



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



Amy R. Wyatt^{a,b}, Patrick Constantinescu^a, Heath Ecroyd^a, Christopher M. Dobson^b, Mark R. Wilson^a, Janet R. Kumita^{b,*}, Justin J. Yerbury^{a,*}

^a Illawarra Health & Medical Research Institute, School of Biological Sciences, University of Wollongong, Northfields Avenue, Wollongong, NSW 2522, Australia

^b Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK

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ABSTRACT

α_2 -Macroglobulin (α_2M) is an extracellular chaperone that inhibits amorphous and fibrillar protein aggregation. The reaction of α_2M with proteases results in an ‘activated’ conformation, where the proteases become covalently-linked within the interior of a cage-like structure formed by α_2M . This study investigates, the effect of activation on the ability of α_2M to inhibit amyloid formation by $A\beta_{1-42}$ and I59T human lysozyme and shows that protease-activated α_2M 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.

Structured summary of protein interactions:

$A\beta_{1-42}$ and $A\beta_{1-42}$ bind by fluorescence technology (View interaction)

I59T lysozyme and I59T lysozyme bind by light scattering (View interaction)

I59T lysozyme and I59T lysozyme bind by fluorescence technology (View interaction)

Alpha-lactalbumin and Alpha-lactalbumin bind by fluorescence technology (View interaction)

I59T lysozyme and I59T lysozyme bind by electron microscopy (View interaction)

$A\beta_{1-42}$ and $A\beta_{1-42}$ bind by electron microscopy (View interaction)

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1. Introduction

α_2 -Macroglobulin is a highly abundant glycoprotein present in blood plasma, cerebrospinal fluid and other extracellular fluids. α_2M 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 α_2M [1]. This reaction results in a conformationally altered form commonly known as “activated” or “fast” α_2M , the latter term relating to enhanced mobility via native gel electrophoresis. Activation of α_2M results in the exposure of a cryptic receptor recognition site for the low-density lipoprotein receptor-related protein (LRP) [1]. In addition to proteases, small nucleophiles can activate α_2M by interacting directly with its thioester bonds [2].

Abbreviations: α_2M , α_2 -macroglobulin; LRP, lipoprotein receptor-related protein; trypsin- α_2M , trypsin-activated α_2M ; (i)trypsin- α_2M , trypsin-activated α_2M treated with small molecule protease inhibitors; ThT, thioflavin T

* Corresponding authors. Fax: +44 (0) 1223 336362 (J.R. Kumita), fax: +61 2 42218130 (J.J. Yerbury).

E-mail addresses: jrk38@cam.ac.uk (J.R. Kumita), jyerbury@uow.edu.au (J.J. Yerbury).

Along with protease trapping, many other biological functions have been proposed for α_2M ; including roles in immunomodulation, cancer progression and extracellular proteostasis [3–5]. α_2M can bind to a range of endogenous disease-associated proteins including the amyloid β peptide ($A\beta_{1-42}$) [6], prion proteins [7] and β_2 -microglobulin [8], which are the main components of deposits found in Alzheimer’s disease (AD), spongiform encephalopathies and dialysis-related amyloidosis, respectively [9]. Moreover, α_2M is found to be co-localized in vivo with amyloid deposits in AD and the spongiform encephalopathies [7,10]. Recent work has shown that native α_2M can act as an ATP-independent molecular chaperone by suppressing stress-induced amorphous protein aggregation [5]. The mechanism by which this occurs appears to involve the formation of stable, soluble complexes between α_2M and the misfolded client proteins [5]. Native α_2M has also been shown to suppress the fibril formation of a range of amyloidogenic proteins and peptides [11,12]. It has been proposed that α_2M can protect against pathogenic misfolded proteins by promoting their removal from the extracellular space [6,13,14]. However, trypsin-activated α_2M (trypsin- α_2M) is reportedly unable to prevent the amorphous aggregation, in vitro, of some proteins [5].

Nevertheless, after binding to misfolded proteins, α_2 M retains the ability to become activated, and α_2 M-trypsin-misfolded protein complexes are recognized by LRP [5], representing a potential route for the targeted disposal of misfolded proteins in vivo.

Activated α_2 M can protect cells from A β toxicity in vitro through specific binding and subsequent LRP mediated uptake and degradation of A β_{1-40} [6,10,15]. While it is clear that activated α_2 M can bind to A β 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 α_2 M on the fibril formation of the amyloidogenic A β_{1-42} 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 [16].

2. Materials and methods

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

2.1. Proteins and peptides

α_2 M was purified from human plasma by zinc chelate affinity chromatography and size exclusion chromatography (SEC) as previously described [5]. Purified α_2 M was stored at 4 °C (for less than 2 months) and routinely examined prior to use 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 [4,17,18]. A β_{1-42} was purchased from Biopeptide Co. Inc. or Bachem AG. Solutions of A β_{1-42} peptide were prepared by a TFA/HFIP dissolution method [19]. The non-natural variant of human lysozyme, I59T, was expressed and purified as previously described [16].

2.2. Preparation of activated α_2 M

Trypsin- α_2 M was prepared by incubating α_2 M with a threefold molar excess of bovine trypsin in PBS (pH 7.4, 25 °C, 45 min). The degree of α_2 M 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 that no cleavage outside the bait region had occurred. To produce enzymatically inactivated trypsin- α_2 M (i.e. (i)trypsin- α_2 M), trypsin- α_2 M 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 α_2 M with 400 mM NH₄Cl in PBS (14 h, 25 °C) and subsequently desalting as described.

2.3. Thioflavin-T assays

A β_{1-42} (5 μ M, PBS (pH 7.4), 50 μ M 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 μ M, 0.1 M citrate buffer (pH 5.0), 25 μ M 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 α_2 M, trypsin- α_2 M, (i)trypsin- α_2 M, or NH₄Cl-activated α_2 M contained a molar ratio of substrate-to- α_2 M of 10:1, based on the

molecular weights of the α_2 M tetramer (720 kDa), the A β_{1-42} monomer (4.5 kDa) or the I59T monomer (14.7 kDa). All experiments were performed in triplicate.

2.4. SDS–PAGE analysis

At the endpoints of the aggregation assays, aliquots were removed and either centrifuged (10 min, 10000 \times g) (I59T lysozyme and A β_{1-42}) or filtered (0.22 μ m filter) (I59T lysozyme). For I59T lysozyme, the pellet fractions were rinsed with dH₂O, centrifuged again (10 min, 10000 \times g) and then dissolved in 10 μ l of 8 M 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 A β_{1-42} , respectively. Additionally, A β_{1-42} labelled with Hilyte™ 488 (AnaSpec) was incubated with 10:1 substrate-to-trypsin- α_2 M (30 min, 25 °C) and centrifuged (10 min, 10000 \times g). The supernatants were separated on 4–12% NuPAGE gels and visualized using a Typhoon Trio Imager (GE Healthcare Ltd).

2.5. Transmission electron microscopy (TEM)

Fibril solutions (5 μ l) 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 analyzed using the SIS Megaview II Image Capture system (Olympus).

3. Results

Native α_2 M has previously been shown to inhibit the amorphous and fibrillar aggregation of a range of proteins by increasing their solubility [5,11,12,16]. To determine if activated α_2 M can also prevent amyloid formation, we compared the effect of native α_2 M and trypsin- α_2 M on the fibril formation of I59T lysozyme and the amyloidogenic peptide A β_{1-42} . Previously reported conditions for generating trypsin- α_2 M vary greatly [2,5,20]; therefore, in this study we used an optimized method to obtain preparations of trypsin- α_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 α_2 M [12,21]. In this study, the kinetics of aggregation show a lag phase of ca. 50 min, followed by a rapid growth phase that reaches a plateau after ca. 150 min (Fig. 1a, black line). α_2 M, present at a molar ratio of 10:1 (lysozyme-to- α_2 M), results in a dramatic decrease in thioflavin-T (ThT) fluorescence over the course of the assay (Fig. 1a; red line). When trypsin- α_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 α_2 M and trypsin- α_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 α_2 M or trypsin- α_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 α_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- α_2 M and shows no evidence of proteolytic degradation (Fig. 1d).

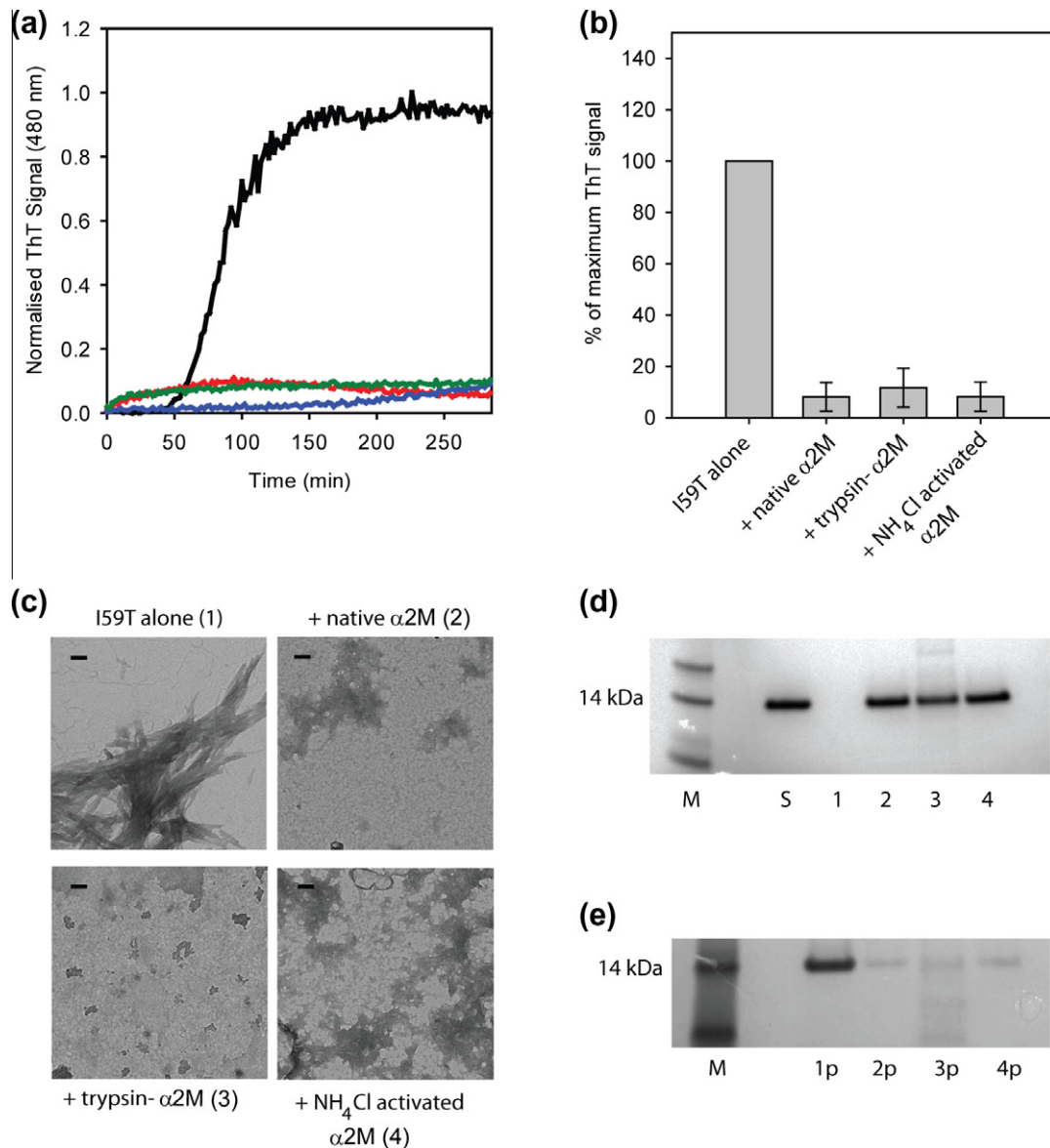


Fig. 1. Effects of α_2M variants on I59T lysozyme fibril formation. (a) In vitro fibril formation of I59T lysozyme incubated alone (black), with native α_2M (red), with trypsin- α_2M (blue) or with NH_4Cl -activated α_2M (green) using α_2M -to-lysozyme molar ratios of 1:10. (b) Percent of maximum ThT 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 α_2M variants, with scale bars representing 100 nm and numbers corresponding to the lanes in gel analysis. (d) 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 α_2M (2), trypsin- α_2M (3) and NH_4Cl -activated α_2M (4). Soluble I59T lysozyme is shown in lane S and lane M shows molecular mass markers. (e) SDS-PAGE of the solubilized endpoint pellets showing a significant quantity of protein present for I59T incubated alone (1p), and also trace quantities of protein present for samples containing native α_2M (2p), trypsin- α_2M (3p) and NH_4Cl -activated α_2M (4p).

Conversely, the pellet fractions (solubilized with 8 M 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 α_2M and trypsin- α_2M (Fig. 1e, lane 2p and 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- α_2M 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% 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- α_2M 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 α_2M and trypsin- α_2M are able to suppress I59T fibril formation predominantly via chaperone action.

We next evaluated whether trypsin- α_2M could also suppress $A\beta_{1-42}$ fibril formation. Under the conditions used here, aggregation of $A\beta_{1-42}$ shows a lag of ca. 70 min, followed by a rapid growth phase and a plateau at ca. 150 min (Fig. 2a, black line). Consistent with previous studies [12], the presence of native α_2M at a 10:1 ($A\beta_{1-42}$ -to- α_2M) molar ratio dramatically reduces the time-dependent increase in ThT fluorescence (Fig. 1a; red line). At the same molar ratio, the presence of trypsin- α_2M 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 α_2M and

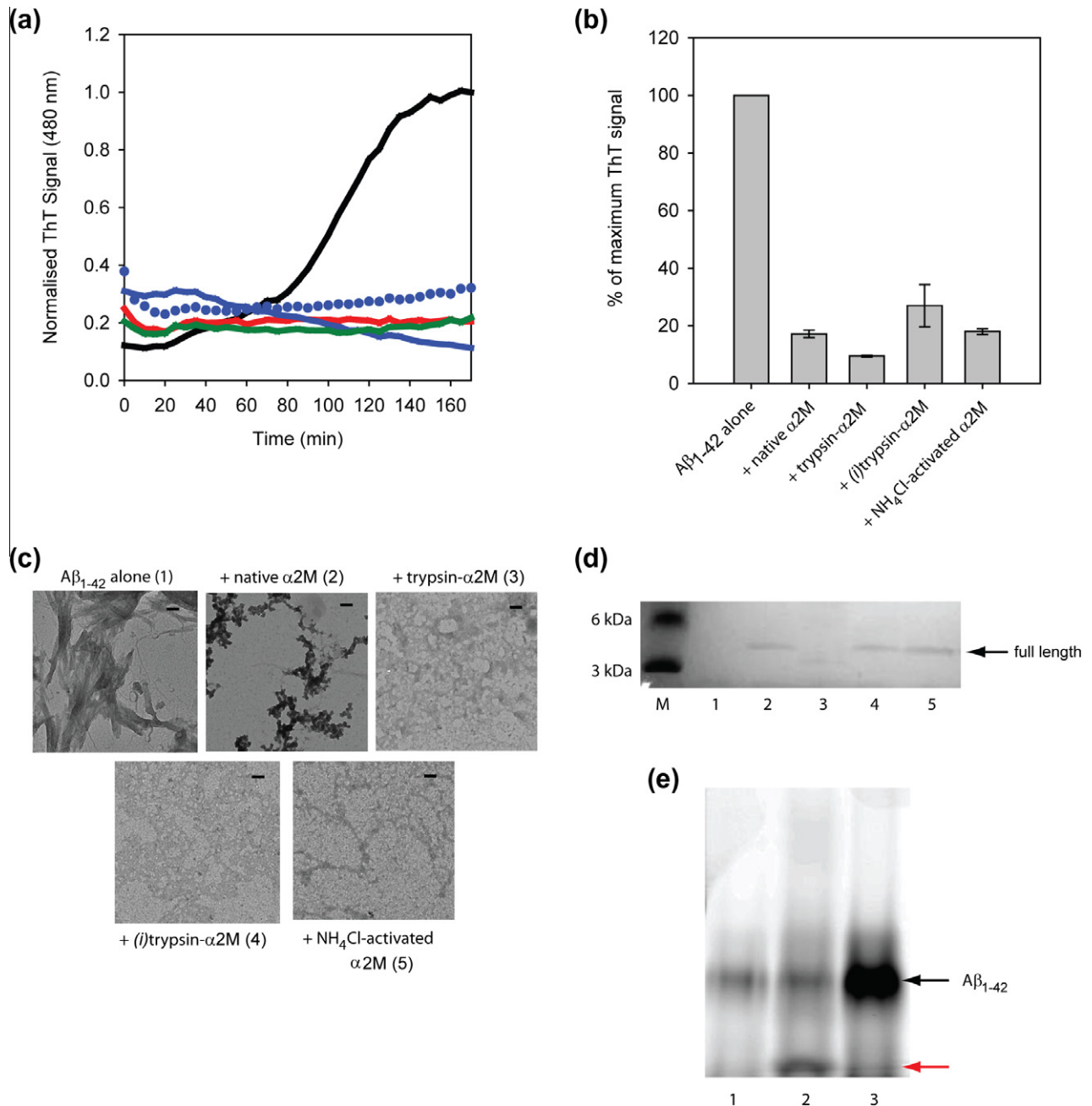


Fig. 2. Effects of α_2 M variants on $A\beta_{1-42}$ fibril formation. (a) In vitro fibril formation of $A\beta_{1-42}$, incubated alone (black), with native α_2 M (red), with trypsin- α_2 M (blue), with (i)trypsin- α_2 M (blue circles) or with NH_4Cl -activated α_2 M (green), using α_2 M-to- $A\beta_{1-42}$ 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 α_2 M variants, with scale bars representing 100 nm and numbers corresponding to the lanes in gel analysis. (d) SDS-PAGE analysis of the endpoint supernatants shows no soluble protein for $A\beta_{1-42}$ incubated alone (1), but soluble peptide present when incubated with native α_2 M (2). Incubation with trypsin- α_2 M (3) results in no full-length $A\beta_{1-42}$ peptide, whereas the incubation with (i)trypsin- α_2 M (4) and NH_4Cl -activated α_2 M (5) have full-length peptide present. Lane M shows molecular mass markers. (e) Fluorescence image of SDS-PAGE analysis of HiLyte-488 labelled $A\beta_{1-42}$, alone (lane 1) and after incubation with trypsin- α_2 M (lane 2) or (i)trypsin- α_2 M (lane 3). The presence of trypsin- α_2 M results in an increase in $A\beta_{1-42}$ fragments (red arrow).

trypsin- α_2 M at the endpoint of the assay (Fig. 2b). In all samples containing $A\beta_{1-42}$ there is a small ThT fluorescence signal at the start of the assay, likely to be 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 α_2 M, but decreases slightly in the presence of trypsin- α_2 M. We suspect that this may be due to the ability of trypsin- α_2 M to degrade these ThT positive species.

TEM images of the ThT assay endpoint samples show that fibrillar aggregates are formed by $A\beta_{1-42}$ incubated alone; however, in

the presence of either native α_2 M or trypsin- α_2 M, the number of well-defined fibrils is reduced and most aggregates appear to be amorphous (Fig. 2c). Analysis of the endpoint supernatants by SDS-PAGE reveals that incubation of $A\beta_{1-42}$ with trypsin- α_2 M results in proteolysis of the peptide (Fig. 2d, lane 3). This result is consistent with previous work showing that α_2 M-trapped proteases remain active against small substrates including $A\beta_{1-42}$ [22]. Therefore, it appears that trypsin- α_2 M prevents $A\beta_{1-42}$ fibril formation, under these conditions at least partly via degradation of the $A\beta_{1-42}$ peptide to form smaller species that remain soluble.

Interestingly, this mechanism for inhibiting fibril formation may not be restricted to just $A\beta_{1-42}$. We have also observed that trypsin- α_2M can suppress the fibril formation of reduced and carbonylmethylated α -lactalbumin by a process which involves proteolysis of the full-length protein (Supplementary Fig. 3).

Given that trypsin- α_2M can degrade polypeptides which can enter the activated α_2M cage, it is necessary to inactivate the bound trypsin to examine, in isolation, whether trypsin- α_2M possesses chaperone activity similar to native α_2M . Trypsin- α_2M and trypsin- α_2M after treatment with a small molecule protease inhibitor (*i*)trypsin- α_2M 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 $A\beta_{1-42}$ with trypsin- α_2M shows that pre-treatment of the latter with protease inhibitors prevents detectable proteolysis of $A\beta_{1-42}$ (Fig. 2e), however, the (*i*)trypsin- α_2M retains the ability to inhibit $A\beta_{1-42}$ aggregation (Fig. 2a, blue circles). Significantly, analysis of the endpoint supernatants reveals that soluble, full-length $A\beta_{1-42}$ is present in the (*i*)trypsin- α_2M sample and no degradation fragments are observed (Fig. 2d, lane 4). Analysis by TEM confirms that no fibrils are present in the $A\beta_{1-42}$ sample containing (*i*)trypsin- α_2M (Fig. 2c).

To confirm that chaperone activity of activated α_2M is not reliant on the presence of the bound protease, we tested the ability of NH_4Cl activated- α_2M to suppress fibril formation. Data from aggregation assays show that NH_4Cl -activated α_2M effectively suppresses the ThT fluorescence associated with fibril formation by $A\beta_{1-42}$ 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 $A\beta_{1-42}$ or the I59T sample containing NH_4Cl -activated α_2M . Furthermore, SDS-PAGE analysis of the endpoint supernatants demonstrates that the presence of NH_4Cl -activated α_2M 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 α_2M can influence the solubility of polypeptides regardless of whether or not it is complexed to a protease molecule.

4. Discussion

In the work presented here, we show that activated α_2M , despite a large conformational change upon activation, retains the ability to suppress fibril formation. From earlier work, it is clear that α_2M has distinct binding sites for proteases and misfolded proteins as the binding of a misfolded client protein does not prevent protease trapping [6]. In the current study, we demonstrate that the presence of a bound protease, regardless of whether or not the protease is pharmacologically inhibited, does not significantly reduce chaperone activity of α_2M . Moreover, α_2M remains an active chaperone after direct activation using small molecules.

In vivo, activated α_2M is rapidly cleared from circulation [2] and typically represents only 0.17–0.7% of the total α_2M in blood plasma of adults [23]. The activated α_2M plasma concentration is, however, increased in many disease states including pancreatitis, multiple sclerosis and sepsis [23–25]. Moreover, the onset of some diseases, such as periodontitis, diabetic retinopathy and inflammatory joint disease results in increased activated α_2M levels in other extracellular fluids [26–28]. Although enhanced concentrations of activated α_2M have been largely attributed to increased protease trapping, it has been reported that interaction with proteases only partially accounts for the total activated α_2M present in synovial fluid [28]; higher levels of both protease-activated and amine-activated α_2M may therefore be significant for facilitating clearance of aberrant clients via LRP. Interestingly, aggregates of $A\beta_{1-40}$ and amylin have been shown to activate the plasmin protease system

[29]. Thus it is possible that concentrations of plasmin-activated α_2M may also be increased in response to the accumulation of misfolded proteins.

In conclusion, we provide evidence that protease-activated α_2M has two distinct mechanisms for inhibiting amyloid formation: (i) via protease- α_2M -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 α_2M is able to inhibit fibril formation via the latter function only. It is tempting to speculate that the chaperone activity of protease-activated α_2M 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 α_2M 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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.febslet.2013.01.020>.

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