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

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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)

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].

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].

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

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Nevertheless, after binding to misfolded proteins, α_2M retains the ability to become activated, and α_2M-trypsin-misfolded protein complexes are recognized by LRP [5], representing a potential route for the targeted disposal of misfolded proteins in vivo.

Activated α_2M can protect cells from Aβ toxicity in vitro through specific binding and subsequent LRP mediated uptake and degradation of $A\beta_{1-40}$ [6,10,15]. While it is clear that activated α_2M 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 α_2M on the fibril formation of the amyloidogenic $A\beta_{1-42}$ 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_2M was purified from human plasma by zinc chelate affinity chromatography and size exclusion chromatography (SEC) as previously described [5]. Purified α_2M 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]. $A\beta_{1-42}$ was purchased from Biopeptide Co. Inc. or Bachem AG. Solutions of $A\beta_{1-42}$ 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_2M

Trypsin-α_2M was prepared by incubating α_2M with a threefold molar excess of bovine trypsin in PBS (pH 7.4, 25 °C, 45 min). The degree of α_2M 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_2M (i.e. (*i*)trypsin-α_2M), trypsin-α_2M 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 (NH_4Cl) activation was performed by incubating α_2M with 400 mM NH_4Cl in PBS (14 h, 25 °C) and subsequently desalting as described.

2.3. Thioflavin-T assays

$A\beta_{1-42}$ (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 α_2M, trypsin-α_2M, (*i*)trypsin-α_2M, or NH_4Cl-activated α_2M contained a molar ratio of substrate-to-α_2M of 10:1, based on the molecular weights of the α_2M tetramer (720 kDa), the $A\beta_{1-42}$ 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×*g*) (I59T lysozyme and $A\beta_{1-42}$) or filtered (0.22 μm filter) (I59T lysozyme). For I59T lysozyme, the pellet fractions were rinsed with dH_2O, centrifuged again (10 min, 10000×*g*) and then dissolved in 10 μl 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 $A\beta_{1-42}$, respectively. Additionally, $A\beta_{1-42}$ labelled with Hilyte™ 488 (AnaSpec) was incubated with 10:1 substrate-to-trypsin-α_2M (30 min, 25 °C) and centrifuged (10 min, 10000×*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 μl) were applied to carbon-coated nickel grids, stained with 2% (w/v) uranyl acetate, and imaged on a FEI Tecnai G_2 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_2M 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_2M can also prevent amyloid formation, we compared the effect of native α_2M and trypsin-α_2M on the fibril formation of I59T lysozyme and the amyloidogenic peptide $A\beta_{1-42}$. Previously reported conditions for generating trypsin-α_2M vary greatly [2,5,20]; therefore, in this study we used an optimized method to obtain preparations of trypsin-α_2M 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_2M [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_2M, present at a molar ratio of 10:1 (lysozyme-to-α_2M), results in a dramatic decrease in thioflavin-T (ThT) fluorescence over the course of the assay (Fig. 1a; red line). When trypsin-α_2M 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_2M and trypsin-α_2M 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_2M or trypsin-α_2M (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_2M, a large majority (>90%) of lysozyme remains soluble (Fig. 1d). The I59T lysozyme also remains in the soluble fraction when incubated with trypsin-α_2M and shows no evidence of proteolytic degradation (Fig. 1d).