Protease-activated alpha-2-macroglobulin can inhibit amyloid formation via two distinct mechanisms

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Abstract
α2-Macroglobulin (α2M) is an extracellular chaperone that inhibits amorphous and fibrillar protein aggregation. The reaction of α2M with proteases results in an 'activated' conformation, where the proteases become covalently-linked within the interior of a cage-like structure formed by α2M. This study investigates the effect of activation on the ability of α2M to inhibit amyloid formation by Aβ1–42 and I59T human lysozyme and shows that protease-activated α2M can act via two distinct mechanisms: (i) by trapping proteases that remain able to degrade polypeptide chains and (ii) by a chaperone action that prevents misfolded clients from continuing along the amyloid forming pathway.

Keywords
distinct, mechanisms, 2, alpha, macroglobulin, can, inhibit, activated, amyloid, protease, formation, via, two, CMMB

Disciplines
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Protease-activated alpha-2-macroglobulin can inhibit amyloid formation via two distinct mechanisms

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Abstract

$\alpha_2$-macroglobulin ($\alpha_2$M) is an extracellular chaperone that inhibits amorphous and fibrillar protein aggregation. The reaction of $\alpha_2$M with proteases results in an “activated” conformation, where the proteases become covalently-linked within the interior of a cage-like structure formed by $\alpha_2$M. This study investigates, for the first time, the effect of activation on the ability of $\alpha_2$M to inhibit amyloid formation of $\text{A}\beta_{1-42}$ and I59T human lysozyme and shows that protease-activated $\alpha_2$M can act via two distinct mechanisms: (i) by trapping proteases still able to degrade polypeptide chains and (ii) by a chaperone action that prevents misfolded clients from continuing along the amyloid forming pathway.

Keywords

$\alpha_2$-macroglobulin, extracellular chaperones, amyloid disease, human lysozyme, $\text{A}\beta_{1-42}$

Abbreviations

$\alpha_2$M, $\alpha_2$-Macroglobulin; LRP, lipoprotein receptor-related protein; trypsin-$\alpha_2$M, trypsin-activated $\alpha_2$M; (i)trypsin-$\alpha_2$M, trypsin-activated $\alpha_2$M treated with small molecule protease inhibitors; ThT, Thioflavin T.
Highlights

- $\alpha_2$M is an extracellular chaperone capable of inhibiting amorphous and fibrillar protein aggregation.

- We show that after activation of $\alpha_2$M by proteases, protease-$\alpha_2$M complexes can inhibit amyloid formation \textit{in vitro} by degrading amyloidogenic substrates or by acting as a chaperone.

- Inhibition of amyloid formation by activated $\alpha_2$M may play an important role in preventing pathological protein deposition \textit{in vivo}.
Introduction

α2-Macroglobulin is a highly abundant glycoprotein present in blood plasma, cerebrospinal fluid and other extracellular fluids. α2M is best known for its ability to trap a broad range of proteases within a cage-like quaternary structure via covalent-linkage of the protease to intramolecular thioester bonds on α2M [1]. This reaction results in a conformationally altered form commonly known as “activated” or “fast” α2M, the latter term relating to enhanced mobility via native gel electrophoresis. Activation of α2M results in the exposure of a cryptic receptor recognition site for the low-density lipoprotein receptor-related protein [1]. In addition to proteases, small nucleophiles can activate α2M by interacting directly with its thioester bonds [2].

Along with protease trapping, many other biological functions have been proposed for α2M; including roles in immunomodulation, cancer progression and extracellular proteostasis [4-6]. α2M can bind to a range of endogenous disease-associated proteins including the amyloid β peptide (Aβ1-42) [7], prion protein [8] and β2-microglobulin [9], which are the main components of deposits found in Alzheimer’s disease (AD), spongiform encephalopathies and dialysis-related amyloidosis, respectively [10]. Moreover, α2M is found to be co-localized in vivo with amyloid deposits in AD and the spongiform encephalopathies [8,11]. Recent work has shown that native α2M can act as an ATP-independent molecular chaperone by suppressing stress-induced amorphous protein aggregation [6]. The mechanism by which this occurs appears to involve the formation of stable, soluble complexes between α2M and the misfolded client proteins [6]. Native α2M has also been shown to suppress the fibril formation of a range of amyloidogenic proteins and peptides [12,13]. It has been proposed that α2M can protect against pathogenic misfolded proteins by promoting their removal from the extracellular space [7,14,15]. However, trypsin-activated α2M (trypsin-α2M) is reportedly
unable to prevent the amorphous aggregation, *in vitro*, of some proteins [6]. Nevertheless, after binding to misfolded proteins, α2M retains the ability to become activated, and α2M-trypsin-misfolded protein complexes are recognized by LRP [6], representing a potential route for the targeted disposal of misfolded proteins *in vivo*.

Activated α2M can protect cells from Aβ toxicity *in vitro* through specific binding and subsequent LRP mediated uptake and degradation of Aβ1-40[7,11,16]. While it is clear that activated α2M can bind to Aβ peptide, its ability to prevent the fibrillar aggregation of amyloid forming peptides or proteins has not been tested. To address this issue, we investigate the effect of activated α2M on the fibril formation of the amyloidogenic Aβ1-42 peptide and of a non-natural variant of human lysozyme (I59T) that possesses many attributes associated with the natural amyloidogenic variants linked to systemic amyloidosis [17].

**Materials and Methods**

Chemicals and reagents were purchased from Sigma-Aldrich Ltd. unless otherwise stated.

**Proteins and peptides**

α2M was purified from human plasma by zinc chelate affinity chromatography and size exclusion chromatography (SEC) as previously described [6]. Purified α2M was stored at 4°C (less than 2 months) and routinely examined by native polyacrylamide gel electrophoresis (PAGE) to ensure that the preparation had not become partially degraded, activated or cross-linked, modifications that can occur with prolonged storage [5,18,19]. Aβ1-42 was purchased from Biopeptide Co. Inc. or Bachem AG. Solutions of Aβ1-42 peptide were prepared by a TFA/HFIP dissolution method [20]. The non-natural variant of human lysozyme, I59T, was expressed and purified as previously described [17].

**Preparation of activated α2M**


Trypsin-α₂M was prepared by incubating α₂M with a 3-fold molar excess of bovine trypsin in PBS (pH 7.4, 25°C, 45 min). The degree of α₂M activation was assessed by NuPAGE Novex 3-8% Tris-acetate gels with Tris-glycine native running buffer (Life Technologies Ltd.). The reaction was allowed to continue for up to an additional 45 min to ensure completion. Unreacted trypsin was removed by SEC and SDS-PAGE analysis using NuPAGE Novex 4-12% Bis-Tris gels with MES running buffer (Life Technologies Ltd.) confirmed no cleavage outside the bait region had occurred. To produce enzymatically inactivated trypsin-α₂M (i.e. (i)trypsin-α₂M), trypsin-α₂M was incubated (2 h, 25°C) with excess Complete™ protease inhibitor cocktail (Roche Diagnostics Ltd.) and samples were desalted using Zeba™ desalting columns (Thermo Fisher Scientific). Ammonium chloride (NH₄Cl) activation was performed by incubating α₂M with 400 mM NH₄Cl in PBS (14 h, 25°C) and subsequently desalting as described.

**Thioflavin-T assays**

Aβ₁₋₄₂ (5 µM, PBS (pH 7.4), 50 µM ThT) was incubated in a 384 well plate (37°C, with shaking) using a FLUOstar OPTIMA fluorescence plate reader (BMG Labtech Ltd.) with excitation and emission wavelengths of 440 nm and 480 nm (slit-widths 10 nm). I59T lysozyme (6.8 µM, 0.1 M citrate buffer (pH 5.0), 25 µM ThT) was incubated with stirring at 60°C in a Cary Eclipse spectrofluorimeter (Agilent Ltd.) and ThT fluorescence intensity was monitored with excitation and emission wavelengths of 440 nm and 480 nm (slit widths 5 nm). All samples incubated with native α₂M, trypsin-α₂M, (i)trypsin-α₂M, or NH₄Cl-activated α₂M contained a molar ratio of substrate-to-α₂M of 10:1, based on the molecular weights of the α₂M tetramer (720 kDa), the Aβ₁₋₄₂ monomer (4.5 kDa) or the I59T monomer (14.7 kDa). All experiments were performed in triplicate.

**SDS-PAGE analysis**

At the endpoint of the aggregation assays, aliquots were removed and either centrifuged (10
min, 10,000g) (I59T lysozyme and Aβ1-42) or filtered (0.22 μm filter) (I59T lysozyme). For I59T lysozyme, the pellet fractions were rinsed with dH2O, centrifuged again (10 min, 10,000g) and then dissolved in 10 μL of 8M Urea solution. The supernatants and solubilised pellets were separated on 4-12% NuPAGE gels under reducing conditions. The gels were stained with Coomassie Brilliant Blue or Sigma ProteoSilver stain kit for I59T and Aβ1-42, respectively. Additionally, Aβ1-42 labelled with Hilyte™ 488 (AnaSpec) was incubated with 10:1 substrate-to-trypsin-α2M (30 min, 25°C) and centrifuged (10 min, 10,000g). The supernatants were separated on 4-12% NuPAGE gels and visualized using a Typhoon Trio Imager (GE Healthcare Ltd).

**Transmission electron microscopy**

Fibril samples (5 μL) were applied to carbon-coated nickel grids, stained with 2% (w/v) uranyl acetate, and imaged on a FEI Tecnai G2 transmission electron microscope (Multi-Imaging Unit in the Department of Physiology, Development and Neuroscience, University of Cambridge, UK). Images were analysed using the SIS Megaview II Image Capture system (Olympus).
Results

Native $\alpha_2$M has previously been shown to inhibit the amorphous and fibrillar aggregation of a range of proteins by increasing their solubility \[6,12,13,17\]. To determine if activated $\alpha_2$M can also prevent amyloid formation, we compared the effect of native $\alpha_2$M and trypsin-$\alpha_2$M on the fibril formation of I59T lysozyme and the amyloidogenic peptide A$\beta_{1-42}$. Previously reported conditions for generating trypsin-$\alpha_2$M vary greatly \[2,6,21\], therefore, in this study we used an optimized method to obtain preparations of trypsin-$\alpha_2$M that were completely activated but not degraded (Supplementary Fig. 1). The aggregation behavior of I59T lysozyme is well established and this system has been used to study the effects on fibril formation of the extracellular chaperones clusterin, haptoglobin and native $\alpha_2$M \[13, 22\]. In this study, the kinetics of the aggregation show a lag phase of ca. 50 minutes, followed by a rapid growth phase that reaches a plateau after ca. 150 minutes (Fig. 1a, black line). $\alpha_2$M, present at a molar ratio of 10:1 (lysozyme-to-$\alpha_2$M), shows a dramatic decrease in thioflavin-T (ThT) fluorescence over the course of the assay (Fig. 1a; red line). When trypsin-$\alpha_2$M is incubated with I59T lysozyme the ThT fluorescence is again, significantly suppressed (Fig. 1a; blue line). At the endpoint of the fibril formation, the presence of both native $\alpha_2$M and trypsin-$\alpha_2$M results in over a 90% decrease in ThT signal relative to the I59T lysozyme sample alone (Fig. 1b).

TEM images of the ThT assay endpoint samples demonstrate that while I59T lysozyme alone forms fibrillar structures there is no evidence for such structures when I59T lysozyme is incubated under the same conditions with native $\alpha_2$M or trypsin-$\alpha_2$M (Fig. 1c). SDS-PAGE analysis of the endpoint supernatants reveals that no detectable I59T lysozyme remains in solution when incubated alone, whereas in the presence of native $\alpha_2$M, a large majority (>90%) of lysozyme remains soluble (Fig. 1d). The I59T lysozyme also remains in the soluble fraction when incubated with trypsin-$\alpha_2$M and shows no evidence of proteolytic
degradation (Fig. 1d). Conversely, the pellet fractions (solublised with 8M urea), shows a large proportion of I59T lysozyme in the I59T alone sample (Fig. 1e, lane 1p) and only trace amounts (less than 10%) of lysozyme present in samples incubated with native α2M and trypsin-α2M (Fig. 1e, lane 2p & 3p). This finding is consistent with the fraction of the maximum ThT signal observed at the aggregation endpoints (Fig. 1b). In separate experiments, incubation of monomeric I59T lysozyme with trypsin or trypsin-α2M does not result in the appearance of any degraded protein in the soluble fractions after 120 min of incubation under the aggregation conditions used (Supplementary Fig. 2a), in addition, trypsin alone has no effect on I59T fibril formation (Supplementary Fig. 2b). However, it is noted that small quantities of protein fragments (less than 5% of total protein) are apparent in the SDS-PAGE analysis of the pellet samples after 300 min incubation. These fragments may be the result of residual trypsin-α2M activity, but they appear to be aggregation prone as they are only apparent in small quantities in the insoluble pellet sample. Taken together these results reveal that, native α2M and trypsin-α2M are able to suppress I59T fibril formation predominantly via chaperone action.

We next evaluated whether trypsin-α2M could also suppress Aβ1-42 fibril formation. Under the conditions used here, aggregation of Aβ1-42 shows a lag of ca. 70 minutes, followed by a rapid growth phase and a plateau at ca. 150 minutes (Fig. 2a, black line). Consistent with previous studies [13], the presence of native α2M at a 10:1 (Aβ1-42-to-α2M) molar ratio dramatically reduces the time-dependent increase in ThT fluorescence (Fig. 1a; red line). At the same molar ratio, the presence of trypsin-α2M also results in a suppression of ThT fluorescence (Fig 2a, solid blue line). This suppression in ThT signal is over 80% for both the presence of native α2M and trypsin-α2M at the endpoint of the assay (Fig 2b). In all samples containing Aβ1-42 there is a small ThT fluorescence signal at the start of the assay, likely due to some ThT positive aggregates being present in the stock peptide solutions. This level
remains constant over the time course for the samples containing native α₂M, but decreases slightly in the presence of trypsin-α₂M. We suspect that this may be due to the ability of trypsin-α₂M to degrade these ThT positive species.

TEM images of the ThT assay endpoint samples show that fibrillar aggregates are formed by Aβ1-42 incubated alone; however, in the presence of either native α₂M or trypsin-α₂M, the number of well-defined fibrils is reduced and aggregates appear amorphous (Fig. 2c). Analysis of the endpoint supernatants by SDS-PAGE reveals that incubation of Aβ1-42 with trypsin-α₂M results in proteolysis of the peptide (Fig. 2d, lane 3). This result is consistent with previous work showing that α₂M-trapped proteases remain active against small substrates including Aβ1-42 [23]. Therefore, it appears that trypsin-α₂M prevents Aβ1-42 fibril formation, under these conditions at least partly via degradation of the Aβ1-42 peptide to form smaller species that remain soluble. Interestingly, this mechanism for inhibiting fibril formation may not be restricted to just Aβ1-42. We have also observed that trypsin-α₂M can suppress the fibril formation of α-lactalbumin by a process which appears to involve proteolysis of the full-length protein (Supplementary Fig. 3).

Given that trypsin-α₂M can degrade polypeptides which can enter the activated α₂M cage, it is necessary to inactivate the bound trypsin to examine, in isolation, whether trypsin-α₂M possesses chaperone activity similar to native α₂M. Trypsin-α₂M and trypsin-α₂M after treatment with a small molecule protease inhibitor (i)trypsin-α₂M migrate similarly when analyzed by native PAGE, suggesting that protease inactivation does not grossly affect the structure of the covalent complex (Supplementary Fig. 1). Incubation of fluorophore-labelled Aβ1-42 with trypsin-α₂M shows that pre-treatment of the latter with protease inhibitors prevents detectable proteolysis of Aβ1-42 (Fig. 2e), however, the (i)trypsin-α₂M retains the ability to inhibit Aβ1-42 aggregation (Fig. 2a, blue circles). Significantly, analysis of the
endpoint supernatants reveals that soluble, full-length \( \text{A}\beta_{1-42} \) is present in the \( (i) \text{trypsin-} \alpha_2 \text{M} \) sample and no degradation fragments are observed (Fig. 2d, lane 4). Analysis by TEM confirms that no fibrils are present in the \( \text{A}\beta_{1-42} \) sample containing \( (i) \text{trypsin-} \alpha_2 \text{M} \) (Fig. 2c).

To confirm that chaperone activity of activated \( \alpha_2 \text{M} \) is not reliant on the presence of the bound protease, we tested the ability of \( \text{NH}_4 \text{Cl activated-} \alpha_2 \text{M} \) to suppress fibril formation. Data from aggregation assays show that \( \text{NH}_4 \text{Cl-activated } \alpha_2 \text{M} \) effectively suppresses the \( \text{ThT} \) fluorescence associated with fibril formation by \( \text{A}\beta_{1-42} \) peptide or I59T lysozyme (Fig. 1a and Fig. 2a, green lines). TEM images of the endpoint samples show that only traces of fibrillar species are present in either the \( \text{A}\beta_{1-42} \) or the I59T sample containing \( \text{NH}_4 \text{Cl-activated } \alpha_2 \text{M} \). Furthermore, SDS-PAGE analysis of the endpoint supernatants demonstrates that the presence of \( \text{NH}_4 \text{Cl-activated } \alpha_2 \text{M} \) increases the proportion of both client proteins remaining in their soluble, full-length forms at the endpoint of the assays (Fig. 1d, lane 4 and Fig. 2d lane 5). These results confirm that activated \( \alpha_2 \text{M} \) can influence the solubility of polypeptides regardless of whether or not it is complexed to a protease molecule.

**Discussion**

In the work presented here, we show that activated \( \alpha_2 \text{M} \), despite a large conformational change upon activation, retains the ability to suppress fibril formation. From earlier work, it is clear that \( \alpha_2 \text{M} \) has distinct binding sites for protease and misfolded proteins as the binding of a misfolded client protein does not prevent protease trapping [7]. In the current study, we demonstrate that the presence of bound protease, regardless of whether the protease is pharmacologically inhibited, does not significantly reduce chaperone activity of \( \alpha_2 \text{M} \). Moreover, \( \alpha_2 \text{M} \) remains an active chaperone after direct activation using small molecules.
In vivo, activated α2M is rapidly cleared from circulation [2] and typically represents only 0.17-0.7% of the total α2M in blood plasma of adults [24]. The activated α2M plasma concentration is, however, increased in many disease states including pancreatitis, multiple sclerosis and sepsis [24-26]. Moreover, the onset of some diseases, such as periodontitis, diabetic retinopathy and inflammatory joint disease results in increased activated α2M levels in other extracellular fluids [27,28,29]. Although enhanced concentrations of activated α2M have been largely attributed to increased protease trapping, it has been reported that interaction with proteases only partially accounts for the total activated α2M present in synovial fluid [29]; higher levels of both protease-activated and amine-activated α2M may therefore be significant for facilitating clearance of aberrant clients via LRP. Interestingly, aggregates of Aβ1-40 and amylin have been shown to activate the plasmin protease system [30]. Thus it is possible that concentrations of plasmin-activated α2M may also be increased in response to the accumulation of misfolded proteins.

In conclusion, we provide evidence that protease-activated α2M has two distinct mechanisms for inhibiting amyloid formation: (i) via protease-α2M-mediated degradation of amyloidogenic substrates and (ii) by a chaperone action that prevents misfolded clients from continuing along the amyloid forming pathway. In the absence of proteases, activated α2M is able to inhibit fibril formation via the latter function only. It is tempting to speculate that the chaperone activity of protease-activated α2M may target misfolded proteins to the trapped protease, thereby providing a specific mechanism for degradation of amyloidogenic proteins in extracellular fluids. Clearly, further studies are required to substantiate this proposition; however, a greater understanding of the mechanisms by which α2M is able to prevent protein aggregation and facilitate the disposal of misfolded peptide and protein molecules could, in future, provide potential therapeutic targets for amyloidosis.
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Figure 1: Effects of α2M variants on I59T lysozyme fibril formation

a) *In vitro* fibril formation of I59T lysozyme incubated alone (black), with native α2M (red), with trypsin-α2M (blue) or with NH4Cl-activated α2M (green) using α2M-to-lysozyme molar ratios of 1:10. b) Percent of maximum ThT signal at the endpoint of aggregation. Each bar represents an average of three individual experiments. c) TEM analysis of the endpoint samples in the absence or presence of the different α2M variants, with scale bars representing 100 nm and numbering corresponds to the lanes in gel analysis. c) SDS-PAGE of the endpoint supernatants shows no soluble protein in I59T lysozyme incubated alone (1), whereas soluble protein is present for samples containing native α2M (2), trypsin-α2M (3) and NH4Cl-activated α2M (4) and soluble I59T lysozyme is shown in lane S and lane M shows molecular mass markers. d) SDS-PAGE of the solubilised endpoint pellets showing significant protein present for I59T incubated alone (1p), and trace protein present for samples containing native α2M (2p), trypsin-α2M (3p) and NH4Cl-activated α2M (4p).

Figure 2: Effects of α2M variants on Aβ1-42 fibril formation

a) *In vitro* fibril formation of Aβ1-42, incubated alone (black), with native α2M (red), with trypsin-α2M (blue), with (i)trypsin-α2M (blue circles) or with NH4Cl-activated α2M (green), using α2M-to-Aβ1-42 molar ratios of 1:10. b) Percent of maximum ThT signal at the endpoint of aggregation. Each bar represents the average of three individual experiments. c) TEM analysis of the endpoint samples in the absence or presence of the different α2M variants, with scale bars representing 100 nm and numbering corresponds to the lanes in gel analysis. d) SDS-PAGE analysis of the endpoint supernatants shows no soluble protein for Aβ1-42 incubated alone (1), but soluble peptide present when incubated with native α2M (2). Incubation with trypsin-α2M (3) results in no full-length Aβ1-42 peptide, whereas the
incubation with (i)trypsin-α2M (4) and NH₄Cl-activated α2M (5) have full-length peptide present. Lane M shows molecular mass markers. d) Fluorescence image of SDS-PAGE analysis of HiLyte-488 labelled Aβ1-42, alone (lane 1) and after incubation with trypsin-α2M (lane 2) or (i)trypsin-α2M (lane 3). The presence of trypsin-α2M results in an increase in Aβ1-42 fragments (red arrow).
Supplementary Figure 1: Effect of activation on the migration of α₂M by native PAGE analysis
The gel shows the migration of native α₂M (lane 1), trypsin-α₂M (lane 2), (i)trypsin-α₂M (lane 3) and NH₄Cl-activated α₂M (lane 4) on a 3-8% Tris-acetate gel after electrophoresis at 150 V for 1.5 hr.
Supplementary Figure 2:

a) SDS-PAGE analysis of I59T lysozyme samples which were incubated at 37°C or 60°C for 2 hr in the absence (lanes 1 and 4) and presence of trypsin (lanes 2 and 5) or trypsin-α2M (lanes 3 and 6). The molar ratios of trypsin-to-I59T and trypsin-α2M-to-I59T were 1:5 and 1:10 respectively. The gel was run under reducing conditions to confirm that no degradation of full length I59T protein had occurred under either set of conditions. 

b) In vitro fibril formation (60°C, pH 5.0) of I59T lysozyme (solid line) in the absence or presence of trypsin (dashed line) at a 1:10 (trypsin-to-lysozyme) molar ratio as monitored by light scattering. The presence of trypsin does not significantly affect the overall fibril formation of I59T lysozyme.
Supplementary Figure 3: Effects of native and trypsin-α2M on fibril formation by α-lactalbumin

a) *In vitro* fibril formation (37°C, pH 7.4) of α-lactalbumin (α-lact) alone (black) or in the presence of native α2M (red) or trypsin-α2M (blue) as monitored by ThT fluorescence. The molar ratio of α2M-to-α-lact used was 1:10. The experiment was performed in triplicate. b) SDS-PAGE analysis of the endpoint samples. Some soluble protein remains in the sample containing α-lact alone, but more full-length, soluble α-lact is present in the sample containing native α2M. After co-incubation with trypsin α2M, SDS-PAGE analysis shows less full-length α-lact than does a corresponding sample of α-lact after co-incubation with plasmin-α2M.