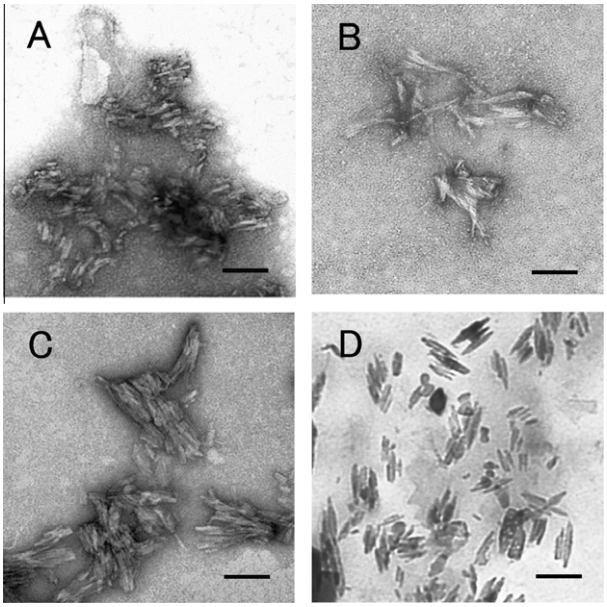

Fig. 2. EM images of ultrasonication-induced fibrils. The amyloid fibrils formed in the presence of 0.5 mM SDS from CL (A) or β2-m (B). The fibrils formed in the presence of 20% (v/v) TFE from CL (C) or β2-m (D). The samples were ultrasonicated for 12 h at 37 °C. The magnification was 30 000 and scale bars represent 100 nm.

formation of partial α-helical structure (Fig. 3A and C). The near-UV CD spectra of CL and β2-m indicated the destruction of the native structure (Fig. 3B and D), consistent with the stabilization of the α-helix-dominated conformation in the presence of TFE [29,30]. These results suggest that CL is more sensitive to solvent-dependent denaturation than β2-m. In addition, thermal unfolding curves of CL and β2-m monitored by CD clearly indicated that β2-m is more stable than CL (Fig. 3C inset). The Gdn-HCl-induced unfolding curves of CL [31] and β2-m [32] were reported previously, and are also consistent with these results.

3.2. Agitation-induced formation of fibrils

β2-m fibrils were also formed by mild agitation and heating in the presence of 1.0 M NaCl at pH 7.0 [17]. In this study, we found that both β2-m and CL form amyloid fibrils by only drastic agitation at 37 °C. We examined the agitation-induced formation of fibrils in the presence of NaCl, Na_2SO_4, and $NaClO_4$. Fig. 4A and B shows ThT fluorescence after 24 h of agitation at various salt concentrations for CL and β2-m, respectively. As for CL, in the presence of $NaClO_4$ or NaCl, ThT fluorescence increased with an increase in the salt concentration and a marked increase was observed at 1.0 M $NaClO_4$. On the other hand, Na_2SO_4 showed a bell-shaped pattern, suggesting that high concentrations of Na_2SO_4 induce aggregation in non-amyloid form, because sulfate ions increase the hydrophobic interactions among protein molecules. The Na_2SO_4-induced aggregation might be similar to those formed upon ammonium sulfate precipitation during protein purification. As for β2-m, $NaClO_4$ and NaCl exhibited a promoting effect on fibril formation monitored by ThT fluorescence. As for β2-m, $NaClO_4$, Na_2SO_4 and NaCl exhibited a promoting effect on fibril formation monitored by ThT fluorescence (Fig. 4D). While the dependence on salt concentration was not clear for $NaClO_4$ and Na_2SO_4, NaCl showed a marked effect at 1.0 M (Fig. 4B).

To understand the role of electrostatic interactions and the effect of ionic co-solutes, especially anions, on amyloid formation, Raman et al. [33] investigated the effects of various salts on the fibril growth of β2-m at pH 2.5. Under acidic conditions, where the monomeric β2-m is disordered, various salts exhibited characteristic bell-shaped dependence with an optimal concentration, where fibril growth is favored. The presence of salts leads to an increase in hydrophobicity of the protein as reported for 8-anilinonaphthalene-1-sulfonic acid, indicating that the anion binding leads to the necessary electrostatic and hydrophobic balance critical for amyloid formation. However, high concentrations of salts tilt the balance towards high hydrophobicity, leading to the partition of protein to amorphous aggregates. The order of anions based on the optimal concentration was $SO_4^{2-} > ClO_4^- > I^- > Cl^-$, consistent with the order of their electroselectivity series [34], suggesting that preferential anion binding, rather than general ionic strength effect, plays an important role in the amyloid fibril growth. Klement et al. [35] examined the effects of salt ions on the aggregation propensity of Alzheimer's Aβ(1–40) and on the structure of the dissolved and of the fibrillar peptide. The salt-dependent aggregation was different from that of β2-m and depended on direct interactions between ions and peptides, correlating with ion-induced change of the surface tension. These results suggest the presence of various ways in which salts affect aggregation and fibril formation of proteins. On the other hand, at pH 7.0, where both β2-m and CL form the native structure, destabilizing the native structure is likely more important than the counterion binding, thus chaotropic effects of $NaClO_4$ is strongest, leading to a series of effectiveness: $NaClO_4$ > NaCl > Na_2SO_4.

Fig. 4C and D shows the time course of ThT fluorescence in the presence of 0.5 M of salt for CL and β2-m, respectively. As for CL in NaCl and Na_2SO_4, the fluorescence increased after about 3 h of lag time and saturated within 12–14 h. With $NaClO_4$, the fluorescence increased faster and saturated within 3 h. In contrast, as for β2-m, ThT fluorescence increased slowly except for Na_2SO_4 and the fluorescence values were smaller than those of CL, indicating that

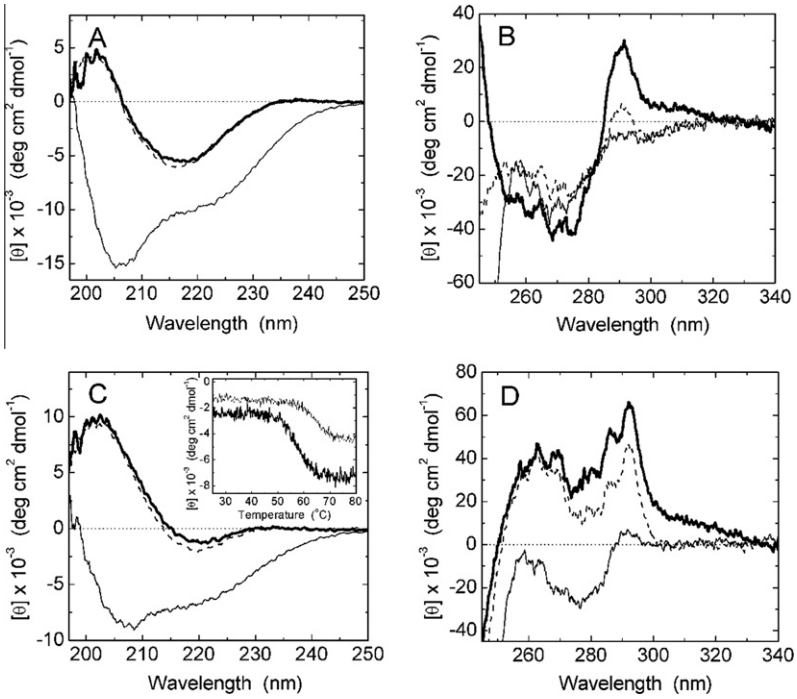

Fig. 3. Far-UV and near-UV CD spectra of intact proteins before ultrasonication treatment. Far-UV CD spectra of intact CL (A) and β2-m (C). Near-UV CD spectra of intact CL (B) and β2-m (D). Samples were incubated at 37 °C without ultrasonication in sodium phosphate buffer containing 100 mM NaCl at pH 7.0 (bold line), in the presence of 0.5 mM SDS (dashed line) or 20% (v/v) TFE (thin line). The thermal unfolding curves of CL (bold line) and β2-m (thin line) monitored at 210 nm and 220 nm, respectively, are presented in the inset of (C).

agitation induced the formation of CL fibrils more effectively than that of β2-m.

The morphology of the fibrils was examined by EM. In the presence of 0.5 M NaCl, CL formed loosely twisted straight fibrils with diameters of 5-35 nm, which were bundles of thin fibrils (Fig. 5A). β2-m formed bundles of long fibrils with diameters of 5–10 nm (Fig. 5B). In the presence of 0.5 M $NaClO_4$, both CL and β2-m formed straight fibrils with diameters of 5–30 nm (Fig. 5E and F). On the other hand, the morphology of fibrils was distinct in 0.5 M Na_2SO_4. CL formed crumbs of short fibrils and β2-m formed

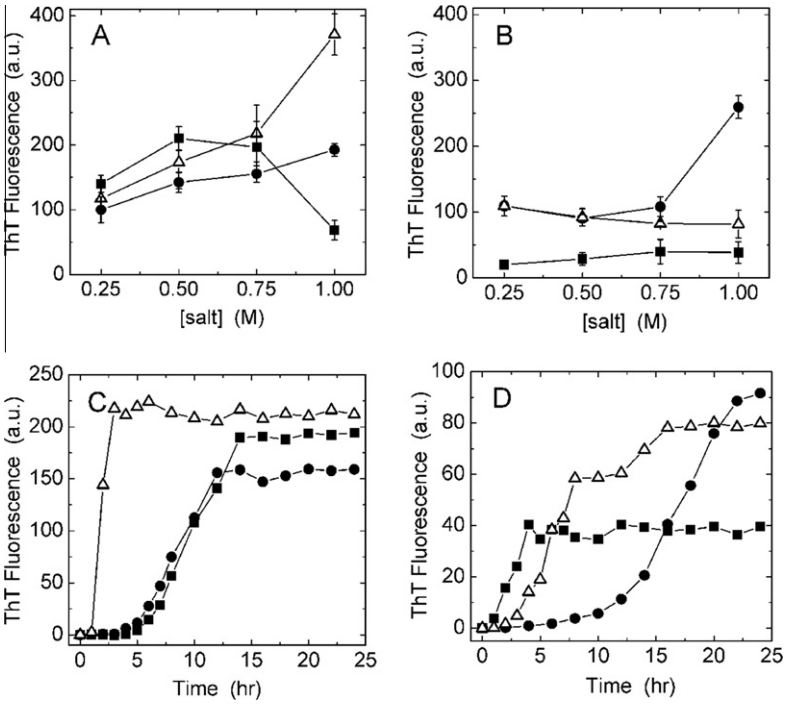

Fig. 4. Agitation-induced formation of fibrils monitored by ThT fluorescence. The increases of ThT fluorescence of CL (A) and β2-m (B) after 24 h of agitation at 37 °C were compared at various concentrations of salts (in the presence of NaCl (●), Na_2SO_4 (■), and $NaClO_4$ (△)). The measurements were performed three times and the error bars depict the standard deviation of the mean. The time course of the fibril extension of CL (C) and β2-m (D), in the presence of 0.5 M NaCl (●), Na_2SO_4 (■), and $NaClO_4$ (△).

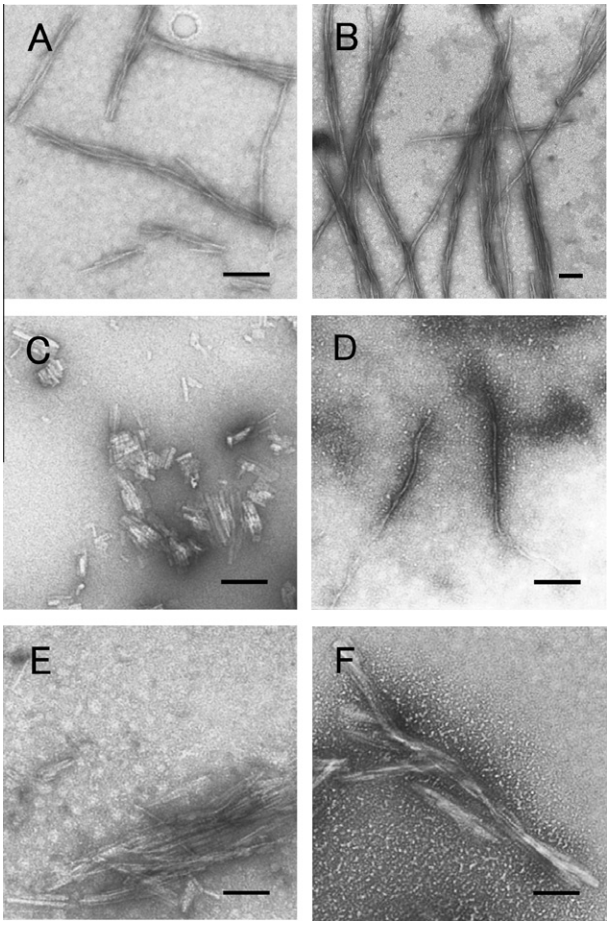

Fig. 5. EM images of the agitation-induced fibrils. The amyloid fibrils formed in the presence of 0.5 M NaCl from CL (A) or β2-m (B). The fibrils formed in the presence of 0.5 M Na_2SO_4 from CL (C) or β2-m (D). The fibrils formed in the presence of 0.5 M $NaClO_4$ from CL (E) or β2-m (F). The samples were agitated for 24 h at 37 °C. The magnification was 30 000; scale bars represent 100 nm.

protofibrils with diameter of 5 nm (Fig. 5C and D), which might be related with the low intensity of ThT fluorescence.

4. Discussion

AL-amyloidosis is usually characterized by the amyloid deposits of VL domain. The cases that amyloid deposits dominantly comprised of CL [10,11] suggested that, although the CL can also form amyloid fibrils, they are usually exposed to solvent and susceptible to proteolysis after or during deposition [10]. However, no in vitro fibril formation of the isolated CL has been reported.

We succeeded in forming amyloid fibrils of CL under the same conditions as used for the fibrils of β2-m at neutral pH. To form the fibrils, we used two methods established to be useful for β2-m. One is ultrasonication-induced fibril formation and the other is agitation-induced fibril formation in the presence of salts. As for the agitation-induced fibril formation, we used a method further developed on the basis of the salt/heat-induced fibril formation established by Sasahara et al. [17], which needs 24 h of mild agitation at 37 °C and subsequent heating to 90 °C. We found that both CL and β2-m form amyloid fibrils only by drastic agitation at 37 °C in the presence of 0.25–1.0 M of NaCl and $NaClO_4$, and incubation. However, fibril formation in Na_2SO_4 was less effective than in other salts probably because high concentrations of Na_2SO_4 induce precipitation of the proteins in their native conformation as expected for the ammonium sulfate precipitation of proteins during purification.

One of the most important aims of this study is comparing the amyloidogenicity of CL and β2-m. As for the ultrasonication-induced fibril formation monitored by ThT assay, β2-m forms fibrils more effectively than CL. On the other hand, as for the high salt-induced fibril formation, the situation seems opposite. Although ThT binding is an important probe for the quantification of the amount of fibrils, it is likely that ThT affinity and fluorescence intensity vary depending on the morphology of fibrils. This is understandable considering that ThT binds to the specifically aligned aromatic groups on the fibrils [36–38]. Moreover, the number of aromatic residues in the protein sequence directly affects ThT fluorescence and has to be taken into account in comparative studies. CL contains 1 Trp, 4 Tyr and 4 Phe residues, while β2-m contains 2 Trp, 6 Tyr and 5 Phe residues. This might contribute to the lower ThT fluorescence intensity of CL fibrils in the case of ultrasonication induced fibril formation, despite the seemingly identical morphology with β2-m fibrils (Fig. 2). Importantly, when monitored by EM, typical types of fibrils were observed abundantly for both ultrasonication and salt-induced fibrils under various conditions. Thus, we can conclude that, under adequate conditions, even isolated CL domain exhibits an amyloidogenicity as high as that of β2-m.

Recently, Feige et al. [12,39] compared the folding and amyloidogenicity of CL and β2-m. They showed that the refolding intermediate of CL exhibits the basic β-barrel topology, yet some strands are distorted. Two short strand-connecting helices conserved in constant antibody domains (Supplementary Fig. S1) assume their completely native structure already in the intermediate, thus providing a scaffold for adjacent strands. By transplanting these helical elements of CL into β2-m, they drastically reduced the amyloidogenicity of β2-m. They propose that minor structural differences in an intermediate can shape the folding landscape decisively to favor either folding or misfolding. However, the significant amyloidogenicity of CL observed here prompts us to reconsider the role of CL in AL-amyloidosis. Although amyloidogenicity under particular conditions was not high, it is possible that amyloidogenicity increases under another conditions, as observed for the different amyloidogenicity of CL and β2-m under distinct conditions. Alternatively, it is still possible that apparent major role of VL in AL-amyloidosis simply results from the susceptibility to protease digestion after fibril formation of the entire light chain.

Finally, it has been proposed that the amyloid formation is a generic property of the polypeptide chains [1–4] suggesting that under proper conditions most of the proteins might form amyloid fibrils. More recently, amyloidogenic segments are proposed to exist in almost all proteins, although not all proteins form amyloid [40]. The prominent amyloidogenicity of CL as revealed here argues the importance of reexamination of amyloidogenicity of other immunoglobulin domains.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.06.019.

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