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Mechanism of Lysophosphatidic Acid-Induced Amyloid Fibril Formation of β_2-Microglobulin *in Vitro* under Physiological Conditions†

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ABSTRACT: β_2-microglobulin- (β2m-) based fibril deposition is the key symptom in dialysis-related amyloidosis. β2m readily forms amyloid fibrils *in vitro* at pH 2.5. However, it is not well understood which factors promote this process *in vivo*, because β2m cannot polymerize at neutral pH without additives even at elevated concentration. Here we show that lysophosphatidic acid (LPA), an *in vivo* occurring lysophospholipid mediator, promotes amyloid formation under physiological conditions through a complex mechanism. In the presence of LPA, at and above its critical micelle concentration, native β2m became sensitive to limited proteolytic digestion, indicating increased conformational flexibility. Isothermal titration calorimetry indicates that β2m exhibits high affinity for LPA. Fluorescence and CD spectroscopy, as well as calorimetry, showed that LPA destabilizes the structure of monomeric β2m inducing a partially unfolded form. This intermediate is capable of fibril extension in a nucleation-dependent manner. Our findings also indicate that the molecular organization of fibrils formed under physiological conditions differs from that of fibrils formed at pH 2.5. Fibrils grown in the presence of LPA depolymerize very slowly in the absence of LPA; moreover, LPA stabilizes the fibrils even below its critical micelle concentration. Neither the amyloidogenic nor the fibril-stabilizing effects of LPA were mimicked by its structural and functional lysophospholipid analogues, showing its selectivity. On the basis of our findings and the observed increase in blood LPA levels in dialysis patients, we suggest that the interaction of LPA with β2m might contribute to the pathomechanism of dialysis-related amyloidosis.

The development of amyloid disorders in the human body, such as Alzheimer's and Parkinson's diseases, or dialysis-related amyloidosis have been shown to be accompanied by an elevated concentration of the pathogenic protein (*1*, *2*). Although increased protein concentration seems to be a prerequisite for amyloid formation, it is not sufficient by itself to explain the progress and symptoms of the diseases. A great interest has emerged recently in searching for specific molecules that may affect the malicious aggregation and amyloid propagation of disease-related proteins.

β_2-Microglobulin (β2m)[1] is the light chain of the type I major histocompatibility complex. After dissociating from the complex, it appears in monomeric form in blood. In patients undergoing long-term hemodialysis, an elevated level of β2m was observed due to insufficient clearance of the protein, accompanied by amyloid deposition in the osteoarticular system (*3*). β2m is a 99-residue protein which has a native, monomeric structure at pH 7.5, composed of two β-sheets connected by a disulfide bridge. At pH 2.5, β2m is acid denatured and readily forms amyloid fibrils in a nucleation-dependent manner *in vitro* (*4*−*8*). However, β2m forms no fibrils at neutral pH, even at protein concentrations 1000-fold higher than the physiological one (*9*), indicating that specific factors facilitate the amyloid formation of β2m *in vivo*. Numerous studies reported the possible role of glycosaminoglycans (*10*−*13*), apolipoprotein E (*14*), serum amyloid protein (*15*), and collagen (*13*, *16*−*18*) in fibrillogenesis of β2m. *In vitro*, amyloid formation of β2m at neutral pH occurs in the presence of

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[1]Abbreviations: β2m, β_2-microglobulin; CMC, critical micelle concentration; DRA, dialysis-related amyloidosis; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; SDS, sodium dodecyl sulfate; S1P, sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; ThT, thioflavin T.

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copper ions (*19*−*21*) or requires the addition of seeds (e.g., seeds prepared from fibrils preformed at pH 2.5 and stabilized by heparin or other additives) in the presence of trifluoroethanol or SDS (*12*, *22*).

The nucleation-dependent amyloid formation of β2m in the presence of SDS has been characterized in detail (*7*, *22*, *23*). Very recently, Ookoshi et al. have shown that some lysophospholipids, especially lysophosphatidic acid (LPA), also induce the formation of β2m amyloid fibrils at neutral pH (*24*). LPA is an amphiphilic molecule with a negatively charged headgroup and a hydrophobic tail of one alkyl chain; thus it is structurally similar to SDS. It is present in numerous biological fluids including blood (*25*). LPA is a widespread lysophospholipid mediator with a plethora of biological effects. In cellular physiology, LPA promotes proliferation, survival, motility, and differentiation of cells (*26*). Furthermore, it plays important roles in the cardiovascular and reproductive biology and in the development of the nervous system. On the other hand, LPA takes part in different pathological processes such as cancer and atherosclerosis (*27*, *28*).

In this work, we studied the mechanism of LPA-induced amyloid fibril formation of β2m at physiological pH, temperature, and ionic strength. We found that micellar LPA destabilizes the structure of monomeric β2m, promotes seed-dependent amyloid formation, and stabilizes preformed amyloid fibrils. Physiologically relevant and structurally related lysophospholipids showed no significant effects, underlining the specificity of the β2m−LPA interaction.

EXPERIMENTAL PROCEDURES

Materials. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL), and other chemicals were from Sigma-Aldrich. Recombinant β2m was expressed in *Escherichia coli* and purified as described previously (*21*, *29*).

Polymerization Reaction under Physiological Conditions. Seeds were prepared by sonication of 100 μL aliquots from amyloid fibril solutions using a Microson sonicator (Misonix, Farmingdale, NY) at intensity level 2, with 50 1-s pulses on ice. In the first polymerization cycle, seeds grown at pH 2.5 were used at a concentration of 20 μg/mL for inducing fibril extension in the presence of LPA. For subsequent cycles, seeds were obtained from the previous cycle. Seeds obtained from the fifth cycle were used for the experiments. The mixtures were incubated at 37 °C without agitation for 1−7 days. Fibril growth was monitored by thioflavin T (ThT) fluorescence. The reaction mixture contained 0.1 mg/mL β2m, 10 μg/mL seeds, 50 mM sodium phosphate or HEPES buffer (pH 7.5), 100 mM NaCl, and various concentrations of LPA in the range of 50−700 μM. Experiments in the presence of lipids other than LPA were carried out at 500 μM lipid concentration. The required quantities of lipids were dried from 10 mM methanolic stock solutions into test tubes and redissolved in the reaction mixture by vigorous vortexing and sonication. S1P was directly diluted into the reaction buffer from 10 mM methanolic stock solution.

Thioflavin T Fluorescence Measurements. Five microliter aliquots were taken from the samples and mixed with 1.0 mL of 5 μM ThT in 50 mM glycine and 100 mM NaCl, pH 8.5 (*30*). Fluorescence intensity of ThT was monitored at 485 nm with excitation at 445 nm at 25 °C, using a Fluoromax instrument (SPEX Industries, Edison, NJ). Excitation and emission bandwidths were set to 5 and 10 nm, respectively.

Circular Dichroism Spectroscopy. CD measurements were carried out on a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). A cylindrical water-jacketed quartz cell of 0.1 cm path length was used. CD spectra in the range of 200−240 nm were recorded at a scanning speed of 10 nm/min in continuous mode using a bandwidth of 1 nm at a temperature of 37 °C. The secondary structure composition of β2m monomers and amyloid fibrils was examined by the Dicroprot (*31*) and GlobalWorks (Olis Inc., Bogart, GA) software packages.

Limited Proteolysis by Chymotrypsin. β2m (0.2 mg/mL) was incubated in either 50 mM sodium phosphate or 50 mM HEPES buffer, pH 7.5, containing 100 mM NaCl at 37 °C in the absence or presence of various contrentrations of LPA and LPC (25−300 μM). After 1 h preincubation, bovine α-chymotrypsin (Worthington Biochemical Co., Lakewood, NJ) was added to the reaction mixture at 1:100 enzyme to substrate molar ratio. Proteolytic digestion was allowed to proceed for 30 min at 37 °C and stopped by the addition of 2× reducing SDS sample buffer and heating at 95 °C for 5 min. Proteolytic fragments were separated by 16.5% SDS−PAGE in Tris/Tricine buffer (*32*, *33*). Gels were fixed in 50% methanol and 9% acetic acid for 30 min and stained with Brilliant Blue G in 5% methanol and 7% acetic acid overnight.

Electron Microscopy. Samples were applied directly to 300-mesh Formvar/carbon-coated copper grids, allowed to adhere for 1 min, and stained for 40 s with 1% uranyl acetate. Grids were examined by a JEOL 100 CX II transmission electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 60 kV.

Differential Scanning Calorimetry. Experiments were carried out on a VP-DSC instrument (MicroCal Inc., MA). Protein solutions (0.2 mg/mL) were examined in the presence of various concentrations of different lipids in 50 mM sodium phosphate and 100 mM NaCl, pH 7.5. Thermal unfolding profiles were analyzed by Origin 7.0 for DSC (MicroCal Inc., MA).

Heat-induced Dissociation of β2m Fibrils at pH 2.5. Amyloid fibrils were grown at pH 2.5 in 50 mM sodium citrate buffer and 100 mM NaCl. Samples contained 0.1 mg/mL fibrils in the same buffer and 500 μM different lipids or SDS. Twenty microliter aliquots were incubated in 200 μL thin-walled PCR tubes, for better heat transfer, at various temperatures in the 25−99 °C range using a Hybaid PCR Sprint thermal cycler (Mandel Scientific, Canada). After 5 min, the reactions were stopped by placing the tubes on ice. Fibril dissociation was monitored by the remaining ThT fluorescence.

Depolymerization of β2m Fibrils at pH 7.5. The tenth generation of fibrils were grown as described above in the presence of 300 μM LPA. The lipid was removed by repeated cycles of centrifugation and washing with LPA-free buffer. Fibrils were resuspended at a concentration of 0.05 mg/mL in buffer containing various concentrations of LPA in the range of 5−300 μM and in LPA-free buffer for reference and incubated at 37 °C for up to 2 months. The amount of fibrils was monitored by ThT fluorescence.

Isothermal Titration Calorimetry. Critical micelle concentrations (CMC) of LPA and LPC were determined by isothermal titration calorimetry (ITC) using a VP-ITC instrument (MicroCal Inc., MA) at 37 °C in a buffer containing 50 mM sodium phosphate and 100 mM NaCl at pH 7.5, similarly to Li et al. (*34*). Micellar solutions (2−10 μL) of 1 mM LPA or 500 μM LPC were injected repeatedly into the calorimeter cell containing only the buffer solution at the beginning. Reaction heat was corrected

with appropriate buffer−buffer measurements. The CMC value was defined as the lipid concentration in the cell that corresponds to a heat effect less than 5% of the initial maximal value.

Interaction of native, monomeric β2m with LPA was also examined by ITC. Ten microliter aliquots of β2m at a concentration of 470 μM in a buffer of 50 mM sodium phosphate and 100 mM NaCl, pH 7.5, were injected into the ITC cell containing 300 μM LPA in the same buffer. A time interval of 1 h was applied between injections. Titration curves were analyzed, and the binding energetics were characterized using the Origin software provided by MicroCal.

RESULTS

At neutral pH, β2m is not capable of amyloid formation without additives even in the presence of fibril seeds (*14*, *22*, *23*, *35*). Moreover, fibrils formed at low pH will dissociate at neutral pH (*14*, *23*). We studied the effect of LPA on the fibril formation of β2m at pH 7.5 in either sodium phosphate or HEPES buffers at 37 °C. LPA is an acronym for a set of glycerophospholipids in which the apolar hydrocarbon chain can differ in length and saturation. Additionally, it can be coupled to the glycerol backbone by an ester, ether, or vinyl−ether bond. Naturally occurring LPA is usually a mixture of these varieties. In the majority of studies, oleoyl-LPA evokes a similar response to the natural mixture, so it is considered as the model compound for the biological actions of LPA. Thus, we used oleoyl-LPA throughout the present study to investigate the biologically relevant effects of LPA on the structure and amyloidogenicity of β2m. Moreover, in the comprehensive work of Ookoshi et al. (*24*), oleoyl LPA was proven to be one of the lysophospholipids that effectively induced elongation of seeds prepared from fibrils grown in the presence of SDS at neutral pH.

Fibril Formation in the Presence of LPA under Physiological Conditions. We studied the effect of LPA on the fibril formation of β2m at pH 7.5 by ThT fluorescence (Figure 1A). β2m monomers were incubated in the presence of 500 μM oleoyllysophosphatidic acid (LPA), oleoyllysophosphatidylcholine (LPC), sphingosine 1-phosphate (S1P), sphingosylphosphorylcholine (SPC), or sodium dodecyl sulfate (SDS) for 24 h (see the structures in Figure 1B). Polymerization was induced by seeds prepared from amyloid fibrils grown at pH 2.5. In the presence of LPA and SDS, a significant increase in ThT fluorescence intensity was observed, indicating the aggregation of β2m. The other lipids exerted no detectable increase in fluorescence intensity, meaning they do not induce polymerization. However, the initial high level of ThT fluorescence first decreased fast in the presence of all lipids, indicating a depolymerization of the seeds. In the presence of SDS this effect was much less pronounced (Figure 1A inset), suggesting a different stabilizing effect of SDS on amyloid fibrils.

Fibril Formation as a Function of LPA Concentration and Incubation Time. β2m monomers were incubated in the presence of fibril seeds and various concentrations of LPA at pH 7.5. For these experiments seeds were prepared from the fifth generation of fibrils grown at pH 7.5 in the presence of 300 μM LPA. We found that the kinetics of fibril growth depended on LPA concentration (Figure 2). Fibril growth was unambigously detectable at 100 μM LPA concentration. The growth rate and ThT fluorescence intensity exhibited maximal values around 300 μM LPA. Above this concentration both the growth rate and maximum fluorescence values decreased. Utilizing seeds grown at different LPA concentrations led to similar results, exhibiting growth maxima around 300 μM LPA (data not shown).

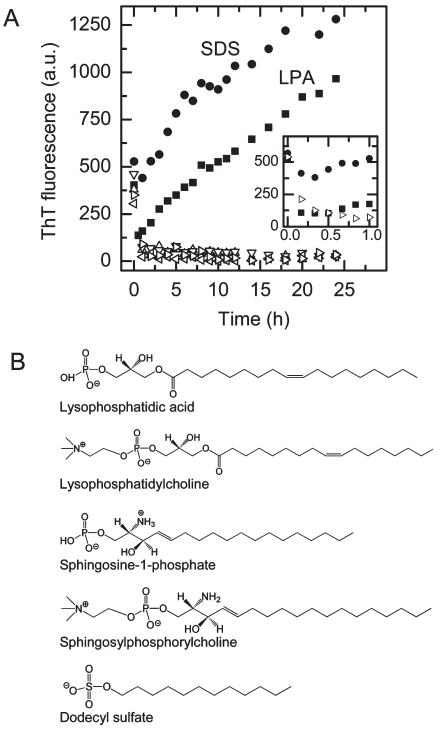

FIGURE 1: Effect of LPA and related analogues on the fibril formation of β2m at pH 7.5. Fibril growth was monitored by ThT fluorescence. (A) β2m monomers at a concentration of 0.1 mg/mL were incubated in the presence of 500 μM LPA (■), LPC (△), S1P (left-pointing triangle), SPC (▽), and SDS (●) in 50 mM sodium phosphate and 100 mM NaCl, pH 7.5 at 37 °C. Samples were inoculated with 20 μg/mL fibril seeds prepared from fibrils formed at pH 2.5. Reaction mixtures were incubated for 24 h at 37 °C. For comparison, ThT fluorescence of β2m was examined in the absence of additives under the same conditions (right-pointing triangle). The first hour of the progress curves for SDS, LPA, and the lipid-free control are depicted in the inset of panel A. Typical experiments of five determinations are shown. The experimental errors were less than 10%. (B) Structures of the additives used in the experiments (LPA, LPC, S1P, SPC, and SDS).

Fibril Morphology Studied by Electron Microscopy. The morphology of β2m aggregates in samples exhibiting high ThT fluorescence intensity was studied by electron microscopy. Figure 3 shows electron micrographs of fibrils formed in a seeded reaction in the presence of 300 μM LPA at pH 7.5. The fibrils consisted of several protofibrils and showed twisted morphology. Generally, the thickness of fibrils was proven to be in the range of 20−50 nm at pH 7.5, with a few exceptions showing 8−10 nm diameter. For fibrils formed at pH 2.5, a smaller thickness of 5−15 nm has been reported (*8*).

Effect of LPA on the Secondary Structure of Native β2m Studied by CD Spectroscopy. Figure 4 shows alterations in the secondary structure of native, monomeric β2m upon interaction with various concentrations of LPA at pH 7.5. Far-UV CD spectra were recorded every 5 min for 2 h, and a final spectrum was recorded after 24 h. Above 150 μM LPA concentration, significant changes in the CD spectra were observed, indicating destabilization of the native conformation with a decrease in the extent of ordered secondary structure (Figure 4D−H). The spectral changes were fully completed within 1−2 h with amplitudes depending on LPA concentration. Measurements at 24 h incubation showed no additional changes compared to the 2 h

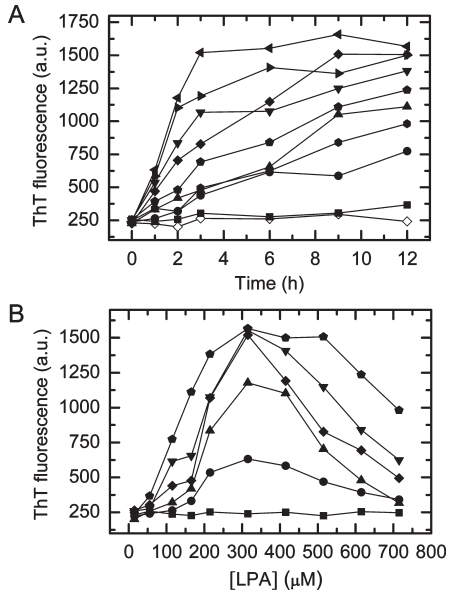

FIGURE 2: Amyloid fibril formation of β2m as a function of LPA concentration at physiological pH. β2m monomer solutions containing different concentrations of LPA were inoculated with seeds prepared from the fifth generation of fibrils formed at pH 7.5 in the presence of 300 μM LPA. The samples contained 0.1 mg/mL β2m monomer and 10 μg/mL seeds in 50 mM sodium phosphate and 100 mM NaCl, pH 7.5, and were incubated at 37 °C. As a reference, a sample without lipid was also examined. Fibril growth was monitored by ThT fluorescence. The data set of amyloid fibril formation is shown as a function of incubation time (A) in the presence of 0 μM (◇), 50 μM (■), 100 μM (●), 150 μM (▲), 200 μM (▼), 300 μM (left-pointing triangle), 400 μM (right-pointing triangle), 500 μM (◆), 600 μM (solid ⬠), and 700 μM LPA (⬢), as well as of LPA concentration (B) at 0 h (■), 1 h (●), 2 h (▲), 3 h (♦), 6 h (▼), and 12 h (solid ⬠). A typical experiment of three determinations is shown. The experimental error was less than 10%.

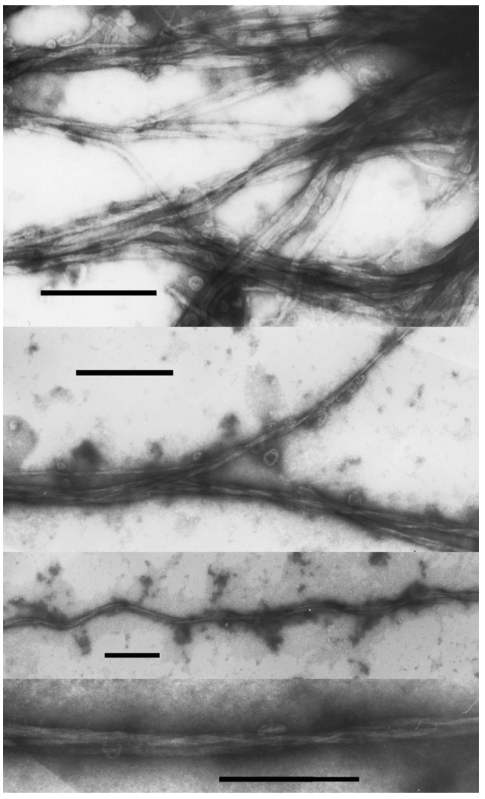

FIGURE 3: Electron microscopy of β2m fibrils formed in the presence of LPA. Amyloid fibrils were formed by incubation of 0.1 mg/mL β2m with 300 μM LPA in 50 mM sodium phosphate and 100 mM NaCl, pH 7.5, for 24 h at 37 °C. Mature fibrils were stained with 1% uranyl acetate and examined by transmission electron microscopy. Scale bars represent 300 nm. The typical morphology is a bundle of numerous fibrils twisted around one another.

time points (Figure 4A−H, dotted lines). In the presence of 500 μM LPC, no change in the secondary structure was detected similarly to the lipid-free control (Figure 4A,B). Figure 4I illustrates the kinetics of β2m−LPA interaction as a function of LPA concentration by monitoring the ellipticity change at 205 nm. The rate of conformational change is accelerated at higher LPA concentrations. At 700 μM LPA, the reaction is completed within 30 min. We estimated the secondary structure content of the different forms of monomeric β2m using the algorithms of SELCON3 (*36*), VARSLC (*37*), CDSSTR (*38*), CONTIN (*39*), CONTINLL (*40*, *41*), and K2D (*42*). The results are presented in Supporting Information Table 1. Although the different prediction algorithms provided different values, we could observe clear tendencies in the secondary structure changes. At 300 μM LPA, a decrease in β-sheet and an appearance of α-helix were observed compared to the native β2m. At 500 μM LPA, a further decrease in β-sheet content was detected with a possible additional small increase of the α-helical structure. The changes in the other secondary structural elements were not unambigously estimated; however, some of the algorithms suggested an increase in the unordered structure.

Cross-Linking of LPA-Destabilized β2m. Native β2m is a monomeric protein. Cross-linking experiments were carried out to verify if β2m remains monomeric upon partial unfolding from the native state induced by LPA. Dithiobis(succimidyl propionate) (DTSP), an amine-reactive cross-linker, was used to stabilize the dimers present, if any, in samples incubated with LPA. The possible dimerization of β2m was examined by SDS−PAGE under nonreducing conditions. The results showed no detectable dimerization in the presence of LPA under the conditions used in the present work as explained and presented in detail in Supporting Information Figure 2.

CD Spectroscopy of Amyloid Fibrils Formed in the Presence of LPA. Figure 5 shows amyloid fibril formation of β2m in the presence of LPA using amyloid seeds grown at pH 7.5. Before the seed-induced fibril formation, β2m monomers were preincubated for 2 h at 37 °C in the presence of either 300 or 500 μM LPA. After addition of seeds, CD spectra were recorded at various incubation times. Consistently with ThT fluorescence measurements, fibril generation was detected only in the presence of both LPA and fibril seeds. After 48 h, intensive amyloid-like CD spectra of high β-sheet content with single negative maxima at 221 and 218 nm were observed at 300 and 500 μM LPA concentrations, respectively. Note the isodichroic point in the case of 300 μM LPA, implicating that two conformational species of β2m are present in the solution, i.e., amyloid fibrils and partially unfolded monomers. Figure 5C shows the representative CD spectra of the different monomeric and fibrillar forms of β2m. Fibril samples showed precipitates of associated fibrils after 1 week of incubation. To resuspend and homogenize these samples, we applied slight ultrasonication. This process increased the amplitude of the CD spectra significantly, indicating that the larger aggregates of fibrils are not detectable by CD spectroscopy.

Limited Proteolysis of Monomeric β2m in the Presence of LPA. β2m was subjected to limited proteolysis by chymotrypsin at pH 7.5 to determine the lowest LPA concentration that

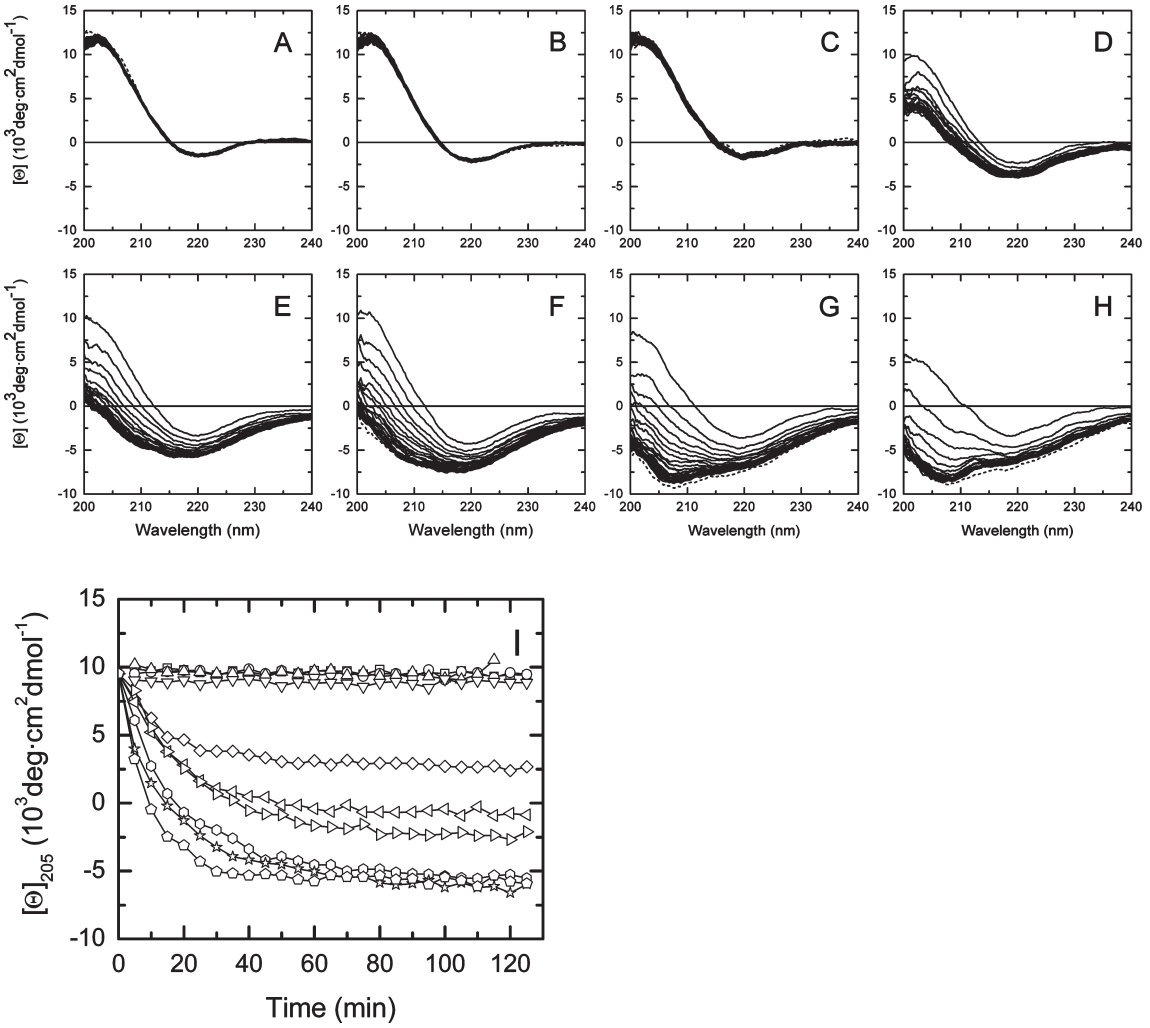

FIGURE 4: Effect of different LPA concentrations on the secondary structure of native β2m. β2m monomers were incubated at 37 °C for 2 h in the absence of any additives (A), in the presence of 500 μM LPC (B), and in the presence of 150 μM (C), 200 μM (D), 300 μM (E), 400 μM (F), 500 μM (G), and 700 μM (H) LPA. Far-UV CD spectra were recorded at every 5 min. Reaction mixtures contained 0.1 mg/mL monomeric β2m in 50 mM sodium phosphate and 100 mM NaCl, pH 7.5. Dotted lines represent spectra at an incubation time of 24 h. Note that spectra obtained at 2 and 24 h are overlapping. (I) Kinetics of the mean residue ellipticity ([θ]) at 205 nm as a function of LPA concentration: (□) 0 μM, (○) 100 μM, (▽) 150 μM, (◇) 200 μM, (left-pointing triangle) 300 μM, (right-pointing triangle) 400 μM, (⬡) 500 μM, (☆) 600 μM, (⬠) 700 μM LPA, and (△) 500 μM LPC.

leads to destabilization of the native structure. Limited proteolysis can be used as a sensitive probe for protein unfolding (*43*). Chymotrypsin was chosen because native β2m is relatively resistant against chymotryptic degradation. Chymotrypsin cleaves at the carboxyl sides of aromatic or bulky nonpolar side chains, which are mostly buried in the native structure. The phosphate moiety in sodium phosphate buffer might compete with the phosphate headgroup of LPA, which would result in an apparent elevation of the threshold for protein destabilization; therefore, we conducted these experiments in HEPES buffer, as well.

Our results indicate that LPA destabilizes the native β2m as proteolytic products appear in the presence of LPA (Figure 6). In 50 mM HEPES buffer, cleavage products appeared at 25 μM and higher LPA, while they were unambiguously observable from 50 μM LPA in 50 mM sodium phosphate buffer. LPC did not affect the proteolytic cleavability of β2m (see Supporting Information Figure 1).

Effect of Monomer Conformation on the Polymerization Reaction. The destabilizing effect of LPA on the structure of native β2m monomers takes place in a time scale of 1 h as presented above. To study whether the native molecule or the partially unfolded species elongate the preformed seeds, additional polymerization experiments were carried out (Figure 7). In one set of samples, β2m monomers were preincubated at 37 °C for 70 min with 300 μM LPA before inducing the fibril formation by seeds. In the other set of samples, the monomeric protein and seeds were added to the lipid solution simultaneously. Seeds grown at pH 7.5 were used for both cases, and fibril growth was monitored by the increase of ThT fluorescence. Without preincubation, a significantly lower growth rate was observed for about 20 min, compared to the preincubated sample (Figure 7). This indicates that a partially unfolded intermediate is required for fibril extension.

Effect of LPA on the Stability of Monomeric β2m Revealed by DSC. To investigate the effect of LPA on the structural stability of native monomeric β2m, DSC measurements were carried out at different LPA concentrations Figure 8). Without additives, β2m exhibits a cooperative unfolding transition with a melting temperature of 64.6 °C. The addition of either 50 μM LPA or 500 μM LPC has no significant effect on the thermal unfolding of the protein. In the presence of 100 and 200 μM LPA, the height of the unfolding peaks decreases significantly, entailing the loss of more than 75% of the peak

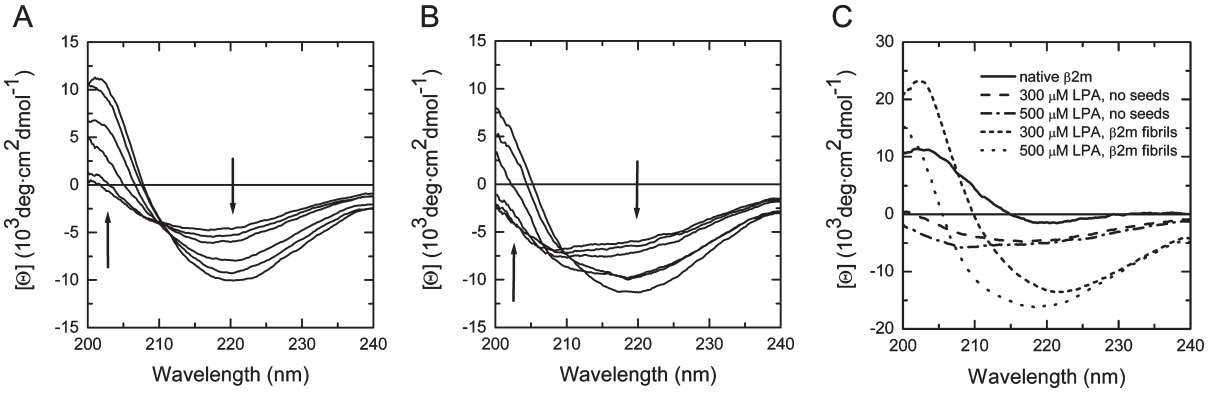

FIGURE 5: Far-UV CD spectra of β2m amyloid fibrils formed in the presence of LPA and pH 7.5 seeds. CD spectra were recorded after 0, 1, 4, 20, 30, and 48 h of incubation at 37 °C in the presence of 300 μM (A) and 500 μM (B) LPA. The reaction mixture contained 0.1 mg/mL monomeric β2m in 50 mM sodium phosphate and 100 mM NaCl, pH 7.5. Seed concentration was 10 μg/mL. Before the seed-induced fibril formation, β2m monomers were preincubated for 2 h in the presence of the lipid. Arrows show the direction of changes. (C) Comparison of the secondary structure of different conformations of β2m. The figure shows the mean residue ellipticity of native β2m (—), β2m incubated for 24 h in the presence of 300 μM (− −) and 500 μM (− · −) LPA with no seeds, and β2m samples inoculated with 10 μg/mL seeds prepared from fifth generation fibrils and incubated for 1 week in the presence 300 μM (- - -) and 500 μM (· · ·) LPA.

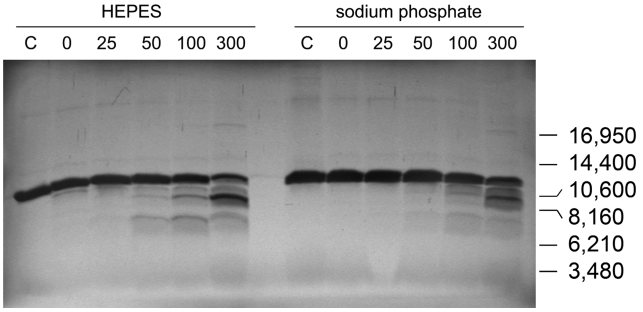

FIGURE 6: Limited proteolysis of monomeric β2m by chymotrypsin as a function of LPA concentration. β2m (0.2 mg/mL) was preincubated in either 50 mM HEPES or 50 mM sodium phosphate, pH 7.5, containing 100 mM NaCl and different concentrations of LPA, as indicated in the figure, at 37 °C for 1 h. Chymotryptic proteolysis was conducted for 30 min; the enzyme:β2m molar ratio was 1:100. Proteolysis was analyzed by running the samples in 16.5% Tris/Tricine SDS−polyacrylamide gels. Lanes "C" show undigested controls.

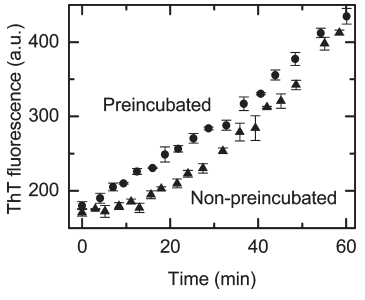

FIGURE 7: Seeded polymerization reaction of β2m with and without preincubation of the monomers with LPA. (●) β2m monomers were preincubated at 37 °C for 70 min in the presence of 300 μM LPA before inducing fibril formation by the addition of seeds. (▲) Slower initial fibril growth was observed when LPA and seeds were added to the β2m solution simultaneously. Samples contained 0.1 mg/mL monomer β2m in 50 mM sodium phosphate and 100 mM NaCl, pH 7.5. Ten μg/mL seeds grown at pH 7.5 were used. Fibril growth was monitored by ThT fluorescence. Data points are mean ± standard error of three independent measurements.

area at 200 μM LPA. A decrease in the peak area, i.e., in calorimetric enthalpy, without significant change in the melting temperature suggests that the number of native molecules

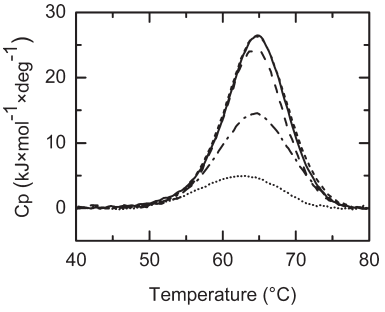

FIGURE 8: Calorimetric melting profile of β2m in the presence of LPA and LPC. Samples containing 0.1 mg/mL β2m and 0 μM (—), 50 μM (- - -), 100 μM (− · −), 200 μM (· · ·) LPA, and 500 μM LPC (− −) in 50 mM sodium phosphate and 100 mM NaCl, pH 7.5, were heated at a rate of 1 °C/min.

decreases with increasing LPA concentration. Because no other cooperative unfolding peaks appeared in the heating profile, a plausible interpretation is that LPA destabilizes the structure of β2m and the LPA-bound protein exhibits no cooperative unfolding transition. A slight decrease (about 2 °C) in the melting temperature was observed at 200 μM LPA concentration, further indicating that LPA destabilizes the protein's native structure.

ANS Binding of Monomeric β2m. As presented in detail in Supporting Information Figure 3, LPA-destabilized β2m exhibited enhanced ANS fluorescence. The time course of the fluorescence increase is in good agreement with the CD spectroscopy results regarding the kinetics of the structural changes. Native β2m showed no ANS fluorescence. Enhancement of ANS fluorescence is one hallmark of the molten globule-like state of proteins.

Effect of LPA on the Stability of β2m Fibrils. In another work, we found that amyloid fibrils dissociate at elevated temperatures, and SDS protects them from thermal-induced depolymerization.[2] Here, we examined the stabilization effect of LPA on the fibrils against heat treatment at pH 2.5. Under this nonphysiological condition, fibrils could be produced in the absence of additives (*4*−*8*), making it possible to study the effect of each lipid separately. Moreover, monomeric β2m is disordered

[2]J. Kardos, A. Micsonai, E. Petrik, H. Naiki, L. Gráf, and Y. Goto, unpublished results.

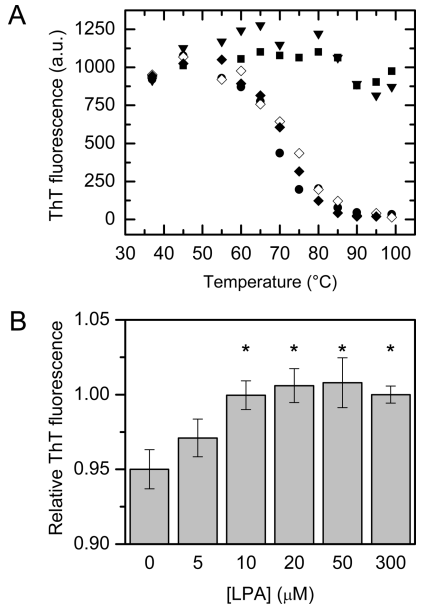

FIGURE 9: Effect of LPA on the stability of amyloid fibrils. (A) Effect of LPA and related analogues on the thermal stability of β2m fibrils. Amyloid fibrils grown at pH 2.5 were heat-treated at different temperatures for 5 min in the presence of SDS (▼), LPA (■), LPC (●), and SPC (◆). The remaining fibril content was monitored by ThT fluorescence. The initial fibril concentration was 0.1 mg/mL. The reaction mixture contained 50 mM sodium citrate, 100 mM NaCl, pH 2.5, and 500 μM different additives. LPA and SDS stabilized the fibrils preventing them from dissociation, while the other lipids exhibited no effect on fibril stability compared to the lipid-free control (◇). A typical experiment of three determinations is shown. The experimental error was less than 10%. (B) Depolymerization of fibrils in the presence of LPA. Amyloid fibrils, grown at pH 7.5, at a concentration of 0.05 mg/mL, were incubated at 37 °C for 2 months in the presence of different concentrations of LPA as indicated below the columns. The remaining fibril content was monitored by ThT fluorescence. LPA completely prevented the depolymerization of fibrils at a concentration as low as 10 μM. Columns represent mean ± standard error of five independent measurements. Asterisks mark significant differences as compared to the control (no LPA) experiment as revealed by Student's t test ($p < 0.05$).

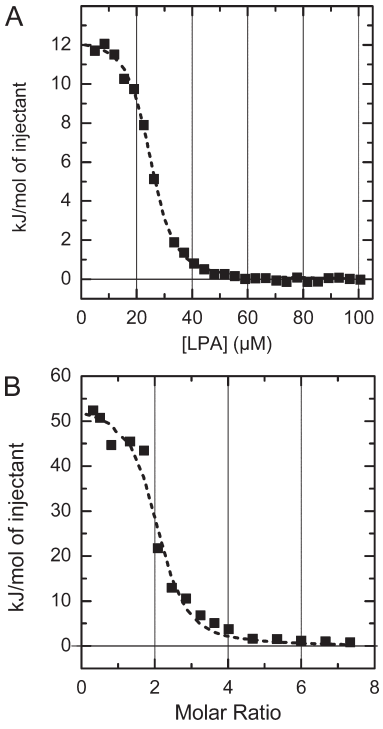

FIGURE 10: Micelle formation of LPA and micelle−β2m interaction investigated by ITC. (A) Determination of critical micelle concentration of LPA. Enthalpy changes corresponding to the dissociation of micelles were followed by repeated injections of 1 mM LPA into the calorimeter cell at 37 °C. A buffer solution containing 50 mM sodium phosphate and 100 mM NaCl, pH 7.5, was used in both the cell and injection syringe. (B) LPA micelle−β2m interaction was measured by injecting 470 μM β2m into the cell containing 300 μM LPA. The line shows a fitted saturation curve assuming the binding of two β2m molecules to one LPA micelle, yielding a dissociation constant of 520 ± 200 nM.

at this low pH, giving the opportunity to selectively study fibril stability. Preformed fibrils were incubated at various temperatures for 5 min, and the remaining ThT fluorescence was measured (Figure 9A). It was found that 500 μM LPA prevented fibrils from heat-induced dissociation, while the other lipids (LPC and SPC) had no effect.

We also studied the effect of LPA on the stability of fibrils under physiological conditions following the depolymerization of tenth generation fibrils, formed at pH 7.5 and 300 μM LPA. After removal of LPA by repeated washing, these fibrils exhibited surprising stability, showing only slow depolymerization. Thus we monitored the remaining fibril content for a prolonged period of time. The fibrils were resuspended in a lipid-free buffer or in the presence of 5−300 μM LPA and incubated at 37 °C. The stabilizing effect of LPA was elucidated after 2 months of incubation (Figure 9B). As can be seen, LPA completely prevented β2m fibrils from depolymerization even at a concentration as low as 10 μM, well below CMC.

Interaction of β2m with LPA Micelles Studied by ITC. Similarly to SDS, lysophospholipids in water-based buffer solutions form micelles in a concentration-dependent manner. To better understand the interaction of LPA with β2m, it is essential to examine whether the observed effect is caused by LPA monomers or the micellar form. Critical micelle concentrations (CMC) of LPA and LPC were determined under the same conditions applied in our other experiments. A solution containing 1 mM LPA, which is expected to exceed CMC, was successively injected into the calorimeter cell that contained only buffer at the beginning. At concentrations fairly below CMC, the injected lipid micelles dissociated via an endothermic reaction taking up significant heat. With LPA concentrations increasing above CMC in the cell, the injected micelles remained intact, exhibiting no further heat effect. The results indicated a CMC value of approximately 50 μM for LPA (Figure 10A). Using another definition for CMC introduced by Li et al. (*34*), i.e., the concentration belonging to the inflection point of the ITC profile, the CMC value was approximated as 25 μM. The CMC value of LPC, measured by the injection of 500 μM LPC into the ITC cell, was proven to be 5−10 μM (data not shown). The 25−700 μM concentration range of LPA, that was shown to be effective on monomeric β2m in the present work, falls into the micelle-forming region. This suggests that the nature of the interaction between LPA and monomeric β2m is a lipid micelle−protein interaction.

To further characterize the interaction of β2m with LPA micelles, titration experiments were carried out by injecting monomeric β2m into the ITC cell containing 300 μM LPA (Figure 10B). To estimate the concentration of micelles in the ITC cell, we assumed that a micelle consists of approximately 60 LPA molecules, based on the work of Tanford (*44*) and the fact that micelles have a narrow size distribution. Figure 10B shows

the titration with an acceptable saturation curve fit using an estimated micelle concentration of 5 μM. Suprisingly, the titration curve indicates that LPA micelles can be saturated by β2m molecules with a 1:2 stoichiometric ratio. The positively charged side chains of β2m are enriched on one side of the molecule (see Protein Data Bank entry 2yxf, published in ref (*45*)). It is a reasonable assumption that the negatively charged surface of one LPA micelle can be surrounded by two β2m molecules facing with their positive sides to the micelle. The 520 ± 200 nM dissociation equilibrium constant indicates high-affinity binding of monomeric β2m to LPA micelles. The reaction is entropy driven with an endothermic enthalpy change of 54 ± 3 kJ/mol. This value is comparable to the 175 kJ/mol unfolding ΔH of native β2m at 37 °C (*46*), suggesting a considerable disruption of the interactions that stabilize the native structure of β2m.

DISCUSSION

An elevated concentration of β2m, observed in DRA, is insufficient for amyloid formation *in vitro* at neutral pH (*9*). Various molecules were identified which might contribute to the amyloid formation of β2m at neutral pH, as presented in the introduction. These molecules can, on one hand, destabilize the structure of monomeric β2m which seems to be essential for amyloid formation (*9*, *47*−*52*). One the other hand, they can stabilize the preformed fibrils and prevent them from depolymerization (*11*−*14*, *22*). However, the detailed mechanism of fibril formation still remained elusive.

The role of lipid−protein interactions in amyloid fibril formation has been reviewed by Gorbenko and Kinnunen (*53*); however, they primarily focused on the role of membrane lipids. Gellermann et al. in a comparative study determined the chemical composition of the lipid components from disease-associated amyloid deposits including β2m fibrils (*54*). They found that these amyloid deposits contain significant amounts of lipids, an average of 7% in the case of β2m deposits. Hagihara et al. showed that anionic lysophospholipids induce aggregation of β_2-glycoprotein (*55*). Ookoshi et al. (*24*) screened for lysophospholipids that have an effect on amyloid formation of β2m. Varying the headgroup and the length and saturation of the hydrophobic tail, they found lysophosphatidic acids to be the most effective. In the present work, we investigated the mechanism of LPA action on the process of β2m amyloid formation. For comparison, we included LPC, the precursor lipid of LPA abundant in blood, as well as the structurally and physiologically related blood-born sphingolipid mediators S1P and SPC.

LPA Induces Amyloid Formation of β2m at Neutral pH. We carried out experiments under physiological conditions concerning pH, ionic strength, and temperature, as well as β2m concentration in patients with end-stage DRA (*56*, *57*). We demonstrated that LPA induces amyloid fibril formation at neutral pH in a nucleation-dependent manner as revealed by ThT fluorescence measurements, CD spectroscopy, and electron microscopy (Figures 1, 3, and 5). On the contrary, the other lipids had no effect on amyloid fibril formation.

LPA-induced fibril extension was significantly faster using seeds prepared from fibrils grown at neutral pH in the presence of LPA compared to seeds prepared from low-pH fibrils (Figures 1 and 2). Additionally, the final ThT intensity reached the end value measured in polymerization at low pH. This indicates a complete polymerization where nearly all β2m molecules are converted into amyloid fibrils, similarly to the reaction at pH 2.5, where a remaining monomer concentration around 1% of the initial value was observed (*46*). We observed another important difference between fibrils grown at neutral pH in the presence of LPA compared to the low-pH fibrils. Using seeds preformed at pH 2.5, fibril formation at pH 7.5 showed an initial depolymerization. This was not observable when seeds formed at pH 7.5 were used; furthermore, these fibrils depolymerize unexpectedly slowly after the removal of LPA. These findings indicate that the molecular organization of fibrils formed under physiological conditions differs from that of fibrils formed at pH 2.5.

Studying the effect of lysophospholipids on the amyloid formation of β2m, Ookoshi et al. (*24*) used seeds prepared from fibrils grown in the presence of SDS. They observed less than 50% of the ThT fluorescence intensity compared to that of SDS-induced growth, indicating that only a part of β2m monomers were incorporated into fibrils. The fact that seeds grown in the presence of LPA can induce complete polymerization suggests that SDS and LPA have different effects on amyloid formation.

The Amyloidogenic Intermediate Is a Partially Unfolded Molecule. In the absence of amyloid seeds fibril formation was not initiated by LPA during the time course of our experiments (up to 1 week). However, LPA destabilizes the structure of native monomeric β2m as revealed by far-UV CD spectra (Figure 4), limited proteolysis (Figure 6), and DSC (Figure 8). Cross-linking studies have proved that LPA-destabilized β2m remains monomeric; it does not form dimers in detectable amount (Supporting Information Figure 2). Additionally, the LPA-bound form exhibited no cooperative unfolding transition in DSC experiments and showed increased ANS fluorescence (Supporting Information Figure 3), similarly to a partially unfolded state such as a molten globule. Structural changes elicited by LPA reached an equilibrium within 1−2 h (Figure 4I). Accordingly, fibril formation was instant when polymerization was initiated after about 1 h preincubation of β2m monomers with LPA. On the contrary, when seeds and LPA were added simultaneously, a lower growth rate was observed in the first 20−30 min (Figure 7). These results prove that native β2m is not capable of extending the seeds; rather, partially unfolded forms have amyloidogenic properties.

Amyloid fibril formation was most efficient around 300 μM LPA (Figure 2). The presence of an isodichroic point in the CD spectra at this concentration (Figure 5A) implicates that two conformational species of β2m dominate in the solution, i.e., amyloid fibrils and partially unfolded monomers. At 500 μM LPA, no isodichroic point can be observed, indicating the presence of more than two conformational states (Figure 5B). Secondary structure estimation from CD spectra of monomeric β2m (Supporting Information Table 1) shows a decrease in the β-sheet content at 300 μM LPA. Above this LPA concentration, the structure of monomers becomes more unfolded, whose form is less favorable for fibril extension.

In conclusion, the amyloidogenic intermediate of β2m is a moderately unfolded species that is most populated around 300 μM LPA concentration. These observations are consistent with previous studies reporting that amyloid formation proceeds via partially unfolded intermediates (*9*, *47*−*52*). We can find a notable similarity between TFE, SDS, and LPA in the context that they are most effective at moderately denaturing concentrations where the tertiary structure of native β2m is destabilized, resulting in a partially unfolded state of the molecule (*12*, *22*, *58*). However, TFE exhibits its effect at significantly higher concentrations as a cosolvent, by altering the solvent properties and

modulating the balance of hydrogen bonds and hydrophobic interactions.

We suppose that LPA can bind β2m through electrostatic interactions. In this respect it is similar to collagen, but in that case the positively charged residues on the collagen fibril surface might be involved in the interaction with β2m (*17*, *18*). In contrast, the headgroup of LPA is negatively charged and may interact primarily with lysine and arginine residues of β2m. Copper ions also induce fibril formation under physiological conditions. Copper binding is localized to His31 and Trp60 of native β2m. Unlike LPA, copper does not cause significant changes in the CD spectrum of the native protein; however, it destabilizes β2m against thermal and urea denaturation (*19*−*21*) and catalyzes oligomerization (*48*, *59*).

LPA Stabilizes the Amyloid Fibrils. Below 100 μM LPA the stable native conformation of β2m is predominant as revealed by CD spectroscopy (Figure 4), and only a low level of the partially unfolded form is present according to limited proteolysis (Figure 6). The fact that amyloid fibril growth proved to be significant at this concentration (Figure 2) suggests that LPA exhibits a stabilizing effect on the amyloid fibrils.

Based on the observation that, at pH 2.5, β2m fibrils dissociate at high temperature,[2] the effect of LPA on fibril stability was first probed by incubating preformed fibrils at elevated temperatures. These experiments revealed that LPA, similarly to SDS, stabilizes fibrils, preventing them from dissociation. In contrast, the other investigated lipids exhibited no detectable effect (Figure 9A).

Furthermore, we observed that β2m fibrils, grown at pH 7.5, exhibited surprising resistance against depolymerization after removal of LPA (Figure 9B) at 37 °C. Nevertheless, the slow dissociation in the absence of LPA could be completely blocked by the presence of only 10 μM LPA (Figure 9B). These results also depict the difference between β2m fibrils grown at low and physiological pH, the existence of which has been an open question (*8*, *60*). Our observations emphasize the importance to study the amyloid formation of β2m at physiological pH.

β2m Interacts with an Aggregated Form of LPA. At an LPA concentration of approximately 25−50 μM and above, the micellar form of the lipid is present *in vitro* (Figure 10). Fibril formation induced by LPA was observed above the CMC, showing that β2m monomers interact with LPA micelles *in vitro*. Based on the estimate of an average of 60 lipids per micelle, the concentration of micelles is in the range of low micromoles. By ITC measurements, we identified a high-affinity interaction between LPA micelles and β2m *in vitro* (K_D = 520 ± 200 nM). In contrast to LPA, the effective concentration range of SDS to induce β2m amyloid fibril formation is below its CMC (*22*, *23*), further emphasizing a difference in the mechanism of action of LPA and SDS.

Physiological Relevance of the Amyloidogenic Effect of LPA. LPA is present in biological fluids, raising a possible *in vivo* relevance of our findings for DRA. According to a recent study, the average serum concentration of LPA in healthy individuals is about 3 μM (*61*). On the contrary, in some ovarian cancer patients concentrations as high as 15−20 μM were detected (*61*). Presumably, these values can be manifold higher locally at the site of production, reaching the effective concentration range in this work. About a 3-fold elevation of plasma LPA (from 0.5 to 1.4 μM, taken before dialysis) was reported in patients undergoing long-term hemodialysis compared to healthy controls (*24*, *62*). It is well-known that the contact with dialysis membrane activates several blood cells including platelets (*63*, *64*). The activated platelets produce LPA (*65*), which should further increase its concentration. Future studies are needed to measure the peak LPA concentrations in patients following the dialysis procedures. Gellermann et al. observed the presence of LPC, the precursor of LPA, in β2m amyloid deposits in patients, though LPA was not measured (*54*). They proposed that the lipid enrichment represents an additional factor affecting the clinical deposition of amyloid.

In our experiments, the polymerization of β2m is completed in hours or days, while the development of DRA is a slow process taking several years. This might be the consequence of the *in vivo* average lower level of LPA compared to our *in vitro* model system. The temporary and local bursts in the concentration of β2m-destabilizing agents such as LPA and the low depolymerization rate of the fibrils formed at physiological conditions together with the potent stabilizing effect of LPA on the amyloid fibrils might give an explanation for the development of DRA.

In summary, LPA induces amyloid formation through a dual mechanism. It is an amyloidogenic factor that destabilizes the structure of monomeric β2m, inducing a partially unfolded intermediate state. This form is capable of fibril extension in the presence of preformed fibril seeds. On the other hand, LPA stabilizes the β2m amyloid fibrils. Although many factors can interplay in the development of DRA, we propose that LPA might be one among the physiologically relevant molecules exhibiting synergistic effect on fibril formation.

SUPPORTING INFORMATION AVAILABLE

We estimated the secondary structure content of the different forms of monomeric β2m from their CD spectra using various methods. The results are presented in Supplementary Table 1. Although different prediction algorithms provided different values, we could observe clear tendencies in the secondary structure changes. LPC did not affect the proteolytic cleavability of β2m, which was verified by SDS−PAGE and shown in Supplementary Figure 1. Cross-linking studies have proved that LPA-destabilized β2m is monomeric. The results are presented in Supplementary Figure 2. Monomeric β2m in the presence of LPA showed increased ANS fluorescence indicating the formation of a molten globule-like intermediate (Supplementary Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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