Protease Activation of α_2-Macroglobulin Modulates a Chaperone-like Action with Broad Specificity

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ABSTRACT: α_2-Macroglobulin (α_2M) is a major human blood glycoprotein best known for its ability to inhibit a broad spectrum of proteases by a unique trapping method. This action induces an "activated" conformation of α_2M with an exposed binding site for the low-density lipoprotein receptor, facilitating clearance of α_2M/protease complexes from the body. This report establishes that protease activation also modulates a potent chaperone-like action of α_2M that has broad specificity for proteins partly unfolded as a result of heat or oxidative stress. Protease-mediated activation of α_2M abolishes its chaperone-like activity. However, native α_2M is able to form soluble complexes with stressed proteins and then subsequently become activated by interacting with a protease, providing a potential mechanism for the in vivo clearance of α_2M/stressed protein/protease complexes. We propose that α_2M is a newly discovered and unique member of a small group of abundant extracellular proteins with chaperone properties that patrol extracellular spaces for unfolded/misfolded proteins and facilitate their disposal.

α_2-Macroglobulin (α_2M)[11] is a major human blood glycoprotein assembled from four identical 180 kDa subunits into a 720 kDa tetramer. The 180 kDa subunits are disulfide bonded to form dimers, which noncovalently interact to yield the final tetrameric quaternary structure (*1*). α_2M is well known for its ability to inhibit a broad spectrum of proteases, which it accomplishes using a unique trapping method. When exposed to a protease, α_2M undergoes limited proteolysis at its bait region leading to a large irreversible conformational change, physically trapping the protease within a steric "cage" (*2*). The trapped protease forms a covalent linkage with α_2M by reacting with an intramolecular thiol ester bond to yield "activated" α_2M (α_2M*), which exposes a receptor recognition site for low-density lipoprotein receptor related protein (LRP); in vivo, the α_2M*/protease complex is cleared by LRP-mediated endocytosis and subsequently degraded (*2*). By directly interacting with the thiol ester bond, small nucleophiles such as methylamine can also activate α_2M (*3*).

Although human α_2M is best known for its protease inhibitor function, it has also been shown to bind to and promote clearance of other endogenous and exogenous molecules, consistent with a broader protective function. α_2M is known to bind to cytokines and growth factors (without converting to α_2M*), including transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), interleukin 8 (IL-8), platelet-derived growth factor-BB (PDGF-BB), nerve growth factor-β (NGF-β), and vascular endothelial growth factor (VEGF) (reviewed in refs *4* and *5*). The affinity of α_2M for most cytokines is higher in the activated state, and while in this state α_2M can deliver them via receptor-mediated endocytosis to lysosomes for degradation (*6*). In addition, α_2M has been shown to bind to the pathogen *Trypanosoma cruzi* and promote its phagocytosis (*7*). α_2M is also known to bind to endogenous disease-associated proteins, including the Aβ peptide associated with Alzheimer's disease (*8*, *9*), β_2-microglobulin which forms insoluble deposits in dialysis-related amyloidosis (*10*), and prion protein associated with plaques in Creutzfeldt−Jakob disease (*11*). Interestingly, α_2M has been shown to suppress the aggregation of Aβ and in association with the α_2M receptor, LRP, protect cells from its toxicity (*8*, *12*−*14*). Previous work has indicated that α_2M complexed to either protein or peptide ligands is immunogenic (*15*−*18*). α_2M-bound peptides are internalized by LRP, and fragments of the peptide are subsequently re-presented on the cell surface. This response is identical to the one elicited by peptides chaperoned by intracellular heat shock proteins (*19*). Collectively, the abilities of α_2M to bind many diverse ligands (*6*), inhibit Aβ aggregation, and influence the immune response to peptides prompted us to examine whether α_2M might be a novel member of a small group of abundant extracellular proteins with chaperone properties ("extracellular chaperones"; ECs) that have been proposed as major elements of a quality control system for the folding state of proteins in extracellular body fluids (*20*). There are a number of similarities between α_2M and the previously identified ECs clusterin (*21*) and haptoglobin (*22*). α_2M, clusterin, and haptoglobin are all secreted glycoproteins with distant evolutionary relationships to complement (*23*−*25*). In addition, all three are (i) structurally composed of disulfide linked subunits (*1*, *21*, *24*), (ii) abundant in human plasma (α_2M 2−4 mg/mL (*2*), clusterin 50−370 μg/mL (*26*), and

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[1] Abbreviations: α_2-Macroglobulin, α_2M; *N*-α-benzoyl-DL-arginine-*p*-nitroaniline, BAPNA; bovine serum albumin, BSA; 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, bisANS; citrate synthase, CS; creatine phosphokinase, CPK; glutathione-*S*-transferase, GST; lysozyme, Lys; ovotransferrin, Ovo; propidium iodide, PI; phenylmethylsulfonylfluoride, PMSF; superoxide dismutase, SOD.

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haptoglobin 0.3−1.9 mg/mL (*24*)), (iii) mediate ligand degradation by receptor-mediated endocytosis (*27*−*29*), and (iv) are known to co-localize with Aβ deposits in Alzheimer's disease (*13*, *30*, *31*).

We hypothesized that, like clusterin and haptoglobin, α_2M might have the ability to bind to a wide variety of partly unfolded stressed proteins to prevent their aggregation and keep them soluble. Results presented here establish that α_2M does indeed have such an activity and that this activity is modulated by protease activation. A model is also presented to describe the potential physiological significance of these findings.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), citrate synthase (CS, porcine), 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bisANS), lysozyme (Lys, chicken), creatine phosphokinase (CPK, rabbit), superoxide dismutase (SOD, bovine), ovotransferrin (Ovo, chicken), trypsin (type 1, bovine), soybean trypsin inhibitor (type 1), *N*-α-benzoyl-DL-arginine-*p*-nitroaniline (BAPNA), phenylmethylsulfonylfluoride (PMSF), propidium iodide (PI), and methylamine hydrochloride were purchased from Sigma. Triton X-100 (TX-100) was from Ajax Chemicals. Glutathione-*S*-transferase (GST) from *Schistosoma japonicum* was prepared as previously described (*32*). A plasmid encoding a GST-RAP fusion protein (RAP is an inhibitor of ligand binding to low-density lipoprotein family receptors) was obtained as a kind gift from Dr Y. Li (Washington University School of Medicine, St. Louis, MO) and purified in the same way as GST. CS, CPK, and Lys were biotinylated using NHS-LC-biotin (Pierce) as per the manufacturer's instructions. Streptavidin-agarose was purchased from Calbiochem. Streptavidin-Alexa 488 was from Invitrogen. Rabbit polyclonal anti-α_2M and anti-DNP antibodies (IgG fractions) were obtained from Dako Cytomation; rabbit-anti-trypsin antibody (IgG fraction) was from Abcam. Horseradish peroxidase and fluorescein conjugates of sheep-anti-rabbit IgG antibody (SaRIgG-HRP and SaRIgG-FITC, respectively) were from Chemicon.

Purification and in Vitro Activation of α_2M. α_2M was purified from heparinized human plasma using a Zn^{2+} HiTrap chelate-affinity column (GE Healthcare) followed by size exclusion chromatography (SEC) as previously described (*3*). The concentration of α_2M was determined by absorbance at 280 nm (extinction coefficient for a 1% solution = 8.93 (*33*)). Native α_2M was converted to activated α_2M (α_2M*) by incubation with either methylamine or trypsin (*34*). Native α_2M (4.85 μM) was incubated overnight at room temperature (RT) with 0.15 M methylamine HCl in 0.5 M Tris, pH 8.2, and then dialyzed against phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4). α_2M/trypsin complexes were formed by incubating α_2M (4.85 μM) in 25 mM Tris, pH 8.0, with a 6-fold molar excess of trypsin at 37 °C for 2 h; unbound trypsin was inactivated with excess soybean trypsin inhibitor. α_2M/trypsin complexes were purified by SEC using a Biosep-SEC-S4000 column equilibrated in PBS. Successful activation of α_2M was confirmed by native PAGE and trypsin binding assay (performed as described in refs *35* and *34*, respectively); α_2M* species were stored in PBS, pH 7.4, at −20 °C.

Protein Precipitation Assays. Individual solutions of α_2M or α_2M* (0.3−7 μM) and CS (6 μM) in 50 mM Tris, 2 mM EDTA, pH 8, or mixtures of CS and α_2M or α_2M* (at the same final concentrations) were heated at 43 °C for 4 h in a 384 well plate, and using the classic technique employed in most chaperone studies (e.g., ref *22*), precipitation was measured as turbidity (absorbance at 360 nm, A^{360}). Absorbance readings were acquired every 4 min using a FLUOstar plate reader (BMG Labtech). In similar experiments, individual solutions of CPK (25 μM), α_2M or α_2M* (0.3−3.5 μM), or mixtures of CPK and α_2M or α_2M* (at the same final concentrations) in PBS were heated at 43 °C for 3 h, and precipitation was measured as described above. In related experiments, α_2M was preincubated for 1 h at RT with a 2:1 molar ratio of bisANS/α_2M before being added to CS and CPK aggregation reactions (like those just described) at final α_2M concentrations of 3.5 and 0.7 μM, respectively. In control experiments, 7 μM bisANS alone was added to aggregation reactions. In addition, individual solutions in PBS of GST (8 μM) and Ovo (12.5 μM) were incubated with or without α_2M (0.7−7 μM) at 60 °C, and the precipitation was measured as above. Last, individual solutions of Lys (70 μM), α-syn (70 μM), and α_2M or trypsin-activated α_2M* (at 0.7−7 μM), or mixtures of Lys or α-syn and α_2M or α_2M* (at the same final concentrations) were incubated in wells of a 96-well plate (100 μL/well) at 37 °C in oxidative stress buffer (OSB; 100 μM $CuSO_4$, 4 mM H_2O_2 in PBS); changes in A^{360} were measured in a SpectraMax Plus^{384} microplate reader (Molecular Devices). As controls, the effects of SOD and BSA on protein precipitation induced by oxidative and heat stress were tested in similar assays.

Precipitation of Proteins in Whole Human Serum. α_2M was selectively depleted from normal human serum (NHS) using a Zn^{2+} HiTrap chelate-affinity column (GE Healthcare). Approximately 2.0−2.5 mg of α_2M and about 0.5 mg of contaminant proteins were recovered per milliliter of serum; the latter were separated from α_2M by Superose 6 SEC, concentrated by ultrafiltration, and then added back to the α_2M depleted serum (α_2MDS) to reconstitute them to approximately their original concentrations. A sample of double depleted serum (DDS) was also prepared by immunoaffinity depletion of clusterin from α_2MDS, as previously described (*36*); depletion of α_2M and clusterin from sera was confirmed by immunoblotting (Supporting Information Figure S3). To allow for small dilution effects during the depletion steps, the A^{280} values of α_2MDS, DDS, and a "matched" sample of NHS (the same batch of serum from which the depleted sera had been prepared) were determined and used to "normalize" the total protein concentration of each sample; the maximum dilution factor was less than 7% and adjustments to the total protein concentration were made by adding PBS. Aliquots (100 μL) of NHS, α_2MDS, and DDS were diluted 1:4 with PBS (supplemented with 7.5 mM NaN_3, 1 mM PMSF) and incubated at 37 °C for 7 days. Under these mild conditions, the extent of protein precipitation is such that it is difficult to resolve differences between samples by turbidometry; instead, for these experiments we separated the precipitated proteins and quantified them directly. Precipitated proteins were recovered using 0.45 μm ULTRAFREE centrifugal filtration units (Millipore), which were washed with PBS before the precipitate was solubilized with 2 M guanidine hydrochloride in PBS for 2 h at 60 °C

and quantified using a bicinchoninic acid microprotein assay (*37*).

Detection and Purification of α_2M/Stressed Protein Complexes. (i) Immunoprecipitations. The following solutions in PBS were incubated for 3 h at either 43 °C or RT: α_2M (3.5 μM), biotinylated CS (CS-b; 6 μM), or mixtures of α_2M and CS-b at the same final concentrations; α_2M (0.7 μM), CPK-b (25 μM), or mixtures of α_2M and CPK-b at the same final concentrations. Similarly, α_2M (3.5 μM), Lys-b (70 μM), or mixtures of α_2M and Lys-b at the same final concentrations were incubated in OSB for 13 h at 37 °C. All samples were then centrifuged (5 min at 10000*g*) to remove insoluble material and incubated with shaking for 1 h at RT with streptavidin-agarose (50-μL packed volume; Calbiochem). After being washed with 0.1% TX-100 in PBS, the agarose beads were boiled in SDS-PAGE sample buffer and the eluted proteins analyzed by SDS-PAGE under reducing conditions.

(ii) Purification of Complexes. α_2M/CS and α_2M/CPK complexes were purified by anion exchange chromatography. A mixture of CS-b (6 μM) and α_2M (3.5 μM) or CPK-b (25 μM) and α_2M (0.7 μM) was incubated at 43 °C for 3 h, centrifuged at 10000*g* for 5 min, and dialyzed against 20 mM TAPS buffer, pH 9, overnight at 4 °C. The samples were then applied to a 1-mL HiTrap Q fast-flow Sepharose column (GE Healthcare) equilibrated with 20 mM TAPS, pH 9, and eluted with a linear gradient of 0−1 M NaCl in the same buffer. The presence of complex in fractions shown by SDS-PAGE to contain both α_2M and the substrate protein was verified by immunoprecipitation, as described earlier. Complexes were stored at −20 °C.

Preparation of α_2M/Stressed Protein/Trypsin Complexes. Using conditions identical to those already described, we subsequently incubated heated mixtures of α_2M and either CS-b or CPK-b with 1.4 μM trypsin for 10 min before adding 2.8 μM soybean trypsin inhibitor. These mixtures were then subjected to immunoprecipitation analysis using streptavidin-agarose (as described earlier). To prepare purified α_2M/stressed protein/trypsin complexes for use in cell surface binding experiments, ion-exchange-purified α_2M/CS-b or α_2M/CPK-b complexes (0.75 mg/mL in PBS) were incubated with a 3-fold molar excess of trypsin for 2 h at 37 °C. Any unbound trypsin was inactivated with excess soybean trypsin inhibitor and subsequently removed by Superose-6 SEC. SDS-PAGE analyses indicated that the stressed protein complexed with α_2M was not significantly degraded during incubation of α_2M/stressed protein complexes with trypsin (data not shown).

Cell Culture and Flow Cytometry. The JEG-3 (human adenocarcinoma) cell line expressing LRP was obtained from ATCC and cultured in Dulbecco's Modified Eagle Medium: F-12 (Invitrogen) supplemented with 5% (v/v) fetal calf serum (Thermotrace), incubated at 37 °C and 5% (v/v) CO_2. Adherent cells were detached using 5 mM EDTA in PBS and then washed by centrifugation at 300*g* for 10 min in Hank's binding buffer (HBB; 0.14 M NaCl, 5 mM KCl, 6 mM glucose, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 20 mM HEPES, 0.1% (w/v) BSA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4). Cells were incubated for 30 min on ice with either α_2M, trypsin-activated α_2M*, or trypsin-activated α_2M/(biotinylated) stressed protein complexes, all at 200 μg/mL in HBB. To detect bound α_2M or α_2M*, the cells were subsequently incubated with rabbit anti-α_2M or (control) anti-DNP antibodies (diluted 1:500), and finally with SaRIgG-FITC (diluted 1:50). To detect bound complexes incorporating biotinylated stressed proteins, cells were incubated with streptavidin-Alexa 488 (5 μg/mL). To confirm the specificity of binding, similar experiments were undertaken in which cells were first preincubated with either an inhibitory rabbit polyclonal anti-LRP antibody (200 μg/mL; kindly donated by S. K. Moestrup, University of Aarhus, Denmark) or GST-RAP (100 μg/mL; RAP is an inhibitor of ligand binding to low-density lipoprotein family receptors, see Materials). Immediately before analysis using an LSR II flow cytometer (Becton Dickinson), dead cell nuclei were stained using 1 μg/mL of PI. Excitation was at 488 nm, and fluorescence emissions were collected at 515 ± 10 nm (FITC) and 695 ± 20 nm (PI). Electronic gating was used to exclude dead cells from the analyses. Data was collected using FACS Diva software (v4.0; Becton Dickinson) and analyzed using FloJo v6.4.1 (Treestar Inc.). The significance of differences in binding was assessed using the Student's *t* test.

RESULTS

α_2M Protects Proteins from Heat and Oxidative Stress-Induced Precipitation. Heating of CS or CPK at 43 °C and Ovo or GST at 60 °C resulted in their gradual precipitation over 50−240 min (Figure 1A). Oxidative stress induced slow precipitation of Lys over 800 min and a more rapid precipitation of α-synuclein (α-syn) over 200 min (Figure 1A). In all cases, the addition of α_2M produced a dose-dependent inhibition of stress-induced precipitation. Under the conditions tested, there was no significant aggregation measured for α_2M alone (Supporting Information Figure S1). Effects corresponding to concentrations of α_2M up to 7 μM are shown; under the conditions tested, higher concentrations of α_2M gave still greater inhibition of protein aggregation (data not shown). In all cases, the addition of control proteins SOD or BSA had no significant effect on protein precipitation (Supporting Information Figure S1). When aliquots of NHS, α_2MDS, and serum depleted of both α_2MDS and clusterin (DDS) prepared from the same original batch of serum (diluted 1 in 4 in PBS) were incubated at 37 °C for 7 days, the α_2MDS contained significantly more aggregated protein than NHS but less than that in DDS (Figure 1B). Adding purified α_2M or clusterin back to the depleted sera returned the level of protein aggregation back to that of the corresponding undepleted samples (Figure 1B). Similar results were obtained (albeit with greater amounts of protein precipitated) when the same sera were heated at 43 °C for 72 h (data not shown). Although we have not yet identified the serum protein(s) protected by α_2M or clusterin, collectively the results indicate that, like clusterin and haptoglobin (*22*, *36*), α_2M specifically inhibits the stress-induced aggregation and precipitation of a broad range of purified proteins and unfractionated proteins in whole human serum.

α_2M Does Not Protect Enzymes from Heat-Induced Loss of Activity or Independently Promote Protein Refolding. To test whether α_2M had any inherent refolding activity, we analyzed the enzyme activity of CS and GST before and after heat stress in the presence and absence of α_2M and ATP. At concentrations sufficient to suppress most protein precipitation, and regardless of the presence or absence of ATP, α_2M had no significant effect on the loss of activity

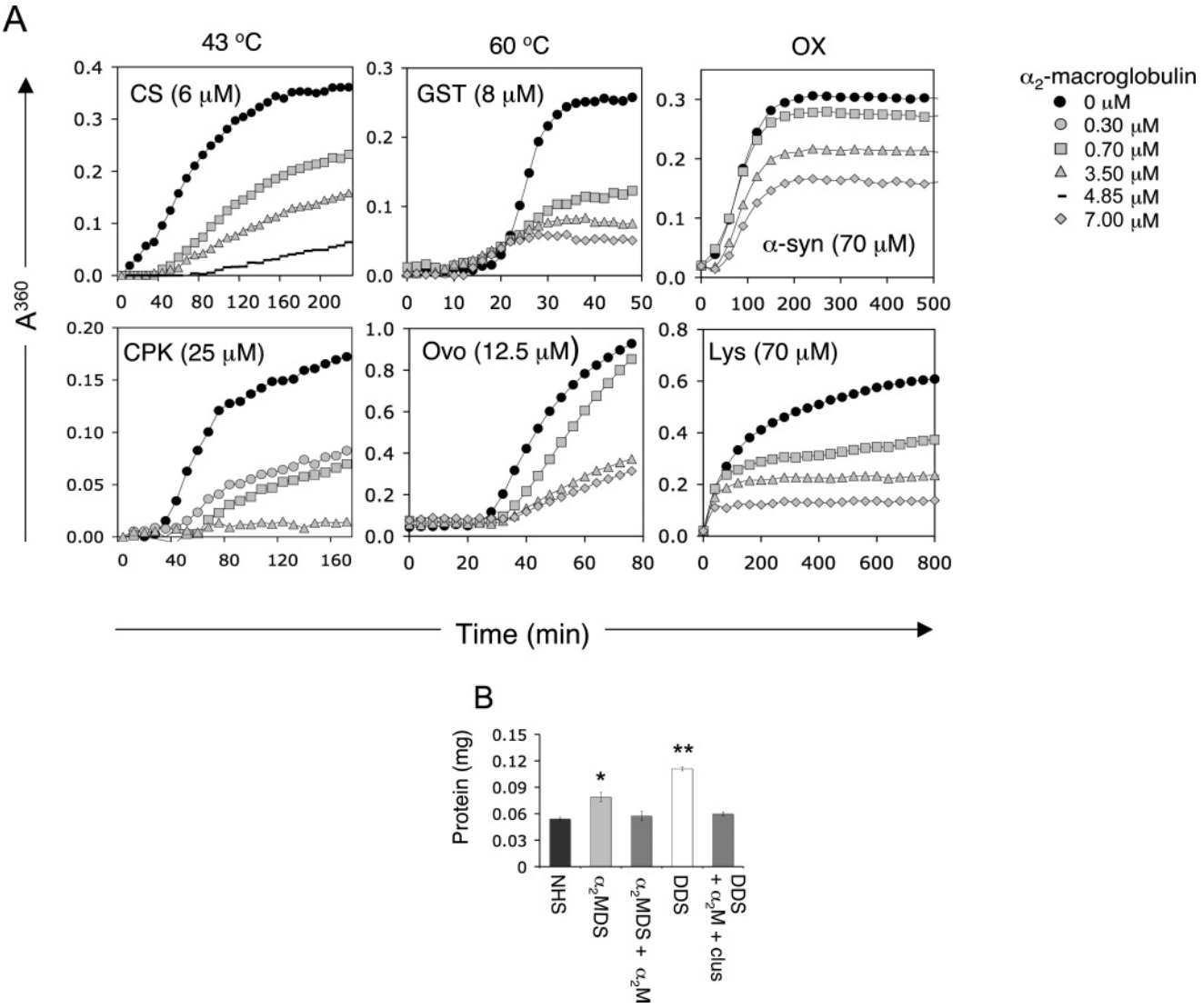

FIGURE 1: α_2M inhibits stress-induced protein precipitation. (A) CS (6 μM) and CPK (25 μM) were incubated at 43 °C, Ovo (12.5 μM) and GST (8 μM) were incubated at 60 °C, and α-syn (70 μM) and Lys (70 μM) were subjected to oxidative stress in the presence of various concentrations of α_2M (indicated in the key). The turbidity associated with protein precipitation was detected as an increase in absorbance (A^{360}). In all cases, the control proteins SOD and BSA had negligible effects on protein precipitation (Supporting Information Figure S1). Data points shown are individual measurements and are representative of at least three independent experiments. (B) Histogram showing the total protein precipitated from 100-μL aliquots of NHS, α_2MDS, serum depleted of both α_2M and clusterin (DDS), and depleted sera supplemented with purified α_2M ± clusterin (indicated), diluted 1 in 4 in PBS and incubated at 37 °C for 7 days. Data points represent the means ($n = 3$) ± standard errors (SE). Asterisks denote significant differences ($p < 0.05$, Student's t test) when compared with NHS (∗) or both NHS and α_2MDS (∗∗). The results are representative of two independent experiments.

of either CS or GST and did not promote the recovery of enzyme activity when measured at up to 5 h after heat stress (Supporting Information Methods and Figure S2).

Chaperone-like Action of α_2M Is Inhibited by bisANS. To test whether the chaperone-like action of α_2M was dependent upon hydrophobic interactions, we examined the effects of bisANS, which binds to solvent-exposed regions of hydrophobicity, on the ability of α_2M to inhibit heat-induced aggregation of substrate proteins. Under the conditions used, the aggregation of CS and CPK was little affected by bisANS but was partially inhibited by α_2M (Figure 2). Under these same conditions, preincubating α_2M with bisANS abolished its ability to inhibit the aggregation of CS and CPK (Figure 2). A trypsin-binding assay and native PAGE analysis confirmed that α_2M was not activated by bisANS under these conditions (data not shown).

α_2M Forms Stable Complexes with Stressed Proteins. We investigated whether α_2M, like clusterin (*38*) and haptoglobin (*22*), forms stable complexes with denatured proteins. We used native gel electrophoresis (Supporting Information Methods) to analyze stressed and nonstressed mixtures of CS, CPK, and Lys, with and without added α_2M. In all cases tested, following heat or oxidative stress but not otherwise, samples containing both α_2M and substrate produced a band of unique electrophoretic mobility suggesting the formation of a complex (data not shown). This interpretation was confirmed by using streptavidin-agarose to affinity adsorb biotinylated (-b) proteins from solutions containing α_2M, or α_2M together with one of CS-b, CPK-b, or Lys-b. The proteins in these solutions had been either untreated or exposed to stresses previously described. Adsorbed proteins were eluted and analyzed by SDS-PAGE. Whether untreated,

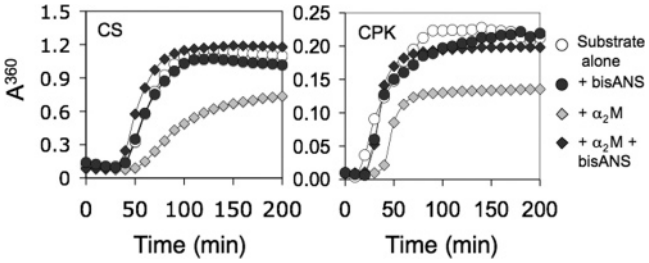

FIGURE 2: Chaperone action of α_2M depends on hydrophobic interactions. Time-dependent changes in turbidity (measured as A^{360}) of heat-stressed CS (6 μM) and CPK (25 μM), either alone or in the presence of α_2M (at 3.5 or 0.7 μM, respectively) or bisANS (7 μM), or α_2M (at the same final concentrations) preincubated with bisANS (see Materials and Methods). The data points shown are individual measurements. The results shown are representative of at least three independent experiments.

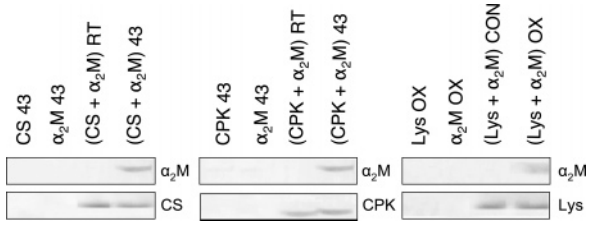

FIGURE 3: α_2M forms stable complexes with stressed proteins. Image of sections of Coomassie blue stained 10% SDS-PAGE gels (run under reducing conditions) showing protein affinity adsorbed by streptavidin-agarose in samples (heated to 43 °C or incubated at RT, as indicated) containing α_2M alone, CS-b or CPK-b alone, or mixtures of α_2M and CS-b or CPK-b. α_2M alone, Lys-b alone, or mixtures of α_2M and Lys-b were also analyzed under oxidative (OX) and control (CON) conditions. Note that, in the absence of α_2M, under the stress conditions used (43 °C or oxidative conditions) most of the CS-b, CPK-b, and Lys-b precipitated from solution and was thus unavailable to bind to streptavidin-agarose. The identity of the bands was established by comparison with molecular mass standards (not shown). The results shown are representative of two independent experiments.

heated, or exposed to oxidizing conditions, α_2M did not bind to the streptavidin-agarose beads (Figure 3). In unstressed samples containing only a substrate protein (CS-b, CPK-b, or Lys-b) together with α_2M, only the biotinylated substrate protein was recovered (Figure 3). For samples that had been exposed to heat or oxidative stress, and that contained a substrate protein and α_2M, both proteins were detected in the bead eluate. Little or no detectable substrate protein was recovered from similarly stressed solutions of substrate alone, because in the absence of α_2M most of the substrate precipitated from solution under these conditions (Figure 3). Collectively, these results demonstrate that α_2M forms stable noncovalent complexes with substrate proteins under conditions of heat and oxidative stress.

Preactivation with Trypsin Abolishes the Chaperone-like Action of α_2M. Under the conditions tested, α_2M potently inhibited the heat-induced precipitation of CS and CPK (Figure 4). However, under the same conditions, trypsin-activated α_2M* had little or no effect on substrate protein precipitation (Figure 4), demonstrating that protease activation of α_2M effectively abolishes it chaperone-like activity. The kinetics and extent of precipitation of CS and CPK shown in Figure 4 differ somewhat from those shown in Figure 1A; this resulted from the use of different commercial batches of the two proteins in the different experiments.

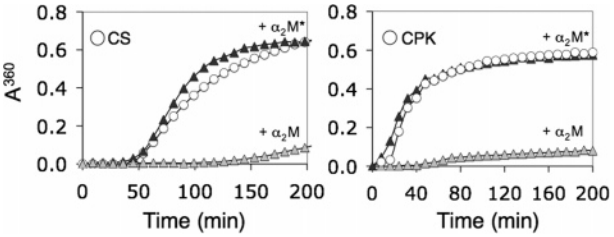

FIGURE 4: Trypsin activation abolishes the chaperone activity of α_2M. Time-dependent changes in turbidity (measured as A^{360}) of heat-stressed CS and CPK, either alone or in the presence of α_2M or trypsin-activated α_2M* (indicated on plots). The data points are individual measurements; the results shown are representative of at least three independent experiments.

α_2M/Stressed Protein Complexes Retain Protease Trapping Activity. Immunoprecipitation analyses were used to test whether trypsin could interact with α_2M/stressed protein complexes. This indicated that the only conditions tested under which the immunoprecipitate contained trypsin was when a heated mixture of α_2M and substrate protein had been subsequently incubated with trypsin (Figure 5A). This establishes that under these conditions a complex is formed that contains all three molecular species (α_2M, heat-stressed protein, and trypsin). Under the conditions used (nonreduced 10% SDS-PAGE gel), trypsin detected in the immunoblots migrated to essentially the same position as native α_2M (i.e., above a 250 kDa molecular mass marker and near the top of the gel, where resolution between molecules of different mass is very limited). This suggests that when trypsin interacts with α_2M/stressed protein complexes, it undergoes the trapping reaction to become covalently linked with α_2M. SDS-PAGE analyses indicated that, under the conditions used, when complexed with α_2M, CS and CPK were not degraded by trypsin (data not shown). This is consistent with results demonstrating that when complexed with prion protein, α_2M protected it from degradation by proteinase K (α_2M does not directly inhibit proteinase K) (*11*). The interaction of α_2M/stressed protein complexes with a protease was also probed using the classic trypsin trapping assay. Interaction with trypsin converts α_2M to α_2M* and coincidentally traps the protease in a steric "cage" (*39*). In this situation, trypsin remains able to cleave substrates less than about 10 kDa, which are small enough to diffuse into the cage (*40*, *41*). In contrast, α_2M* is unable to bind trypsin (*41*). A molar excess of trypsin was incubated with purified complexes formed between α_2M and heat-stressed CS or CPK and any unbound trypsin subsequently inactivated using soybean trypsin inhibitor (which is sterically unable to access and inactivate trypsin bound to α_2M) (*41*). Residual trypsin activity, attributable to complexation with α_2M, was measured using the low molecular weight substrate BAPNA. Purified α_2M/CS and α_2M/CPK complexes showed dose-dependent trypsin binding activity, which, on a mass basis, was very similar to that measured for native α_2M (Figure 5B). This indicates that α_2M remains in its native form when complexed with heat-stressed proteins and suggests that the mass of the complexes tested was dominated by α_2M. SEC analyses supported this interpretation; Superose 6 chromatography was unable to resolve α_2M/CS and α_2M/CPK complexes from native α_2M (data not shown). The resolving power of SEC is limited at these high molecular masses and limited further by the fact that α_2M migrates as a broad peak.

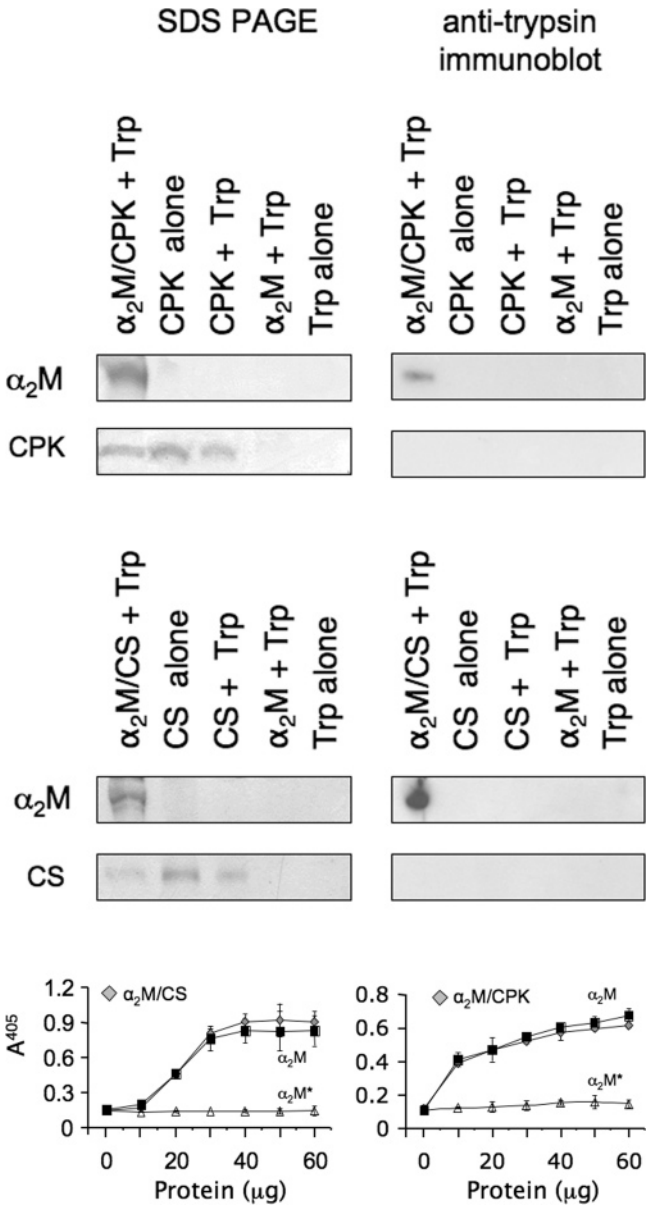

FIGURE 5: α_2M/stressed protein complexes retain the ability to trap trypsin. (A) Image of sections of nonreduced Coomassie blue stained 10% SDS-PAGE gels (left panels) and corresponding immunoblots probed with an anti-trypsin antibody (right panels), showing proteins affinity absorbed by streptavidin-agarose from samples incubated with trypsin containing CS-b or CPK-b alone, or α_2M alone (all at room temperature), or mixtures of α_2M and either CS-b or CPK-b, which had been heated at 43 °C before being mixed with trypsin (i.e., α_2M/CS and α_2M/CPK). On the SDS-PAGE gels, the identity of bands was established by comparison with molecular mass standards (not shown); where detected on the immunoblots, trypsin migrated to the same position as α_2M (left lanes). Results for heated CS or CPK alone, and for unheated mixtures of α_2M and either CS or CPK, are not shown here (see Figure 3). The results shown are representative of two independent experiments. (B) Native α_2M, methylamine-activated α_2M*, and purified α_2M/CS and α_2M/CPK complexes (indicated on plots) were assayed for trypsin binding activity. Data points represent means (n = 3) ± SE; the results shown are representative of three independent experiments.

However, it suggests that the complexes consist of a single 720 kDa α_2M tetramer bound to one or a small number of the much smaller substrate protein molecules.

α_2M/Stressed Protein Complexes Bind to LRP after Proteolytic Activation. Native α_2M and α_2M/(heat-stressed)

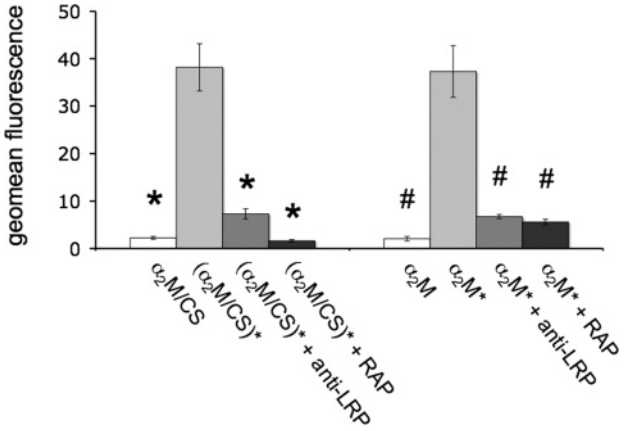

FIGURE 6: Trypsin activates α_2M/stressed protein complexes to expose an LRP binding site. Histogram plot showing the average geometric mean fluorescence (n = 3, ±SE, arbitrary units) for immunochemical detection of the binding to JEG-3 cells of α_2M, trypsin-activated α_2M*, native α_2M/CS complexes, and trypsin-activated α_2M/CS complexes (i.e., (α_2M/CS)*). In some cases, cells were preincubated with an inhibitory anti-LRP antibody or GST-RAP (indicated below the *x*-axis). The values shown have been corrected for the fluorescence associated with cells stained with negative control antibody. The results shown are representative of three independent experiments. Significant differences ($p < 0.05$) are indicated by ∗ (vs (α_2M/CS)*, left) and # (vs α_2M*, right).

CS complexes showed little binding to JEG-3 cells; in contrast, following activation with trypsin, both α_2M* and trypsin-activated α_2M/CS complexes (i.e., (α_2M/CS)*) showed substantial binding (Figure 6). This binding was primarily to the cell surface receptor LRP because it was strongly inhibited by both an anti-LRP antibody and GST-RAP (Figure 6). Similar results were obtained using CPK as the heat-stressed substrate protein (data not shown).

DISCUSSION

Previous work showed that α_2M could suppress the formation of Aβ fibrils and reduce their toxicity (*12*). In addition, α_2M was recently compared with intracellular chaperones because of its ability to behave as an immunogen when complexed to peptides (*19*). Two key properties of chaperones are selective binding to non-native protein conformations to form stable complexes, and inhibition of the irreversible aggregation of non-native protein conformations (*42*). The possibility that α_2M might have chaperone properties with broad substrate specificity has not been tested before. The intrinsic stability of globular mammalian proteins means that none of these will normally unfold and aggregate under "physiological conditions" (i.e., neutral pH, 37 °C) at an experimentally convenient rate. Clearly, this is a physiological necessity, otherwise our bodies would routinely be choked with pathological protein aggregates. Protein aggregation in vitro requires solution conditions (such as increased temperature or oxidative conditions) such that the native structure is partially or completely disrupted but under which interactions such as hydrogen bonding are not completely inhibited. Using such conditions protein aggregates formed in vitro are indistinguishable from those aggregates found in vivo (*43*). Furthermore, although different proteins may require a greater or lesser heat stress to unfold at comparable (experimentally convenient) rates, the pathways of unfolding remain the same regardless of the temperature (*44*).

Our data show for the first time that α_2M can inhibit the aggregation and precipitation of a broad variety of proteins induced by heat or oxidative stress (Figure 1A). During inflammation and carcinoma, the in vivo levels of H_2O_2 and Cu^{2+} can approach those used in this study to exert oxidative stress (*45*, *46*); millimolar levels of H_2O_2 are known to have negligible effects on the structure and function of α_2M (*47*, *48*). It was also shown that the selective removal of α_2M from whole human serum renders proteins in this fluid more susceptible to precipitation, even at 37 °C (Figure 1B). Collectively, these results indicate that α_2M has potent in vitro chaperone properties that are likely to be relevant in vivo. Like clusterin (*38*), α_2M can inhibit protein precipitation even when present at substoichiometric levels (Figure 1A). This is likely to result from the stabilization of a small fraction of the total pool of substrate molecules which are misfolded and which would otherwise act as aggregation nuclei. The substrate-specific variation in the ratio of α_2M/substrate required to suppress precipitation is typical of the behavior seen with other proteins having chaperone properties, including the small heat shock proteins (*49*), clusterin (*38*), and haptoglobin (*22*), and probably results from substrate-specific differences in the proportion of misfolded substrate molecules present under set conditions. These same chaperone-active proteins share with α_2M the ability to irreversibly bind to misfolded proteins in vitro. The demonstration that endogenous α_2M and clusterin significantly inhibit the spontaneous precipitation of proteins in unfractionated human serum incubated at 37 °C may have important medical implications. Abundant extracellular proteins with this type of chaperone property may act as an important line of defense against inappropriate extracellular protein aggregation, which underpins a variety of serious human diseases (*20*). The effects of α_2M and clusterin on plasma protein precipitation are additive (Figure 1B), suggesting that even though they are promiscuous in their interactions with different substrate proteins, they may provide complementarity with respect to the endogenous extracellular proteins they protect.

In the case of two enzymes tested, when acting alone, α_2M did not protect enzymes from heat-induced loss of activity or promote recovery of this activity following heat stress, regardless of the presence or absence of ATP (Supporting Information Figure S3). Moreover, a motifscan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and a BLAST search failed to identify any known ATPase motifs or sequence similarity to any known ATPases, respectively. The demonstration that the hydrophobic probe bisANS suppressed the ability of α_2M to inhibit heat-induced substrate protein aggregation (Figure 2) indicates that it binds to stressed proteins, at least in part, via hydrophobic interactions. Previous work has shown that, overall, α_2M contains more surface-exposed hydrophobicity after it has been activated (*50*). However, this does not exclude the possibility that specific region(s) of exposed hydrophobicity on α_2M important in the chaperone-like action are sterically more accessible to stressed proteins before protease activation. It is also possible that there are other unknown determinants required for binding to stressed proteins that are affected by the conformational changes associated with protease activation.

Last, we used immunoprecipitation analyses (Figure 3) to show that α_2M forms stable soluble complexes with stressed proteins. Collectively, the above functional characteristics demonstrate that α_2M possesses chaperone properties similar to those of the small heat shock proteins (sHSPs), clusterin (*49*) and haptoglobin (*22*). Although we have not excluded the possibility that, like some of the sHSPs (*42*), α_2M may hold stressed proteins in a refolding-competent state, there are no known chaperones with established refolding activity present at significant levels in extracellular body fluids (*20*) and thus any such activity would be of questionable physiological relevance.

Two major types of binding reactions that have been previously described for α_2M are the protease trapping and thiol ester/covalent linking reactions, which are involved in α_2M activation (*33*). α_2M also binds to a range of nonprotease ligands, independently of its activation, including concanavalin A, phytohemagglutinin, aspartate aminotransferase, myelin basic protein, histone H4, endotoxin (*33*), Aβ, cytokines, and growth factors (*9*). α_2M is known to have discrete binding sites for Aβ, cytokines, and LRP (*9*). The relationship between binding sites on α_2M for misfolded proteins versus the many other ligands remains to be defined. However, whatever binding mechanism(s) underlie the chaperone-like action of α_2M, they are clearly prevented if the molecule is first activated by undergoing a protease trapping reaction (Figure 4).

It had previously been shown that a lysozyme/α_2M/elastase complex could be formed when, using a 100-fold molar excess of lysozyme, all three were incubated together at room temperature (*15*). The association with lysozyme appears to result from it becoming nonspecifically trapped within the protease "cage" following elastase-mediated cleavage of the α_2M bait region, rather than specific binding to α_2M. Thus, although this previous study demonstrated formation of a trimolecular complex with α_2M, it is not directly comparable to the stressed protein/α_2M/trypsin complexes described here, which are formed at low stoichiometries. Data presented here establishes that the protease trapping action of α_2M prevents it from subsequently exerting a chaperone-like action but that if it first complexes with a stressed protein, it remains able to later trap proteases forming a stressed protein/α_2M/protease complex (Figure 5A,B). This establishes α_2M as the first known mammalian protein with both chaperone-like and protease inhibitor activities. We also demonstrated that following interaction with a protease (trypsin), but not otherwise, α_2M incorporated into complexes with heat-stressed proteins exposed binding site(s) for LRP, consistent with it adopting an "activated" conformation (Figure 6). Conversion to α_2M* was also indicated by the demonstration of a covalent association between trypsin and α_2M in these complexes (Figure 5A). It follows that if one important function of α_2M is to bind to and solubilize extracellular proteins with nonnative conformations, and subsequently mediate their clearance by LRP, then interaction with a protease may be one in vivo switch to trigger LRP-mediated uptake of α_2M/stressed protein complexes. At physiological locales such as sites of inflammation, this process would be facilitated by the relative abundance of proteases. Uptake of nonactivated α_2M/stressed protein complexes via other currently unknown cell surface receptors is also possible (Figure 7).

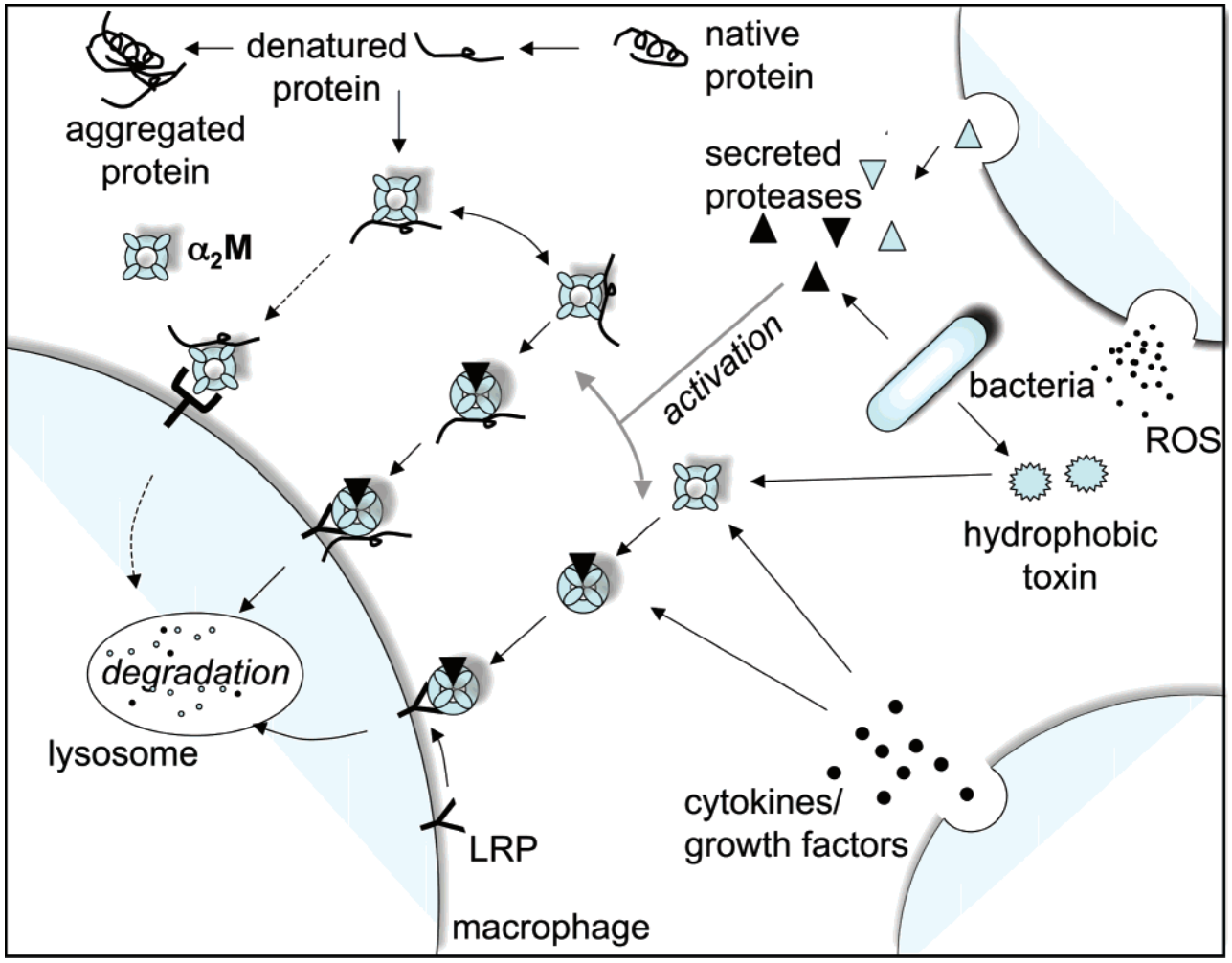

FIGURE 7: Proposed model for α_2M function. At sites of inflammation, factors such as elevated temperature, reactive oxygen species (ROS), and lowered pH may cause damage to extracellular proteins inducing them to partially unfold. Bacteria present at the site may secrete pathogenic proteases and toxins, while host immune cells are known to secrete proteases in an attempt to destroy invading pathogens. In addition, a variety of cell types may locally secrete cytokines or growth factors. Native α_2M may exert a broad anti-inflammatory action by binding to and promoting the clearance of (i) endogenous or exogenous proteases and (ii) other ligands such as denatured proteins, hydrophobic toxins (such as endotoxin), and cytokines. The α_2M-mediated clearance of nonprotease ligands can occur via LRP following the activation of α_2M/ligand complexes by interaction with proteases, which are likely to be abundant at sites of inflammation. Clearance of native α_2M/ligand complexes might also occur via other cell surface receptors, independently of protease activation.

The affinity of α_2M for most cytokines (e.g., TGFβ) is higher when it is in the activated state, and while in this state α_2M delivers the ligands to LRP for uptake and subsequent degradation (*6*). Thus, the model we propose (Figure 7) substantively expands a previous paradigm that α_2M is a scavenger/disposal vehicle for a variety of extracellular proteins. In addition to α_2M, mice also express a structurally and functionally closely related protein, murinoglobulin-1. The only study to ablate the expression of both these proteins in mice did not directly examine their role in clearing extracellular misfolded proteins. However, the results of this study implied a general anti-inflammatory action for the proteins, which is consistent with such a role (*51*). We propose that α_2M is a newly discovered and unique member of a small group of abundant proteins with chaperone properties that patrol extracellular spaces for unfolded/misfolded proteins and facilitate their disposal. Such an activity would contribute to the important anti-inflammatory actions of α_2M in vivo.

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SUPPORTING INFORMATION AVAILABLE

A description of methods and corresponding figures relating to protein precipitation controls, enzyme activity assays, and depletion of α_2M from human serum. This material is available free of charge via the Internet at http://pubs.acs.org.

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