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Inhibition of β_2 -Microglobulin Amyloid Fibril Formation by α_2 -Macroglobulin^{*[5]}

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The relationship between various amyloidoses and chaperones is gathering attention. In patients with dialysis-related amyloidosis, α_2 -macroglobulin (α_2 M), an extracellular chaperone, forms a complex with β_2 -microglobulin (β_2 -m), a major component of amyloid fibrils, but the molecular mechanisms and biological implications of the complex formation remain unclear. Here, we found that α_2 M stoichiometrically inhibited the β_2 -m fibril formation at a neutral pH in the presence of SDS, a model for anionic lipids. Binding analysis showed that the binding affinity between α_2 M and β_2 -m in the presence of SDS was higher than that in the absence of SDS. Importantly, SDS dissociated tetrameric α_2 M into dimers with increased surface hydrophobicity. Western blot analysis revealed that both tetrameric and dimeric α_2 M interacted with SDS-denatured β_2 -m. At a physiologically relevant acidic pH and in the presence of heparin, α_2 M was also dissociated into dimers, and both tetrameric and dimeric α_2 M interacted with β_2 -m, resulting in the inhibition of fibril growth reaction. These results suggest that under conditions where native β_2 -m is denatured, tetrameric α_2 M is also converted to dimeric form with exposed hydrophobic surfaces to favor the hydrophobic interaction with denatured β_2 -m, thus dimeric α_2 M as well as tetrameric α_2 M may play an important role in controlling β_2 -m amyloid fibril formation.

A number of proteins and peptides can misfold into β -sheet-rich structures, called amyloid fibrils (1). The deposition of amyloid fibrils in intra- and extracellular spaces is responsible for more than 40 serious diseases, including Alzheimer and Parkinson diseases and dialysis-related amyloidosis (DRA)² (1). β_2 -Microglobulin (β_2 -m) is a major structural component of amyloid fibrils in DRA, a common and serious complication in long term hemodialysis patients (2). The formation of β_2 -m

amyloid fibrils is thought to be induced by partial unfolding of β_2 -m (3, 4). Several groups have established conditions under which β_2 -m amyloid fibril formation occurs at a neutral pH (5–11). We found that β_2 -m amyloid fibrils are formed at a neutral pH in the presence of SDS (11). SDS below its critical micelle concentration unfolds the compact structure of β_2 -m to an amyloidogenic conformer and stabilizes the extended amyloid fibrils. SDS is an anionic detergent that mimics some characteristics of biological membranes and is considered to be a good model for anionic lipids. We recently reported that some lysophospholipids, especially lysophosphatidic acid as well as nonesterified fatty acids induce the extension of β_2 -m amyloid fibrils at a neutral pH by partially unfolding the compact structure of β_2 -m to an amyloidogenic conformer as well as by stabilizing the extended fibrils (6, 8). Although many groups have proposed the mechanisms by which β_2 -m amyloid fibrils are formed under physiological conditions, the biological machineries to inhibit the formation and deposition of β_2 -m amyloid fibrils are poorly understood (12).

α_2 -Macroglobulin (α_2 M), haptoglobin (Hp), and clusterin are abundantly secreted glycoproteins present in human plasma and cerebrospinal fluid and are known as acute phase proteins (13). These glycoproteins have been found to be associated with extracellular amyloid deposits in Alzheimer disease and many other amyloidoses (13). Recently, these glycoproteins have been shown to suppress the amyloid fibril formation and amorphous aggregation of various proteins and have been described as extracellular chaperones (14–18). All of these glycoproteins can interact with prefibrillar species to maintain the solubility of amyloidogenic proteins (15–17). Wilson's group proposed that extracellular chaperones respond to misfolded and aggregated proteins in the extracellular space by binding to their exposed hydrophobic regions, maintaining the solubility of the substrate, and promoting its removal from the extracellular space via receptor-mediated endocytosis and lysosomal degradation (13, 14).

α_2 M is a homotetramer that is formed by noncovalent association of two disulfide-bonded dimers (19). In addition to its interactions with amyloidogenic proteins, α_2 M is known to trap proteases (19). The trapping of proteases is accompanied by a conformational change of α_2 M, which exposes receptor binding domains and consequently undergoes endocytosis by binding to low density lipoprotein receptor-related protein (20, 21). In patients with DRA, α_2 M is identified in the amyloid deposits (22) and forms a complex with β_2 -m in the blood (23). Serum concentrations of the α_2 M: β_2 -m complex are correlated with

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² The abbreviations used are: DRA, dialysis-related amyloidosis; α_2 M, α_2 -macroglobulin; ANS, 8-anilino-1-naphthalenesulfonate; β_2 -m, β_2 -microglobulin; BS³, bis(sulfosuccinimidyl) suberate; Hp, haptoglobin; HSQC, heteronuclear single-quantum coherence; ThT, thioflavin T.

the progression of DRA (23). The complex formation has been thought to prevent the metabolism of β_2 -m in the kidney (24). Therefore, α_2 M may play an active role in the pathogenesis of DRA. However, it remains unclear whether α_2 M directly affects the formation of β_2 -m amyloid fibrils. Moreover, the mechanism by which α_2 M interacts with β_2 -m is poorly understood.

In this study, we first examined the effect of α_2 M on the formation of β_2 -m amyloid fibrils at a neutral pH in the presence of 0.5 mM SDS. We next assessed the interaction of α_2 M with β_2 -m adopting various conformational states (native state, denatured state, and amyloid fibrils). We also examined the structural changes of α_2 M under conditions where α_2 M interacted with β_2 -m. Finally, we examined the effect of α_2 M on the fibril growth at a physiologically relevant acidic pH and in the presence of heparin. Our results provide new insights into the effect of α_2 M on β_2 -m amyloid fibril formation and the mechanism of α_2 M- β_2 -m interaction.

EXPERIMENTAL PROCEDURES

Materials—Human α_2 M, human Hp, bovine serum albumin (BSA), and equine ferritin were obtained from Sigma. Recombinant human β_2 -m was expressed using an *Escherichia coli* expression system and purified as described previously (25). Additional procedures are discussed in the [supplemental Methods](#).

Seed-dependent Growth Reaction of β_2 -m Amyloid Fibrils and Thioflavin T (ThT) Assay—Seed β_2 -m amyloid fibrils used for the growth reaction were prepared from the patient-derived β_2 -m amyloid fibrils by the repeated growth reaction at pH 7.5 with recombinant human β_2 -m, as described elsewhere (26). Seeds (*i.e.* fragmented fibrils) were prepared by sonication of the amyloid fibrils. The reaction mixture containing 30 μ g/ml seeds, 25 μ M β_2 -m, 0–25 μ M α_2 M, Hp, BSA, or ferritin, 50 mM phosphate buffer (pH 7.5), 100 mM NaCl, 0.5 mM SDS, and 0.05% NaN_3 was incubated at 37 °C without agitation. In the presence of heparin, the reaction mixture containing 30 μ g/ml seeds, 25 μ M β_2 -m, 0 or 25 μ M α_2 M, 50 mM phosphate buffer (pH 6.3), 100 mM NaCl, 100 μ g/ml heparin, and 0.05% NaN_3 was incubated at 37 °C in a 96-well plate with moderate stirring (300 rpm) using a Teflon-coated microstirrer bar. The reactions were monitored by fluorescence assay with ThT in which an aliquot of 5 μ l was taken from each reaction tube and mixed with 1 ml of 5 μ M ThT in 50 mM sodium glycine buffer (pH 8.5) (27). The ThT fluorescence was measured using a Hitachi F-4500 spectrofluorometer (Tokyo, Japan) at 25 °C with excitation at 445 nm and emission at 485 nm.

Transmission Electron Microscopy—Sample was spread on carbon-coated grids, negatively stained with 1% phosphotungstic acid (pH 7.0), and examined under a Hitachi H-7650 electron microscope with an acceleration voltage of 80 kV.

Dot-Blot Assay—Samples of α_2 M, Hp, and BSA (1 μ g) were spotted onto nitrocellulose membranes using a dot-blot apparatus (Bio-Rad). The membranes were blocked with 5% skim milk and then incubated for 1 h at 25 °C with 25 μ M β_2 -m in 50 mM phosphate buffer (pH 7.5), 100 mM NaCl, 0 or 0.5 mM SDS and 1.25 μ M BSA. After washing three times with a washing buffer (50 mM phosphate buffer (pH 7.5), 100 mM NaCl, and 0 or

0.5 mM SDS), bound β_2 -m was detected with horseradish peroxidase-conjugated anti-human β_2 -m antibody (1:2,000) (Dako) followed by enhanced chemiluminescence with BM Chemiluminescent Blotting substrate (Roche Applied Science). In a separate experiment, β_2 -m amyloid fibrils (1 μ g) were first spotted on the membrane. The membranes were blocked with 5% skim milk and then incubated for 1 h at 25 °C with 55 nM α_2 M in 50 mM phosphate buffer (pH 7.5), 100 mM NaCl, 0.5 mM SDS, and 1.25 μ M BSA. After washing three times with a washing buffer, bound α_2 M was detected using anti-human α_2 M antibody (1:400) (Sigma) and horseradish peroxidase-conjugated anti-rabbit immunoglobulins antibody (1:2,000) (Dako).

Enzyme-linked Immunosorbent Assay (ELISA)—We used an ELISA plate kit (Sumitomo Bakelite). Each well of a 96-well ELISA plate was first coated with 100 μ l of 27 nM α_2 M dissolved in a coating buffer supplied by the manufacturer. After washing three times with a washing buffer (50 mM phosphate buffer (pH 7.5), 100 mM NaCl), 100 μ l of 0–42 μ M β_2 -m, 50 mM phosphate buffer (pH 7.5), 100 mM NaCl, 0 or 0.5 mM SDS and 1.25 μ M BSA was added to the wells and incubated for 1 h at 25 °C. After washing three times with a washing buffer containing 0.5 mM SDS, bound β_2 -m was detected with horseradish peroxidase-conjugated anti-human β_2 -m antibody (1:1,000) (Dako) followed by color development using 3,3',5,5'-tetramethylbenzidine as the peroxidase substrate (Bio-Rad). The absorbance was measured at 450 nm in a SpectraMax 250 microplate reader (Molecular Devices, Sunnyvale, CA). The binding data were subjected to Scatchard analysis.

Amyloid Fibril Formation from β_2 -m Monomer—The reaction mixture containing 25 μ M β_2 -m, 1.25 μ M α_2 M, Hp, or BSA, 50 mM phosphate buffer (pH 7.5), 100 mM NaCl, 0.5 mM SDS, and 0.05% NaN_3 was incubated at 37 °C in a 96-well plate with moderate stirring (300 rpm) using a Teflon-coated microstirrer bar. ThT assay was performed as described above.

Circular Dichroism (CD) Measurements—Far-UV CD spectra of the mixture containing 0.14 μ M α_2 M, 50 mM phosphate buffer (pH 7.5), 100 mM NaCl, and 0 or 0.5 mM SDS were measured with a Jasco 725 spectropolarimeter (Tokyo, Japan) and a cell of 0.1-cm path length at 25 °C. For the measurement of near-UV region, the CD signals of the mixture containing 0.69 μ M α_2 M, 50 mM phosphate buffer (pH 7.5), 100 mM NaCl, and 0 or 0.5 mM SDS were recorded with a cell of 1-cm path length. The results were expressed in terms of mean residue ellipticity.

Analytical Ultracentrifugation—Sedimentation velocity of α_2 M in the presence or absence of SDS was measured using a Beckman-Coulter Optima XL-A analytical ultracentrifuge with an An-60 rotor and two-channel charcoal-filled Epon cells. The sample solution containing 1 μ M α_2 M, 50 mM phosphate buffer (pH 7.5), 100 mM NaCl, and 0 or 0.5 mM SDS was measured at 25 °C. The data were analyzed using the software UltraScan 9.3.

8-Anilino-1-naphthalenesulfonate (ANS) Binding—ANS (Wako Pure Chemical Industries) was added at a final concentration of 10 μ M to the mixture containing 1.25 μ M α_2 M or 25 μ M β_2 -m, 50 mM phosphate buffer (pH 7.5), 100 mM NaCl, and 0 or 0.5 mM SDS and incubated for 1 h at 25 °C. The ANS fluorescence was measured using a Hitachi F-7000 spectrofluorometer at 25 °C with excitation at 350 nm and emission at 400–600 nm.

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Cross-linking Experiments with Bis(sulfosuccinimidyl) Suberate (BS^3) and Western Blot Analysis—The reaction mixture containing 0 or 12.5 μM β_2 -m, 0 or 0.63 μM $\alpha_2\text{M}$, 50 mM citrate buffer (pH 4–5) or phosphate buffer (pH 6–7.5), 100 mM NaCl, and 0–0.5 mM SDS or 0–100 $\mu\text{g}/\text{ml}$ heparin was incubated for 1 h at 25 °C. BS^3 (Thermo Fisher Scientific), an amine-reactive cross-linking reagent, was then added at a final concentration of 5 mM to the mixture. After a 45-min incubation at 25 °C, the cross-linking reaction was quenched with 50 mM Tris buffer (pH 7.5). Cross-linked products were separated by 5% SDS-PAGE without reducing reagent and heat treatment. After separate SDS-PAGE performed in parallel, the proteins were transferred to PVDF membranes (Bio-Rad). The membranes were blocked with 5% skim milk. After washing three times with a washing buffer (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20), bound β_2 -m was detected with horseradish peroxidase-conjugated anti-human β_2 -m antibody (1:2,000) (Dako) followed by enhanced chemiluminescence with BM Chemiluminescent Blotting substrate.

RESULTS

Effect of $\alpha_2\text{M}$ on Seed-dependent Growth of β_2 -m Amyloid Fibrils—First, we examined the effects of $\alpha_2\text{M}$ and Hp on the seed-dependent growth of β_2 -m amyloid fibrils at pH 7.5 monitored by ThT fluorescence assay (Fig. 1, A and B). Because lysophospholipids, nonesterified fatty acids, and SDS induce the extension of β_2 -m amyloid fibrils by the same mechanisms (6, 8, 11), the experiments were performed mainly using SDS as a model for anionic lipids. In the absence of $\alpha_2\text{M}$, ThT fluorescence increased without a lag phase and proceeded to equilibrium at 24–48 h after initiation of the reaction (Fig. 1A). In the presence of $\alpha_2\text{M}$ at molar ratios of 1:200–1:20 to β_2 -m, a concentration-dependent inhibitory effect was observed. At a molar ratio of 1:20, the fluorescence increase was negligible. Notably, $\alpha_2\text{M}$ exhibited a significant inhibitory effect substoichiometrically even at a molar ratio of 1:200. In the presence of 0.5 mM oleate, $\alpha_2\text{M}$ exhibited similar concentration-dependent inhibitory effects on the fibril growth (supplemental Fig. S1). In contrast, the addition of BSA or ferritin (440 kDa) at a molar ratio of 1:20 to β_2 -m had little effect on the fluorescence increase, suggesting the specific inhibitory effect of $\alpha_2\text{M}$ on the growth of β_2 -m amyloid fibrils. By electron microscopy, clear fibril growth was observed after a 72-h incubation in the absence of $\alpha_2\text{M}$ and in the presence of ferritin (Fig. 1, C, D, and F), whereas no fibril growth was observed in the presence of $\alpha_2\text{M}$ at a molar ratio of 1:20 (Fig. 1, C and E). In the presence of Hp, another extracellular chaperone with an action similar to $\alpha_2\text{M}$ (16), at molar ratios of 1:100–1:1 to β_2 -m, a concentration-dependent inhibitory effect was observed (Fig. 1B). The inhibitory effect of Hp was much lower than that of $\alpha_2\text{M}$ because at a molar ratio of 1:20, Hp inhibited fibril growth only slightly. By electron microscopy, no fibril growth was observed in the presence of Hp at a molar ratio of 1:1 (Fig. 1, C and G). In the presence of 0.5 mM oleate, Hp exhibited similar concentration-dependent inhibitory effects on the fibril growth (supplemental Fig. S1).

Next, to determine whether $\alpha_2\text{M}$ inhibits the seed-dependent fibril growth by binding to β_2 -m monomers or to the

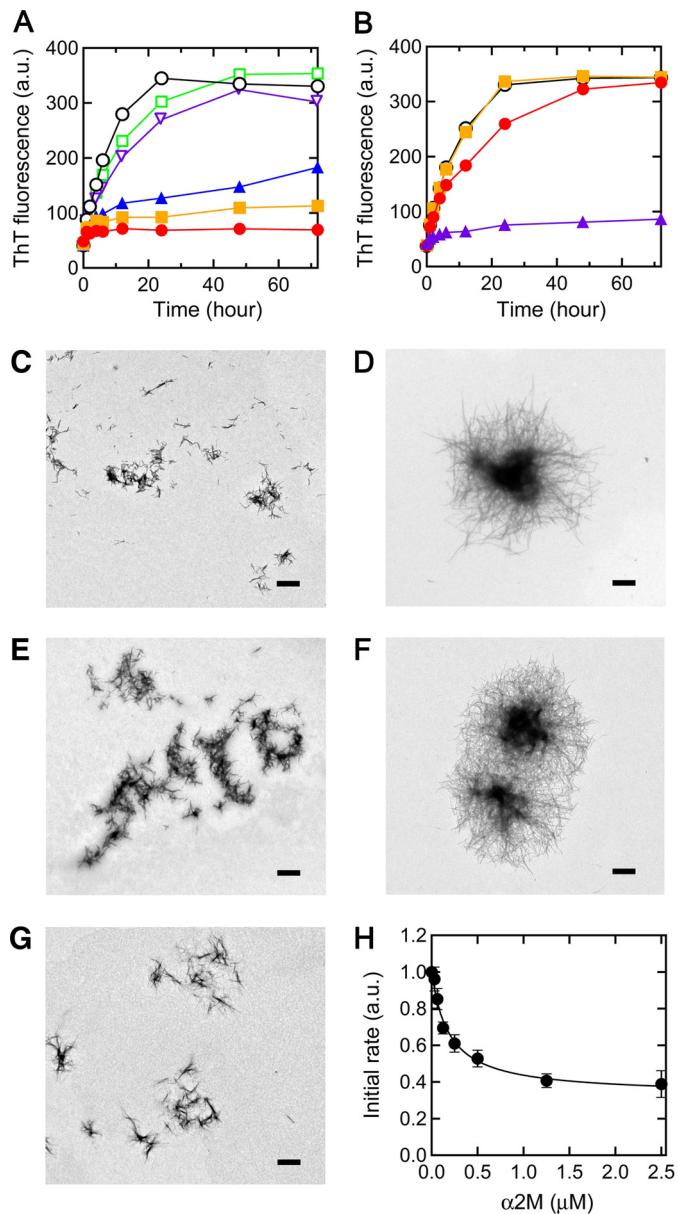


FIGURE 1. Effects of $\alpha_2\text{M}$ and Hp on seed-dependent growth of β_2 -m amyloid fibrils. A, time course of fibril growth monitored by ThT fluorescence in the absence (black) or presence of 1:200 (molar ratio of $\alpha_2\text{M}$ to β_2 -m) (blue), 1:100 (orange), or 1:20 (red) $\alpha_2\text{M}$, or in the presence of 1:20 BSA (green) or ferritin (purple). B, time course of fibril growth in the absence (black) or presence of 1:100 (orange), 1:20 (red), or 1:1 (purple) Hp. Each point represents the average of three ThT measurements from the same sample. S.D. was <5% of each average value. Each figure is a representative pattern of three independent experiments. C–G, electron microscopy images of the samples of seed-dependent growth reaction. The sample prepared in the absence (D) or presence of 1:20 $\alpha_2\text{M}$ (E), 1:20 ferritin (F), or 1:1 Hp (G) was incubated at 37 °C for 72 h. C, seeds prepared by sonication. Scale bars, 1 μm . H, effect of $\alpha_2\text{M}$ concentration on the initial rate of seed-dependent growth. The initial rates of seed-dependent growth were calculated from the increase of ThT fluorescence after 1-h incubation. The data points representing the average of three independent experiments were fitted to the function: Initial rate = $(m + n[\alpha_2\text{M}]) / (m + [\alpha_2\text{M}])$, as reported previously (27) ($r = 0.994$). Error bars, S.D.

growing ends of β_2 -m fibrils, we used two kinetic schemes reported previously (27). The initial rates of seed-dependent growth were calculated from the increase of ThT fluorescence after a 1-h incubation in the presence of 1:800–1:10 $\alpha_2\text{M}$ (supplemental Fig. S2) and normalized by that without $\alpha_2\text{M}$. At a

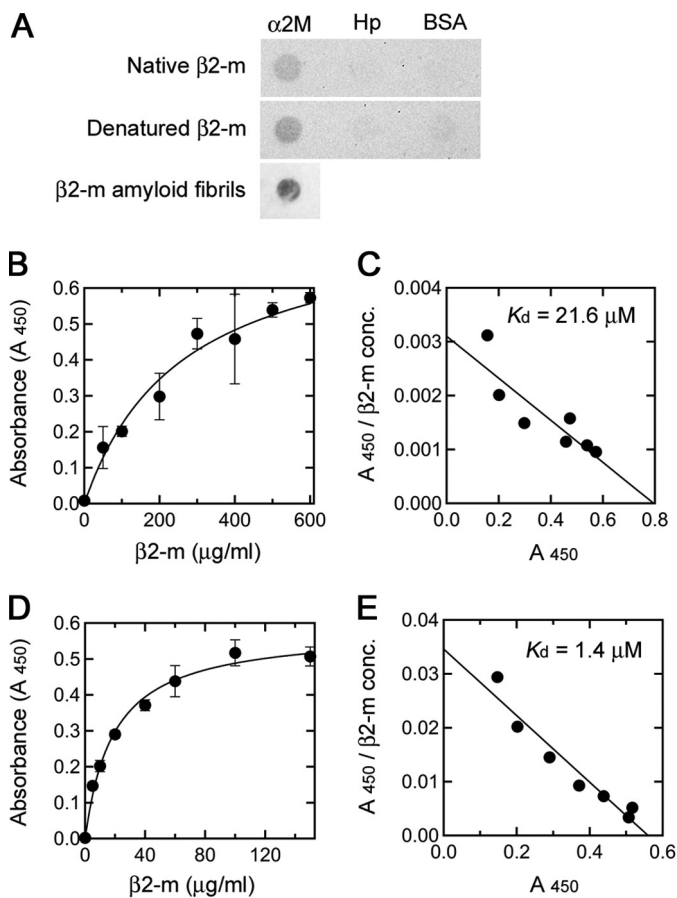


FIGURE 2. Differential binding of α_2 M to β_2 -m adopting various conformational states. *A*, binding of native and SDS-denatured β_2 -m to immobilized α_2 M, Hp, and BSA and binding of α_2 M to immobilized β_2 -m amyloid fibrils were assessed by dot-blot assay as under "Experimental Procedures." *B–E*, binding affinities of native and SDS-denatured β_2 -m to immobilized α_2 M. α_2 M was immobilized on an ELISA plate and incubated with β_2 -m in the absence (*B*) or presence (*D*) of SDS as described under "Experimental Procedures." Bound β_2 -m was detected with horseradish peroxidase-conjugated anti-human β_2 -m antibody, and the absorbance at 450 nm was determined. Each point represents the average of three wells. Error bars, S.D. Each figure is a representative pattern of two independent experiments. *C* and *E*, Scatchard plots of the binding data shown in *B* and *D*, respectively.

constant β_2 -m concentration, the initial rate decreased exponentially as the α_2 M concentration increased (Fig. 1*H*). When these data points were fitted to the function: Initial rate = $(m + n[\alpha_2M]) / (m + [\alpha_2M])$ (27), a good fit was observed ($r = 0.994$). This may indicate that α_2 M inhibits the seed-dependent fibril growth by binding to β_2 -m monomers rather than binding to the growing ends of β_2 -m amyloid fibrils.

Differential Binding of α_2 M to β_2 -m Adopting Various Conformational States—To determine whether α_2 M interacts with β_2 -m adopting various conformational states, including native and denatured states, and amyloid fibrils, we first analyzed the binding of native and SDS-denatured β_2 -m to α_2 M by dot-blot assay. Both native and SDS-denatured β_2 -m were found to bind to α_2 M (Fig. 2*A*). The spot of SDS-denatured β_2 -m seemed to be darker than that of native β_2 -m. In contrast, there was no detectable binding of native and SDS-denatured β_2 -m to Hp and BSA. We observed previously that in the presence of 0.5 mM SDS at 37 °C, β_2 -m is partially unfolded, and this β_2 -m conformer weakly and reversibly aggregates into small oligom-

ers composed of up to five molecules via hydrophobic interactions (11, 26). The far-UV CD spectrum of β_2 -m in the presence of SDS was different from that in the absence of SDS (supplemental Fig. S3). Analytical ultracentrifugation revealed that at 25 °C at which dot-blot assay was performed, SDS-denatured β_2 -m partly aggregated into small oligomers composed of 2–4 molecules (supplemental Fig. S3). This indicates that α_2 M may interact with SDS-denatured monomeric/oligomeric β_2 -m. The binding of α_2 M to β_2 -m amyloid fibrils was also observed (Fig. 2*A*). This is consistent with the previous report of α_2 M identified in amyloid deposits from patients with DRA (22).

To assess quantitatively the binding of native and SDS-denatured β_2 -m to α_2 M, we performed ELISA (Fig. 2, *B* and *D*). The ELISA data were subjected to Scatchard analysis to determine the apparent dissociation constant (K_d) (Fig. 2, *C* and *E*). The apparent K_d values for native and SDS-denatured β_2 -m were 21.6 μ M and 1.4 μ M, respectively, clearly indicating that α_2 M interacts with SDS-denatured β_2 -m much more strongly than native β_2 -m.

To identify the binding sites for α_2 M in the β_2 -m molecule, we performed nuclear magnetic resonance (NMR) experiments. 15 N-Labeled β_2 -m was titrated with increasing concentrations of α_2 M both in the presence and absence of 0.5 mM SDS at 25 °C. Although each peak position did not shift in 1 H- 15 N heteronuclear single-quantum coherence (HSQC) spectra, the intensities of all peaks decreased simultaneously both in the absence and presence of SDS (supplemental Fig. S4). The decrease in signal intensity in the presence of SDS was greater than that in the absence of SDS. In contrast, the peak intensities remained unchanged when β_2 -m was titrated with BSA (supplemental Fig. S4). Although the specific regions of β_2 -m to interact with α_2 M are unidentified, these data show that α_2 M strongly interacts with β_2 -m (see "Discussion").

Effect of α_2 M on Amyloid Fibril Formation from β_2 -m Monomer—Because α_2 M interacted with SDS-denatured β_2 -m, we next examined the effect of α_2 M on the SDS-induced amyloid fibril formation from β_2 -m monomer. When β_2 -m alone was incubated in the presence of 0.5 mM SDS at pH 7.5 and with agitation, ThT fluorescence increased sigmoidally with a 10-h lag time, then proceeded to equilibrium after 30–40 h (Fig. 3*A*). Although final equilibrium levels were varied from experiment to experiment, similar kinetics was observed in the presence of Hp and BSA (Fig. 3*A*). In contrast, no increase in ThT fluorescence was observed during a 40-h incubation when β_2 -m was incubated with α_2 M (Fig. 3*A*). By electron microscopy, clear amyloid fibril formation was observed when β_2 -m was incubated alone or with Hp or BSA (Fig. 3, *B*, *D*, and *E*), whereas only amorphous aggregates were occasionally found in the presence of α_2 M (Fig. 3*C*). These data indicate that α_2 M may inhibit the *de novo* amyloid fibril formation from β_2 -m monomer by binding to SDS-denatured monomeric/oligomeric β_2 -m.

Structural Analysis of α_2 M in the Presence of SDS—Although the structural change of β_2 -m is observed in the presence of 0.5 mM SDS, below the critical micelle concentration (supplemental Fig. S3) (11, 26), it is unclear whether the structure of α_2 M is affected by 0.5 mM SDS. The far-UV CD spectrum of α_2 M in the presence of SDS was slightly different from that in the absence

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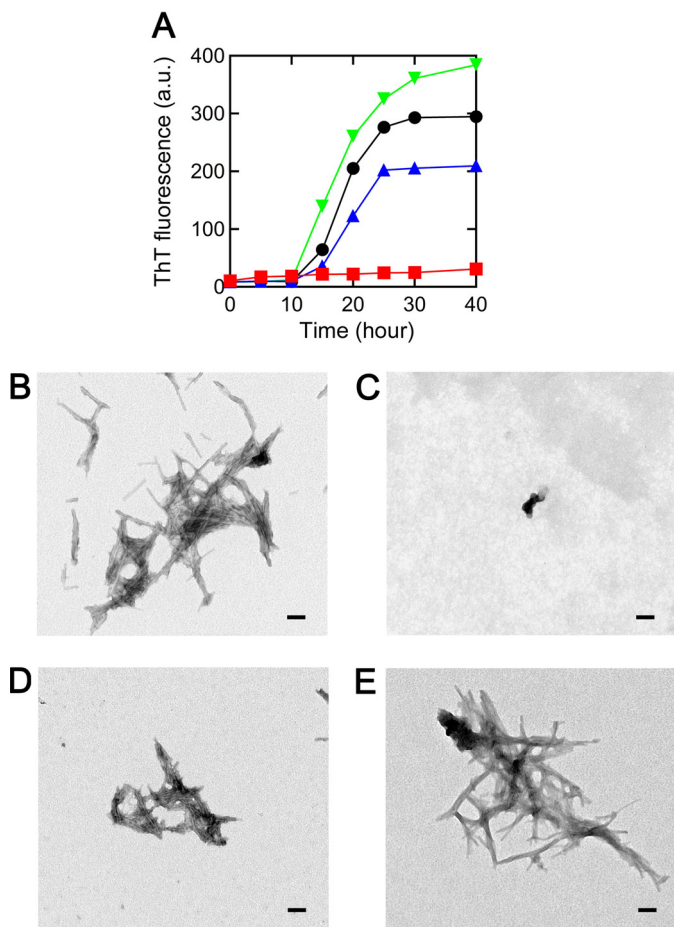


FIGURE 3. Effects of α_2 M and Hp on the amyloid fibril formation from β_2 -m monomer. A, time course of amyloid fibril formation monitored by ThT fluorescence in the absence (black) or presence of 1:20 α_2 M (red), Hp (blue), or BSA (green). Each point represents the average of three ThT measurements from the same sample. S.D. was <5% of each average value. This is a representative pattern of three independent experiments. B–E, electron microscopy images of the amyloid fibrils formed from β_2 -m monomer. Monomeric β_2 -m was incubated at 37 °C for 40 h in the absence (B) or presence of 1:20 α_2 M (C), Hp (D), or BSA (E). Scale bars, 100 nm.

of SDS (Fig. 4A). On the other hand, the near-UV CD spectra of both samples were clearly different from each other in the range of 250–300 nm (Fig. 4B). These data suggest the SDS-induced change in the secondary and tertiary structures of α_2 M.

To investigate whether tetrameric α_2 M is dissociated into dimers in 0.5 mM SDS, we next performed sedimentation velocity analytical ultracentrifugation. In the absence of SDS, the sedimentation coefficient of α_2 M was 17 S, consistent with a tetramer (Fig. 4C). In the presence of SDS, α_2 M existed as two major forms with sedimentation coefficients of 11 S and 17 S, corresponding to a dimer and a tetramer, respectively (Fig. 4D). These results indicate that α_2 M is partly dissociated and exists as both dimeric and tetrameric forms in the presence of 0.5 mM SDS.

The interaction between extracellular chaperones and misfolded proteins can be attributed to hydrophobic interactions (13, 14, 28). We obtained further information about the conformations of α_2 M and β_2 -m by ANS binding experiments, which are used to probe the exposure of the hydrophobic surfaces. Intriguingly, when α_2 M was incubated with 0.5 mM SDS, an

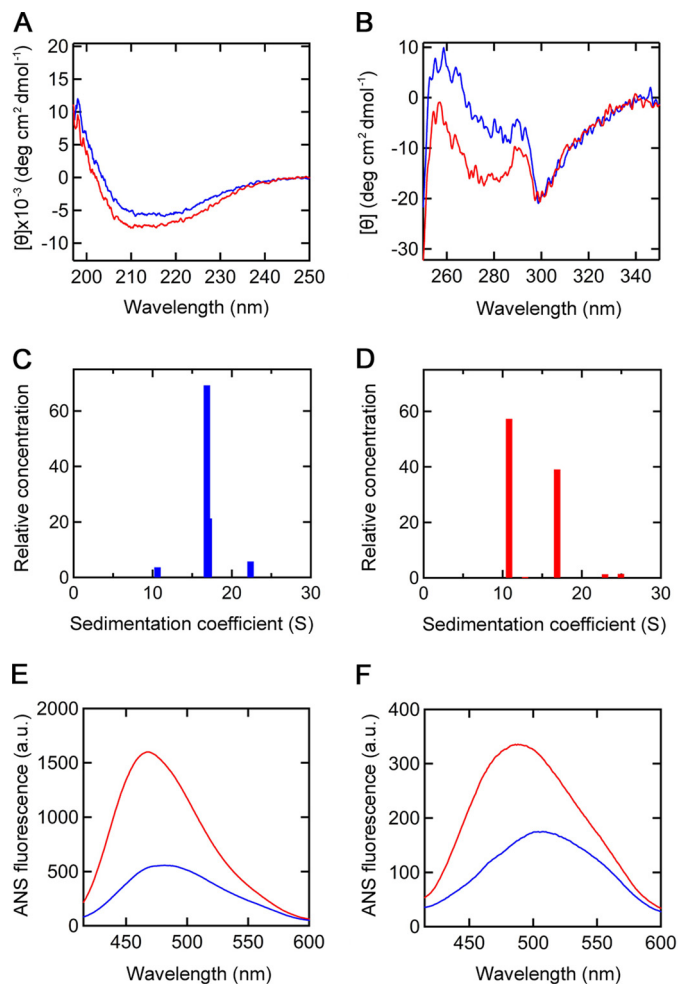


FIGURE 4. Structural analysis of α_2 M and β_2 -m in the absence and presence of SDS. A and B, far (A) and near-UV (B) CD spectra of α_2 M in the absence (blue) or presence (red) of SDS. The results are expressed in terms of mean residue ellipticity. C and D, distribution of sedimentation coefficients obtained from the sedimentation velocity measurements of α_2 M in the absence (C) and presence (D) of SDS. The centrifugation experiments were performed at $30,000 \times g$ (C) and $17,000 \times g$ (D), respectively. E and F, ANS fluorescence emission spectra of α_2 M (E) and β_2 -m (F) in the absence (blue) or presence (red) of SDS. The ANS fluorescence was measured with excitation at 350 nm.

~3-fold increase in ANS fluorescence intensity ($\lambda_{em} = 470$ nm) and a blue shift of the spectrum were observed (Fig. 4E). Jensen *et al.* (29) showed that the surface hydrophobicity of dimeric α_2 M is significantly higher than that of tetrameric α_2 M. Thus, it is reasonable to consider that SDS-induced dimerization of α_2 M (Fig. 4D) may lead to the exposure of the hydrophobic surfaces on α_2 M. Similarly, when β_2 -m was incubated with 0.5 mM SDS, an increase in ANS fluorescence intensity and a blue shift of the spectrum were observed (Fig. 4F), indicating that SDS induced the exposure of the hydrophobic surfaces on β_2 -m. These results clearly indicate that the much stronger binding of α_2 M to SDS-denatured β_2 -m than to native β_2 -m (Fig. 2) may be due to SDS-induced exposure of the hydrophobic surfaces on both α_2 M and β_2 -m.

Interaction of Tetrameric and Dimeric α_2 M with SDS-denatured β_2 -m—To demonstrate the interaction of tetrameric and dimeric α_2 M with SDS-denatured β_2 -m, we performed BS³ cross-linking experiments. When α_2 M was incubated with SDS, two bands corresponding to tetramer and dimer was iden-

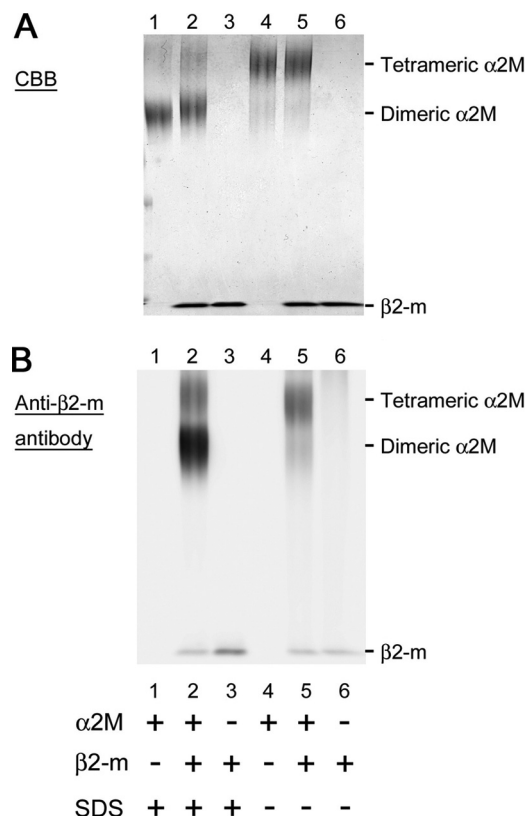


FIGURE 5. Interaction of tetrameric and dimeric α_2 M with β_2 -m as demonstrated by SDS-PAGE (A) and Western blot analysis (B). After α_2 M was incubated with β_2 -m for 1 h in the absence or presence of SDS, BS³ cross-linking reagent was added to the mixture, then SDS-PAGE was performed (A, Coomassie Brilliant Blue (CBB) staining). In Western blot analysis (B) performed in parallel, bound β_2 -m was detected with horseradish peroxidase-conjugated anti-human β_2 -m antibody followed by enhanced chemiluminescence.

tified on SDS-PAGE (Fig. 5A, lane 1). On the other hand, when α_2 M was incubated without SDS, only a band corresponding to tetramer was identified (Fig. 5A, lane 4). When α_2 M was incubated with β_2 -m in the presence of SDS, the dimeric band slightly shifted toward a higher molecular mass (Fig. 5A, lane 2). As shown in Western blot analysis performed in parallel (Fig. 5B, lane 2), this dimeric band harbored β_2 -m, indicating the interaction between SDS-denatured dimeric α_2 M and β_2 -m. Additionally, tetrameric α_2 M also harbored β_2 -m irrespective of the presence of SDS (Fig. 5B, lanes 2 and 5). The difference in the captured β_2 -m amount between SDS-denatured dimeric α_2 M and tetrameric α_2 M (Fig. 5B, lanes 2 and 5, respectively) is consistent with the difference in the apparent K_d value of α_2 M for SDS-denatured and native β_2 -m (1.4 μ M and 21.6 μ M, respectively; Fig. 2). The interaction of ferritin with β_2 -m was not observed in the presence of 0.5–2 mM SDS (supplemental Fig. S5), suggesting that the SDS-induced interaction of α_2 M with β_2 -m is not the generic hydrophobic interaction between SDS-denatured proteins.

Effect of α_2 M on Seed-dependent Growth of β_2 -m Amyloid Fibrils at an Acidic pH in the Presence of Heparin—To analyze the interaction of α_2 M with β_2 -m in other physiologically relevant *in vitro* conditions (30, 31), we next examined the effect of α_2 M on seed-dependent growth of β_2 -m amyloid fibrils at an acidic pH in the presence of heparin. When β_2 -m monomers

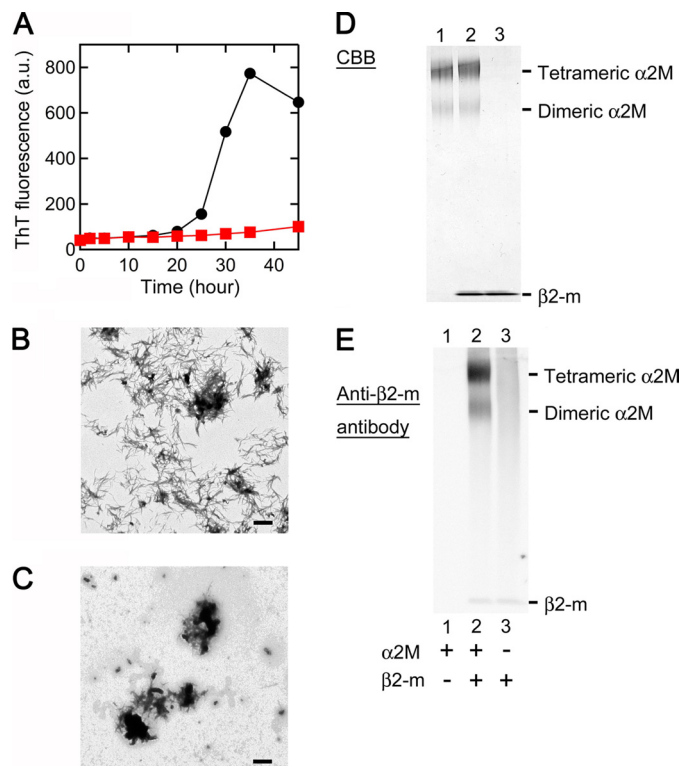


FIGURE 6. Effect of α_2 M on seed-dependent growth of β_2 -m amyloid fibrils at an acidic pH in the presence of heparin. A, time course of fibril growth monitored by ThT fluorescence in the absence (black) or presence (red) of 1:20 α_2 M. Each point represents the average of three ThT measurements from the same sample. S.D. was <5% of each average value. This is a representative pattern of three independent experiments. B and C, electron microscopy images of the samples of seed-dependent growth reaction. The sample prepared in the absence (B) or presence (C) of 1:20 α_2 M was incubated at 37 °C for 45 h. Scale bars, 1 μ m. D and E, interaction of α_2 M with β_2 -m. After α_2 M was incubated with β_2 -m for 1 h at pH 6.3 in the presence of 100 μ g/ml heparin, BS³ cross-linking reagent was added to the mixture, then SDS-PAGE (D) and Western blot analysis (E) were performed.

were incubated with seeds at pH 6.3 in the presence of 100 μ g/ml heparin and with agitation, seed-dependent growth was observed (Fig. 6, A and B). However, when incubated with α_2 M, this reaction was inhibited during a 45-h incubation (Fig. 6, A and C). As shown in SDS-PAGE (Fig. 6D) and Western blot analysis (Fig. 6E), a small proportion of tetrameric α_2 M was dissociated into dimers, and both tetramers and dimers harbored β_2 -m at pH 6.3 in the presence of heparin. As shown in Fig. 7A, pH-dependent dissociation of tetrameric α_2 M into dimers was observed in the absence of heparin. Moreover, both tetramers and dimers harbored more β_2 -m according to the reduction in pH (Fig. 7B).

DISCUSSION

In the mechanism of amyloidogenesis from natively folded proteins such as β_2 -m and transthyretin, their partial unfolding is believed to be a prerequisite for their assembly into amyloid fibrils both *in vitro* and *in vivo* (32). We propose a model of the interaction of α_2 M with β_2 -m under normal and amyloidogenic denaturing conditions (e.g. SDS, lipids, low pH) (Fig. 8). Under normal conditions, because the hydrophobic surfaces of both α_2 M and β_2 -m are not exposed, tetrameric native α_2 M interacts weakly with native β_2 -m (Fig. 8A). Under amyloido-

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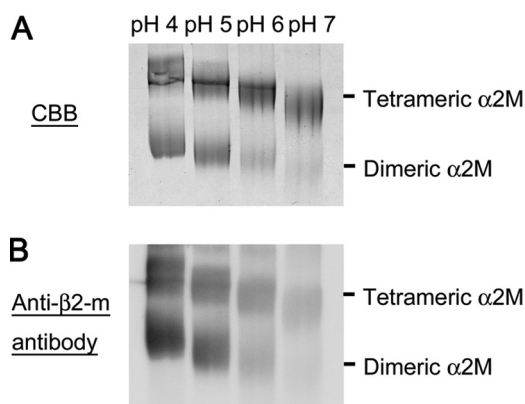


FIGURE 7. **pH-dependent dissociation of α_2 M and interaction with β_2 -m.** After α_2 M was incubated with β_2 -m for 1 h at pH 4–7 in the absence of heparin, BS³ cross-linking reagent was added to the mixture, then SDS-PAGE (A) and Western blot analysis (B) were performed.

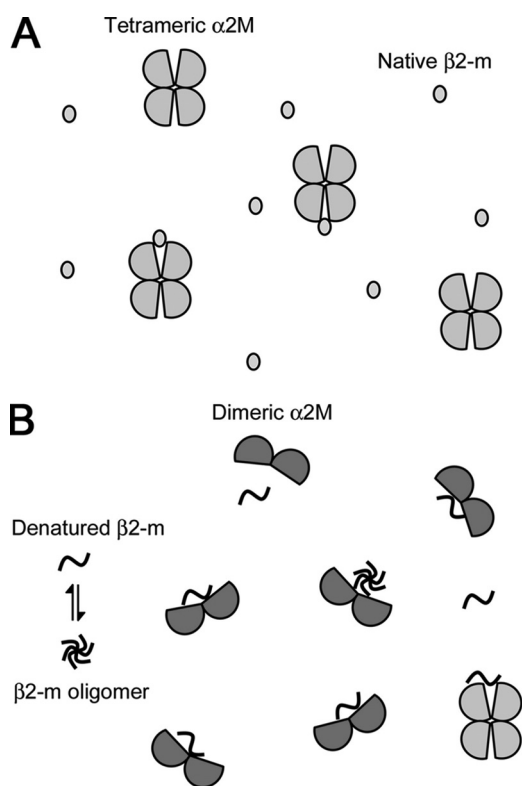


FIGURE 8. **Schematic model of the denaturation-driven interaction of α_2 M with β_2 -m.** A, under normal conditions, tetrameric native α_2 M may interact weakly with native β_2 -m. B, under amyloidogenic denaturing conditions (e.g. SDS, lipids, low pH), tetrameric α_2 M is partly converted to dimeric form. β_2 -m is also partially unfolded, and this β_2 -m conformer may weakly and reversibly aggregate into small oligomers. Because the surface hydrophobicity of dimeric α_2 M is clearly higher than that of tetrameric α_2 M, dimeric α_2 M can strongly interact with denatured monomeric/oligomeric β_2 -m with exposed hydrophobic surfaces. Tetrameric α_2 M may also interact with denatured monomeric/oligomeric β_2 -m. By binding to the denatured monomeric/oligomeric β_2 -m, α_2 M may inhibit the formation of β_2 -m amyloid fibrils.

genic denaturing conditions, tetrameric α_2 M is partly converted to dimeric form (Figs. 4–7). β_2 -m is also partially unfolded, and this β_2 -m conformer may weakly and reversibly aggregate into small oligomers via hydrophobic interactions (11, 26). Because the surface hydrophobicity of dimeric α_2 M is clearly higher than that of tetrameric α_2 M (Fig. 4) (29), dimeric

α_2 M can strongly interact with denatured monomeric/oligomeric β_2 -m with exposed hydrophobic surfaces (Fig. 8B). This scenario is consistent with the finding that the binding affinity between α_2 M and β_2 -m in the presence of SDS is higher than that in the absence of SDS (Fig. 2 and supplemental Fig. S4). At the same time, Figs. 5–7 indicate that tetrameric α_2 M may also interact with denatured monomeric/oligomeric β_2 -m, suggesting that the effect of tetrameric α_2 M on β_2 -m fibrillogenesis is not a minor one. Under amyloidogenic denaturing conditions, tetrameric α_2 M may also be partially denatured, inducing the exposure of the hydrophobic surfaces without dissociating into dimers.

Gouin-Charnet *et al.* (24) reported that several peptides from the β_2 -m sequence (e.g. Phe³⁰-Asp³⁸, Val⁴⁹-Trp⁶⁰, and Leu⁶⁴-Thr⁷³) bind to α_2 M and suggested that the α_2 M binding sites are buried in the native form of β_2 -m complexed with the heavy chain of the class I major histocompatibility complex, and β_2 -m released from the complex binds to α_2 M. Interestingly, these binding sites, especially Phe³⁰-Asp³⁸ and Leu⁶⁴-Thr⁷³, are hydrophobic regions (33). Our data suggested that under conditions where native β_2 -m is denatured, tetrameric α_2 M may also be converted to a dimeric form with exposed hydrophobic surfaces to favor the hydrophobic interaction with denatured β_2 -m, thus dimeric α_2 M as well as tetrameric α_2 M may interact with denatured β_2 -m. Based on this scenario, we attempted to identify the binding sites for α_2 M in the SDS-denatured β_2 -m molecule by NMR experiments (supplemental Fig. S4). However, when ¹⁵N-labeled β_2 -m was titrated with increasing concentrations of α_2 M, the intensities of all peaks in ¹H-¹⁵N HSQC spectra decreased simultaneously. Because α_2 M is thought to bind to a variety of proteins via hydrophobic interactions (14, 16), the result suggests that α_2 M interacts with the broad hydrophobic surfaces of SDS-denatured β_2 -m rather than the specific region of β_2 -m. Alternatively, as α_2 M is much larger than β_2 -m (360 kDa as a dimer *versus* 12 kDa), α_2 M· β_2 -m complex formation makes the tumbling of the β_2 -m molecule much slower, leading to the simultaneous decreases in peak intensities of bound β_2 -m. More elaborate NMR studies are essential to identify the specific region of β_2 -m to interact with α_2 M.

Amyloid deposits, including those found in Alzheimer disease and DRA, contain not only major components of amyloid fibrils but also many other amyloid associated molecules, e.g. glycosaminoglycans, apolipoproteins, and glycoproteins (4), suggesting that these molecules affect the deposition of amyloid fibrils. α_2 M was found in amyloid deposits from patients with DRA (22). Moreover, the complex of α_2 M with β_2 -m was detected in serum obtained from the patients (23). These data suggested that α_2 M favors the formation of β_2 -m amyloid fibrils by modifying the degradation process of β_2 -m. On the other hand, recent studies have shown that α_2 M inhibits the amyloid fibril formation of amyloid β peptide, calcitonin, and lysozyme (16). The amorphous aggregation of proteins with no connections with amyloid diseases is also prevented by α_2 M (14). These results suggest that α_2 M can protect a variety of proteins from the formation of insoluble aggregates.

In the current study, we showed that α_2 M inhibits both seed-dependent growth of β_2 -m amyloid fibrils (Figs. 1 and 6) and

amyloid fibril formation from β_2 -m monomer (Fig. 3). The growth and *de novo* formation of the fibrils were almost completely inhibited even at a 1:20 molar ratio of α_2 M to β_2 -m, suggesting that α_2 M interacts with one or more β_2 -m molecules. The dot-blot assay revealed the interaction of α_2 M with β_2 -m adopting a broad range of conformational states, including the native state, SDS-denatured state, and amyloid fibrils (Fig. 2). In the presence of SDS, β_2 -m exists as denatured monomers and oligomers (11, 26). We confirmed that under the conditions used here, SDS-denatured β_2 -m partly aggregated into small oligomers composed of 2–4 molecules (supplemental Fig. S3). Thus, α_2 M may interact not only with monomers but also with oligomers (Fig. 8B). In fact, Yerbury *et al.* (17) reported that α_2 M interacts with prefibrillar species to maintain the solubility of amyloidogenic proteins. These findings suggest that α_2 M plays an important role in controlling the amyloid fibril formation of β_2 -m by binding to denatured monomeric/oligomeric β_2 -m. In addition, it has been reported that α_2 M protects cells from the toxicity of amyloid β peptide and promotes the uptake of amyloid β peptide in macrophage-like cells (34), suggesting that α_2 M and other extracellular chaperones are important elements of a system of extracellular protein folding quality control that protects against the toxicity and accumulation of amyloid β peptide. When the efficient protein quality control machinery of α_2 M and other extracellular chaperones is overwhelmed, the denatured β_2 -m and other amyloidogenic proteins may form extracellular amyloid deposits *in vivo* (12, 16). Although α_2 M interacted with the amyloid fibrils (Fig. 2A), this complex may be too huge to be removed from the extracellular space via receptor-mediated endocytosis and lysosomal degradation, leading to the co-deposition of α_2 M with amyloid fibrils *in vivo* (22).

Mettenburg *et al.* (35) reported that a chemically stabilized preparation of human α_2 M conformational intermediates binds to natively unfolded amyloid β peptides more strongly than native α_2 M. Interestingly, Haslbeck *et al.* (36) reported that under conditions of heat stress, oligomeric small heat shock proteins display structural changes with increased surface hydrophobicity and exhibit increased chaperone activity by complexing a variety of nonnative proteins. Moreover, Poon *et al.* (37) showed that the chaperone action of clusterin is enhanced as clusterin oligomer is dissociated at mildly acidic pH. These indicate that the conformational change of α_2 M may be critical to bind to denatured proteins. Although the proteolytic cleavage in the “bait region” of α_2 M could prime the conformational changes that by modifying the dimer-dimer interaction would enhance the interaction with unfolded/misfolded proteins, the chaperone activity of α_2 M was reported to be abolished in the conformational change induced by trypsin (14).

α_2 M exists as a tetramer in human plasma and cerebrospinal fluid (13). On the other hand, tetrameric α_2 M can be dissociated into dimers *in vitro* by denaturants (38, 39), oxidative modification (40), or low pH (41, 42). We also showed that dimeric α_2 M is formed by 0.5 mM SDS (Figs. 4 and 5) or at low pH (Fig. 7). Similar to tetrameric α_2 M, dimeric α_2 M retains the ability to bind proteases (38, 42). Interestingly, the receptor binding domain of dimeric α_2 M for receptor-mediated endocytosis is

exposed irrespective of the conformational change induced by the trapping of proteases (43).

In the tenosynovial tissues of DRA patients, infiltrating macrophages into β_2 -m amyloid deposits cause local inflammation, resulting in tissue destruction and further amyloid deposition (44). At sites of inflammation, factors such as elevated temperature, reactive oxygen species, and lowered pH may cause damage to extracellular proteins, inducing them to partially unfold (14). α_2 M expression is increased in response to inflammation (45), and at localized inflammatory sites, the local pH is known to fall to <6 (46–50). This raises the possibility that α_2 M may respond to unfolded/misfolded proteins in acidic environments at inflammatory sites. In fact, pH-dependent dissociation of tetrameric α_2 M into dimers was observed (Fig. 7A) and both tetramers and dimers interacted with more β_2 -m according to the reduction in pH (Fig. 7B). This pH-dependent dissociation of α_2 M and enhancement of the chaperone activity are similar to those of clusterin (37). However, these extracellular chaperones could not prevent the progression of β_2 -m amyloid deposition *in vivo* because the efficient protein quality control machinery of extracellular chaperones might be overwhelmed in the inflammatory environments.

We cannot rule out the possibility that the stronger binding efficiency of α_2 M dimers may not actually show up *in vivo* simply because only the small amounts of dimers are present. Thus, future studies are essential to examine whether tetrameric α_2 M is converted to dimeric form and tetrameric/dimeric α_2 M interact with unfolded/misfolded proteins *in vivo* in the extracellular environment where native proteins are denatured.

In conclusion, we revealed that α_2 M inhibits the formation of β_2 -m amyloid fibrils. Moreover, we demonstrated that tetrameric and dimeric α_2 M interact with denatured β_2 -m. These results suggest that α_2 M plays an important role in controlling the abnormal aggregation of unfolded proteins in the extracellular space. Further understanding of the function of α_2 M may help the development of the therapeutics and prophylaxis of DRA and other protein deposition diseases.

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Inhibition of β_2 -Microglobulin Amyloid Fibril Formation by α_2 -Macroglobulin

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