Nonproteolytic Incorporation of Protein Ligands into Human α_2-Macroglobulin: Implications for the Binding Mechanism of α_2-Macroglobulin†

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ABSTRACT: α_2-Macroglobulin (α_2M) is a complex tetrameric protein of 718 kDa. In native α_2M, each of the four subunits contains a thiol ester between the side chains of Cys^{949} and Gln^{952}. Cleavage of the thiol ester with small nucleophiles destabilizes the native conformation and causes a major conformational change in α_2M, which leads to exposure of receptor binding sites and a change in electrophoretic mobility. Recently it has been shown that nucleophilic cleavage of the four thiol esters in α_2M is a reversible process with energy requirements dependent on the nucleophile [Grøn, H., Thøgersen, I. B., Enghild, J. J., and Pizzo, S. V. (1996) *Biochem. J. 318*, 539−545]. The present study is a further investigation of the properties of α_2M with cleaved thiol esters and the potential for incorporation of protein ligands at the site of the thiol ester. The thiol ester in α_2M was cleaved by NH_3. After removal of excess NH_3, the α_2M derivative was incubated with excess protein ligand (hen egg lysozyme or bovine insulin) at 23, 37, or 50 °C, leading to covalent incorporation of the ligands in α_2M as analyzed by SDS−PAGE, gel filtration, and centrifugal microfiltration. Receptor binding studies and native pore-limit PAGE confirmed that the α_2M derivatives with ligand incorporated remained in the receptor-recognized, "fast" migrating conformation. This is the first demonstration of nonproteolytic, covalent incorporation of protein ligands into receptor-recognized α_2M.

α_2-Macroglobulin (α_2M)[1] is a member of a superfamily of proteins that contains both proteinase inhibitors and complement components (reviewed in refs *1* and *2*). Each subunit of α_2M contains a region that is sensitive to proteolysis, the so-called bait region. Proteolytic bait region cleavage initiates a series of conformational changes that lead to exposure of receptor recognition sites and entrapment of the proteinase involved. During this event internal β-cysteinyl-γ-glutamyl thiol esters are exposed in each subunit, and through these the proteinase can be covalently attached. Although traditionally considered a proteinase inhibitor, α_2M possesses several properties suggesting additional functions. If nonproteolytic proteins are present during the proteolytic event, they can be co-trapped with the proteinase and compete for the thiol esters (reviewed in refs *1* and *3*−*5*). For example, many growth factors bind covalently via the thiol esters, and complexes formed between α_2M and nonproteolytic proteins facilitate antigenic presentation by macrophages to T-cells as well as enhancing in vivo antibody responses (*6*, *7*). In vaccines employing specific proteins rather than whole organisms, the immunogens are often small, specific peptides. However, due to proteolytic degradation, peptides cannot be incorporated into receptor-recognized forms of α_2M during the proteolytic event.

Larger nucleophiles such as polypeptides and proteins cannot access the thiol esters when α_2M is in the native state. However, α_2M can be converted to a receptor-recognized conformation by reaction with small nucleophiles, such as NH_3 or small primary amines, which directly attack the thiol ester, thus omitting a proteolytic step (*8*, *9*). Recently we have shown that the cleavage of thiol esters is a reversible process with energy requirements dependent on the nucleophile (*10*), and we developed methods to drive the equilibrium in either direction. The reversible reaction has at least two intermediates between native α_2M, which contains four intact, protected, thiol esters, and the receptor-recognized form of α_2M (α_2M*), with a closed "trap" and four cleaved thiol esters (*10*). We have taken advantage of the reversibility of the system to further examine the properties of thiol ester-cleaved α_2M* and the potential for incorporation of protein ligands at the thiol ester site. We show here that nonproteolytic ligands can be successfully incorporated into the receptor-recognized form of α_2M* without the use of a proteolytic step by virtue of a nucleophilic exchange mechanism.

EXPERIMENTAL PROCEDURES

Materials. Buffers, 5,5′-dithiobis(2-nitrobenzoate) (DTNB), hide powder azure, NH_4HCO_3, β-aminopropionitrile, iodoacetamide, porcine pancreatic elastase, and bovine insulin were from Sigma (St. Louis, MO). Thiocyanic acid 2,4-dinitrophenyl ester (DNPSCN) was obtained from TCI America

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[1] Abbreviations: α_2M, human α_2-macroglobulin; α_2M*, receptor recognized α_2M; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DNPSCN, thiocyanic acid 2,4-dinitrophenyl ester; cpm, γ-disintegrations per minute; lysozyme, hen egg lysozyme; [^{125}I]lysozyme, [^{125}I]Bolton−Hunter labeled lysozyme; [^{125}I]insulin, [^{125}I]Bolton−Hunter labeled insulin; K_d, dissociation constant.

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(Portland, OR). Bovine serum albumin, RPMI medium, and Earle's balanced salt solution were from Gibco BRL (Grand Island, NY). Hen egg lysozyme was from Boehringer Mannheim. Iodobeads were from Pierce (Rockford, IL), and New England Nuclear (Boston, MA) was the source of [^{125}I]-Bolton−Hunter reagent and Na^{125}I. The electrophoresis reagents were from Bio-Rad Laboratories (Richmond, CA), and frozen, platelet-depleted, outdated human plasma was from the American Red Cross (Charlotte, NC). C57BI/6 mice were obtained from Charles River Laboratories (Raleigh, NC). The spectrophotometers used were either a Shimadzu UV 160U (Columbia, MD) or a Beckman DU640 spectrophotometer (Arlington Heights, IL). Radioactivity associated with protein was determined in an LKB-Wallac 1272 Clinigamma counter (Piscataway, NJ), and gels with labeled proteins were analyzed in a PhosphorImager 410A from Molecular Dynamics (Sunnyvale, CA).

Protein. Human α_2M was purified as previously described (*11*). The concentration of intact thiol ester was determined by titration with DTNB (*11*, *12*). The protein concentration was based on $A_{280nm}^{(1\%/1cm)} = 8.93$, molecular mass 718 kDa (*13*). The DTNB titration confirmed that >95% of the thiol esters in the α_2M preparations were intact.

The thiol ester-cleaved derivative (α_2M*) was prepared by incubating α_2M (2−6 mg/mL) with 0.2 M NH_4HCO_3 (pH adjusted to 8.5 with NH_4OH) for 60 min at room temperature. By this treatment >95% of the thiol esters were cleaved as judged by thiol ester titration with DTNB (*11*, *12*), electrophoretic mobility, and the hide powder azure assay (*11*, *14*, *15*). Excess modifying reagent was removed by gel filtration on a PD-10 Sephadex G-25 column (Pharmacia, Piscataway, NJ). The buffer was, unless otherwise stated, 50 mM Tris/50 mM NaCl, pH 7.5.

Lysozyme was brought into solution in water and diluted into buffer. Insulin was brought into solution at acidic pH and diluted. The purity of insulin and lysozyme was confirmed by reducing and nonreducing SDS−PAGE. The insulin concentration was based on $\epsilon_{280nm} = 5220\ M^{-1}\ cm^{-1}$ (*16*), and $A_{280nm}^{(1\%/1cm)} = 26.5$ was used for lysozyme (*17*). Insulin or lysozyme was incorporated into α_2M by incubating desalted α_2M* with excess ligand at 37 or 50 °C for 5−24 h. In some cases the complexes were separated from free ligand by gel filtration on a Sephacryl S-300-HR column (Sigma). The extinction coefficient used for the complexes was that of free α_2M, which we consider a reasonable estimate well within the experimental error. Proteins were concentrated using Amicon cells or Centricon concentrators from Amicon (Danvers, MA).

Lysozyme was labeled with [^{125}I]Bolton−Hunter reagent, as described by Bolton and Hunter (*18*). For receptor binding studies the proteins were ^{125}I-radio-iodinated using Iodobeads according to the manufacturer's specifications.

Polyacrylamide Gel Electrophoresis (PAGE). SDS−PAGE was performed in 4−20% gradient gels (10 cm × 10 cm × 1.5 mm) using the glycine/2-amino-2-methyl-1,3-propanediol/HCl system described by Bury (*19*). Nondenaturing pore-limit PAGE separates proteins according to their radius of gyration and was carried out as previously described (*11*). When α_2M is treated with NH_3, the thiol ester is cleaved and the conformational changes associated can be monitored by nondenaturing pore-limit PAGE (*8*, *9*, *20*). The electrophoretic mobility of native α_2M is traditionally referred to as "slow" and that of nucleophile-inactivated α_2M* as "fast". In all work presented here the electrophoretic mobility of α_2M and its derivatives will be referred to relative to these two standards. The pore-limit gels described here were 4−15% gradient gels (10 cm × 10 cm × 1.5 mm). In some cases the gels were dried and scanned for radioactive markers in a PhosphorImager.

Receptor Binding Assay. Direct binding studies were performed as described by Imber and Pizzo (*8*). Peritoneal macrophages were obtained from thioglycolate-stimulated C57BI/6 mice as previously described (*21*), plated in 24-well plates (2×10^5 cells/well), and incubated at 37 °C in a humidified CO_2 incubator. After equilibration at 4 °C, the monolayers of cells were rinsed with ice-cold Earle's balanced salt solution, 10 mM Hepes, and 0.2% bovine serum albumin, pH 7.4. Increasing concentrations (0.23−60 nM) of ^{125}I-labeled α_2M* or α_2M* with protein ligand incorporated by incubation for 5 h at 50 °C were added to each well and allowed to incubate with gentle agitation at 4 °C for 16 h. Nonspecific binding was determined in parallel experiments in which binding of radioligand took place in the presence of 10−100-fold molar excess of unlabeled ligand. Radioligand solution was removed from the wells, which were rinsed as described above. The cells were solubilized with 1.0 M NaOH/0.1% SDS and counted in the γ-counter. Specific binding was calculated as total binding minus nonspecific binding, and the dissociation constant (K_d) was determined for each ligand by direct fit to the one-site binding equation, using the nonlinear data program SigmaPlot (Jandel Scientific, San Raphael, CA).

RESULTS

Lysozyme Binds to α_2M at 50 °C.* α_2M* was prepared as described under Experimental Procedures and incubated with a 40-fold molar excess of [^{125}I]Bolton−Hunter labeled hen egg lysozyme at 50 °C. The samples were analyzed by nondenaturing pore-limit PAGE (Figure 1A). The control samples, in the absence of lysozyme, behaved as expected (*10*), reverting to the slow migrating conformation characteristic of native α_2M (Figure 1A, lanes 6−8). However, in the presence of lysozyme there was a distribution of slow and fast migrating α_2M even after 24 h at 50 °C (Figure 1A, lane 5). The gels were dried and scanned for radioactivity on a PhosphorImager (Figure 1B). Radioactivity was identified only in the samples that had been incubated with [^{125}I]lysozyme, and it migrated at the position corresponding to fast, receptor-recognized α_2M* (Figure 1B, lanes 3−5). To further confirm the position of the radioactive band, an aliquot of the complex isolated after 5 h of incubation (see below) was incubated with an excess of porcine pancreatic elastase. Coomassie blue staining confirmed that all of the protein shifted to migrate in the same position as the radioactive band, fast α_2M* (Figure 1, lanes 9 and 10). Studies utilizing increasing concentrations of lysozyme were attempted in an effort to prevent α_2M* from reverting to the slow migrating conformation. However, due to solubility problems it was not possible to drive the reaction to completion, and in all experiments some α_2M* reverted to the slow migrating native conformation with no lysozyme associated. SDS−PAGE analysis confirmed that not all of

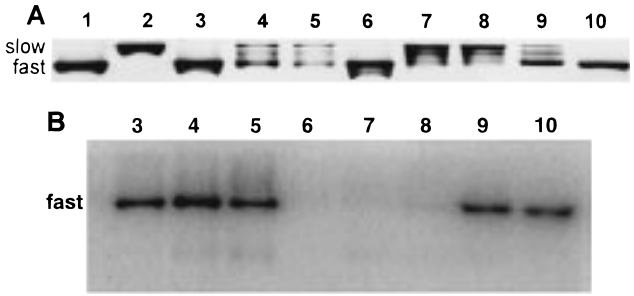

FIGURE 1: Electrophoretic analysis of complex of [^{125}]lysozyme and α_2M* (formed at 50 °C) by nondenaturing 4−15% pore-limit PAGE and PhosphorImager scanning. The complex was prepared at 50 °C by incubating [^{125}I]Bolton−Hunter labeled lysozyme and NH_3-treated α_2M* as described under Experimental Procedures. At the indicated times, aliquots were frozen to be analyzed for electrophoretic mobility by nondenaturing 4−15% pore-limit PAGE (A) and PhosphorImager scanning (B). After 5 h of incubation, an aliquot was gel-filtrated, and the α_2M-containing fractions were pooled (lanes 9 and 10). The sample concentrations were not corrected for precipitation after prolonged exposure at 50 °C. The lanes are as follows: 1, fast migrating α_2M*; 2, slow migrating α_2M; 3−5, α_2M* incubated with [^{125}I]Bolton−Hunter labeled lysozyme at 50 °C for 0, 5, and 24 h, respectively; 6−8, α_2M* alone incubated at 50 °C for 0, 5, and 24 h, respectively; 9, isolated α_2M*−lysozyme complex; 10, isolated α_2M*−lysozyme complex, treated with porcine pancreatic elastase.

the lysozyme associated with α_2M* was covalently incorporated (Figure 2). With the samples that were kept on ice or at room temperature most of the radioactivity was released from α_2M* by heating the sample to 100 °C in the presence of 1% SDS (Figure 2B, lane 4). Covalent incorporation of [^{125}I]lysozyme into α_2M* was observed only after prolonged incubation at 50 °C (Figure 2B, lanes 5 and 6, radioactive band at the position of the 180 kDa subunit of α_2M). A time course study determined optimal conditions for covalent ligand incorporation to be 5 h at 50 °C.

Characterization of the Complex Formed between Lysozyme and α_2M at 50 °C.* To further characterize the complex, α_2M* was incubated with a 40-fold excess of [^{125}I]Bolton−Hunter labeled lysozyme at 50 °C (5 h) as described above. The complex formed was separated from the free ligand by gel filtration on an S-300-HR column. As expected, both fast and slow migrating α_2M were present as determined by nondenaturing pore-limit PAGE (Figure 1A, lane 9). It is not possible to separate the two forms of the macroglobulin by gel filtration, and the stoichiometry presented is based on the mixture of the two forms. The amount of lysozyme incorporated was determined from the total protein concentration (A_{280nm}), the radioactivity incorporated, and the specific radioactivity of the [^{125}I]Bolton−Hunter labeled lysozyme (3000−5000 cpm/pmol). The complex had ≈2.3 mol of lysozyme bound to each mole of α_2M (Table 1). More than 94% of the radioactivity of the complex was precipitated with 25% trichloroacetic acid, indicating that it is all associated with protein. To characterize the stability of the complex, an aliquot was boiled for 30 min, followed by centrifugal microfiltration in Centricon 100 microconcentrators (cutoff at 100 kDa) to isolate any free lysozyme or radioactive label. The filtrate was analyzed for radioactive counts, and <15% of the radioactivity of the complex was released. The level of noncovalent binding was quantified by denaturing the complex in 1% SDS for 30 min at 100 °C, followed by centrifugal microfiltration. Approximately

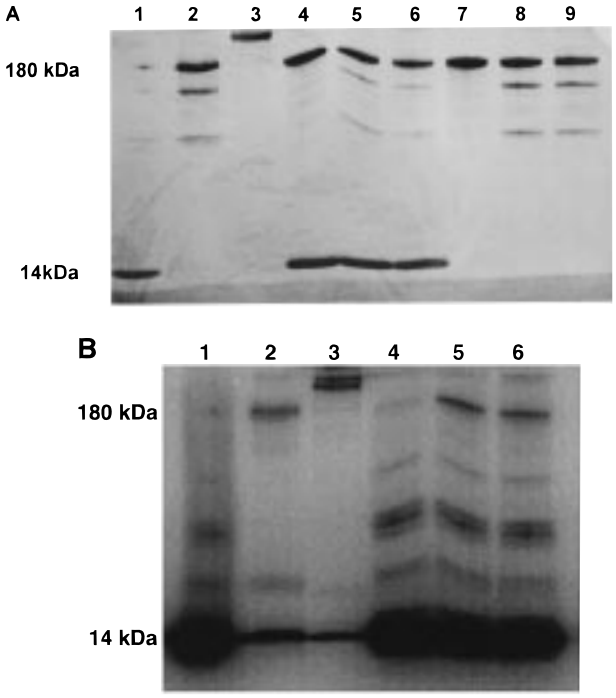

FIGURE 2: Electrophoretic analysis of complex of [^{125}I]lysozyme and α_2M* (formed at 50 °C) by 4−20% PAGE and PhosphorImager scanning. The complex was prepared at 50 °C by incubating [^{125}I]-Bolton−Hunter labeled lysozyme and NH_3-treated α_2M* as described under Experimental Procedures. At the indicated times, aliquots were frozen to be analyzed for electrophoretic mobility by 4−20% SDS−PAGE (A) and PhosphorImager scanning (B). After 5 h of incubation, an aliquot was gel-filtrated, and the α_2M-containing fractions were pooled. The sample concentrations were not corrected for precipitation after prolonged exposure at 50 °C. The lanes are as follows: 1, Bolton−Hunter labeled lysozyme; 2, reduced, isolated α_2M*−lysozyme complex; 3, nonreduced, isolated α_2M*−lysozyme complex; 4−6, α_2M* incubated with Bolton−Hunter labeled lysozyme at 50 °C for 0, 5, and 24 h, respectively; 7−9, α_2M* incubated at 50 °C for 0, 5, and 24 h, respectively.

Table 1: Protein Ligand Binding to α_2M* When Incubated at 37 and 50 °C[a]

	mol of labeled ligand bound/mol of α_2M* [b]			
	lysozyme		insulin	
interaction	37 °C/24 h	50 °C/5 h	37 °C/24 h	50 °C/5 h
covalent and noncovalent	6.6	2.3	nd[c]	nd[c]
Cys^{949} and Gln^{952} mediated (SDS resistant)	1.3	1.4	2.5	3.0
Gln^{952} mediated (SDS and DTT resistant)	1.0	0.6	1.6	0.3

[a] α_2M* was incubated with [^{125}I]Bolton−Hunter labeled lysozyme or insulin, at 37 °C (24 h) or 50 °C (5 h) as indicated in the table. The binding of protein ligand to α_2M* was determined before and after treatment with 1% SDS at 100 °C as described under Results. [b] Each determination is based on three to eight experiments with complexes prepared on independent days. [c] Not determined

60% of the counts remained in the α_2M* complex, indicating that 1.4 mol of lysozyme bound covalently to 1 mol of α_2M* at 50 °C (5 h) (Table 1). Analysis of the complex by SDS−PAGE confirmed the stoichiometry (Figure 2, lanes 2 and 3). Before electrophoresis, the samples were boiled for 10 min in the presence of 1% SDS and, in some cases, 50 mM DTT. After drying, the gels were subjected to imaging on a PhosphorImager. The radioactive bands were quantified either by the program provided with the PhosphorImager or

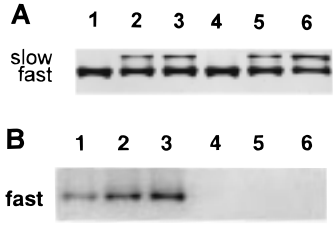

FIGURE 3: Electrophoretic analysis of complex of [^{125}I]lysozyme and α_2M* (formed at 37 °C) by nondenaturing 4−15% pore-limit PAGE and PhosphorImager scanning. The complex was prepared at 37 °C by incubating [^{125}I]Bolton−Hunter labeled lysozyme and NH_3-treated α_2M* as described under Experimental Procedures. At the indicated times, aliquots were frozen to be analyzed for electrophoretic mobility by nondenaturing 4−15% pore-limit PAGE (A) and PhosphorImager scanning (B). The lanes are as follows: 1−3, α_2M* incubated with [^{125}I]Bolton−Hunter labeled lysozyme at 37 °C for 0, 5, and 24 h, respectively; 4−6, α_2M* alone incubated at 37 °C for 0, 5, and 24 h, respectively.

by excising bands from the gels and counting in a γ-counter; both methods gave very similar results. Under nonreducing, denaturing conditions, ≈1.6 mol of [^{125}I]lysozyme remained bound per mole of complex (Figure 2B, lane 3). When 50 mM DTT was present during the SDS treatment, ≈0.6 mol of [^{125}I]lysozyme remained bound to α_2M per mole of complex (Figure 2B, lane 2). The radioactivity migrated at positions corresponding to either the electrophoretic mobility of free lysozyme or the 180 kDa subunit of α_2M.

Temperature-Dependent Incorporation of Lysozyme into α_2M.* The efficiency of the reaction at lower temperatures was investigated. α_2M* was incubated with a 40-fold excess of [^{125}I]lysozyme at 23 and 37 °C, and a time course study was performed. Even after 24 h of incubation at 23 °C, there was no covalent incorporation of lysozyme into α_2M*, as analyzed by SDS−PAGE and centrifugal microfiltration of the SDS-treated, isolated complex (data not shown). As was observed at 50 °C, at 37 °C the time-dependent electrophoretic mobility pattern of α_2M* changed in the presence of lysozyme and less of the macroglobulin reverted to the slow migrating conformation characteristic of native α_2M (Figure 3A, lanes 3 and 6). SDS−PAGE determined the optimal time for covalent incorporation to 24 h. The complex isolated after 24 h at 37 °C had ≈6.6 mol of lysozyme bound to each mole of α_2M (Table 1). The level of noncovalent binding was quantified by denaturing the complex in 1% SDS for 30 min at 100 °C, followed by centrifugal microfiltration. Approximately 1.3 mol of lysozyme remained covalently bound per mole of α_2M* complex (Table 1). Analysis of the complex by SDS−PAGE confirmed the stoichiometry (Figure 4). Under nonreducing conditions ≈1.3 mol of lysozyme remained bound to each mole of macroglobulin. When 50 mM DTT was present during the SDS treatment, 1.0 mol of [^{125}I]lysozyme remained bound per mole of α_2M. It appears that at 37 °C a higher fraction of the covalent binding is resistant to reduction than is at 50 °C.

Reaction between α_2M and Insulin.* The nonproteolytic, covalent incorporation of protein into α_2M* is not limited to lysozyme. The smaller protein insulin behaved very similarly. α_2M* was incubated with a 40-fold excess of [^{125}I]-Bolton−Hunter labeled insulin at 37 or 50 °C for 5 or 24 h. At each condition the complex formed was analyzed by nondenaturing pore-limit PAGE and both fast and slow

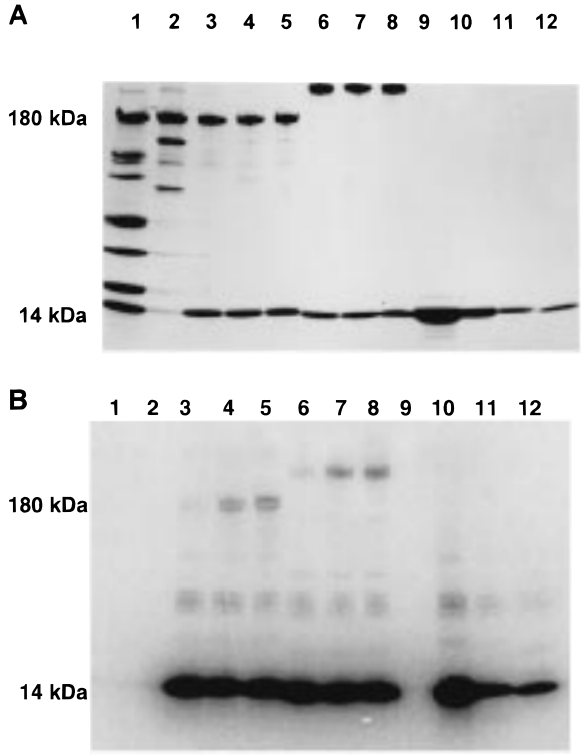

FIGURE 4: Electrophoretic analysis of complex of [^{125}I]lysozyme and α_2M* (formed at 37 °C) by 4−20% PAGE and PhosphorImager scanning. The complex was prepared at 37 °C by incubating [^{125}I]-Bolton−Hunter labeled lysozyme and NH_3-treated α_2M* as described under Experimental Procedures. At the indicated times, aliquots were frozen to be analyzed for electrophoretic mobility by 4−20% SDS−PAGE (A) and PhosphorImager scanning (B). The sample concentrations were not corrected for precipitation after prolonged exposure to 37 °C. The lanes are as follows: 1, molecular weight marker; 2, native α_2M; 3−5, reduced α_2M* incubated with Bolton−Hunter labeled lysozyme for 0, 5, and 24 h, respectively; 6−8, nonreduced α_2M* incubated with Bolton−Hunter labeled lysozyme for 0, 5, and 24 h, respectively; 9, reduced 16 μg of nonlabeled lysozyme; 10, reduced 4 μg of Bolton−Hunter labeled lysozyme; 11, reduced 0.8 μg of Bolton−Hunter labeled lysozyme; 12, nonreduced 0.8 μg of Bolton−Hunter labeled lysozyme.

migrating α_2M was present, as described above. The amount of insulin covalently incorporated was determined by SDS−PAGE in a time course study. The optimal conditions for incorporation were (as for lysozyme) 5 h at 50 °C or 24 h at 37 °C. The complex formed after 5 h of incubation at 50 °C had 3 mol of insulin bound covalently to each mole of α_2M* (Table 1). Under reducing conditions only 0.3 mol of insulin remained bound per mole of α_2M*. As was observed with lysozyme, the complex was more resistant to reduction when formed at 37 °C relative to 50 °C. In the absence of reducing agents 2.5 mol of insulin bound covalently per mole of complex formed at 37 °C (24 h). Under reducing conditions ≈1.6 mol of [^{125}I]insulin remained bound to each mole of α_2M* (Table 1).

Properties of the Covalent Bond between Lysozyme and α_2M.* The covalent bond between lysozyme and fast migrating α_2M* in the complex was further characterized. Native, slow migrating α_2M was incubated with [^{125}I]-lysozyme at 37 °C (24 h) or 50 °C (5 h). The samples were analyzed by SDS−PAGE as described above (gels not shown). At 37 °C the covalent incorporation into native α_2M was <7% of the incorporation into the thiol ester cleaved, fast migrating α_2M*. At 50 °C the covalent incorporation

Table 2: Competition between Lysozyme and Small Ligands for Binding to the Thiol Ester in α_2M* When Incubated at 37 or 50 °C[a]

amino acid residue targeted by competing reagent	% of labeled ligand bound to α_2M* in the presence of competing reagent, relative to conditions when no thiol ester specific reagentis present[b]	
	37 °C/24 h	50 °C/5 h
Gln^{952}	40	40
Cys^{949}	55	30

[a] α_2M* was incubated with [^{125}I]Bolton−Hunter labeled lysozyme in the presence or absence of Gln^{952} or Cys^{949} modifying reagents, at 50 °C (5 h). The samples were analyzed by nonreducing SDS−PAGE, and the binding of lysozyme to α_2M* was determined from the PhosphorImager. [b] Each determination is based on three experiments with complexes prepared on independent days.

into native α_2M was ≈10% of the incorporation into α_2M*. The only chemical difference between native α_2M and thiol ester cleaved α_2M* is the release of free Cys^{949} and the modification of Gln^{952} with $-NH_2$ in α_2M*. The limited incorporation of ligand into native α_2M indicates that the majority of the covalent incorporation of lysozyme into α_2M* is mediated through the components of the thiol ester, either through nucleophilic exchange at Gln^{952} or through thiol−disulfide exchange at Cys^{949}. This was further investigated by examining the incorporation of protein ligand in the presence of competing nucleophiles or thiol specific reagents. In some experiments, incubations of α_2M* and [^{125}I]-lysozyme were carried out in the presence of 150 mM β-aminopropionitrile, a reagent that competes for incorporation into the glutamyl residue of the thiol ester (*22*). Some incubations were carried out in the presence of 0.65 mM DNPSCN or 0.1 mM iodoacetamide, reagents that modify Cys^{949} in α_2M* (*23*−*28*) (at higher concentrations of reagents the protein precipitated during incubation at elevated temperatures). In parallel experiments α_2M* was incubated with either [^{125}I]lysozyme or the modifying reagents alone. The samples were analyzed for radioactive protein incorporation in α_2M* by SDS−PAGE (Table 2). After 5 h at 50 °C, the samples with β-aminopropionitrile present had incorporated ≈40% of the lysozyme incorporated in the absence of β-aminopropionitrile. In the presence of DNPSCN or iodoacetamide, the incorporation represented close to 30%. After 24 h at 37 °C, the samples with β-aminopropionitrile present had incorporated ≈40% of the lysozyme incorporated in the absence of β-aminopropionitrile. In the presence of DNPSCN or iodoacetamide the incorporation was 50−60%. Thus, modification of either Gln^{952} or Cys^{949} in α_2M* reduces the incorporation of protein ligand significantly.

Binding of α_2M and α_2M*−Lysozyme Complex to Macrophages.* α_2M* and α_2M*−lysozyme complex formed by incubation at 50 °C (5 h) were radio-iodinated with Na^{125}I according to the method of chemical oxidation with *N*-chlorobenzenesulfonamide immobilized on polystyrene beads (Iodobeads). The binding of labeled complexes to macrophages was examined as described under Experimental Procedures. The two samples bound to macrophages with similar affinities: $K_d(\alpha_2M^*) = 5 \pm 2$ nM and K_d(complex) $= 8 \pm 2$ nM. In the complex sample, both slow migrating and receptor-recognized α_2M* are present. We did not separate the two forms of the macroglobulin, and the stoichiometry is based on the mixture of the two forms, disregarding the fact that only the receptor-recognized form binds to macrophages. This may explain why the K_d for the complex is slightly higher than for α_2M* itself. However, the observed values are within experimental error for such studies and consistent with our previously reported K_d value for binding of α_2M* to the LRP receptor (*29*).

DISCUSSION

α_2M displays a unique capacity in that it can trap and covalently cross-link nonproteolytic ligands if these are present during proteolytic activation. The reaction is very efficient, and the resulting complexes bind to the α_2M* receptors. The four thiol esters in native α_2M are accessible to cleavage by NH_3 and small primary amines, and such cleavage also causes α_2M to adopt a receptor-recognized conformation and to lose the ability to react with proteinases. This event has generally been considered to be irreversible, and in many experimental procedures nucleophile-treated α_2M is used to simulate proteinase-inactivated α_2M. Recently we reported that nucleophile cleavage of the thiol esters in α_2M is a reversible process (*10*), and in the present study we have further explored the chemistry and microenvironment of the thiol esters. We now find that proteins can also be covalently incorporated into the nucleophile-treated, receptor-recognized α_2M*, thereby omitting the need for proteinases.

Covalent binding of lysozyme during proteinase-induced activation of α_2M is well characterized (*6*, *7*). However, we are unaware of a successful effort to similarly incorporate peptides or protein ligands during nucleophile activation. The data presented here show that lysozyme can incorporate covalently into nucleophile-treated α_2M* when co-incubated at 37 °C (24 h) or 50 °C (5 h). Approximately 6.6 (37 °C) or 2.3 (50 °C) mol of lysozyme were associated with each mole of α_2M. Even samples that were kept on ice (0 h of incubation) had some ligand noncovalently associated with the α_2M*. Lysozyme is a very basic protein, and some basic proteins adhere to α_2M* in a manner distinct from trapping and covalent attachment (*30*). Boiling of the α_2M*−lysozyme complex released 15−25% of the radioactivity incorporated. Boiling in the presence of 1% SDS released significantly more, indicating that at 50 °C (5 h) or 37 °C (24 h) ≈1.4 mol of lysozyme incorporated covalently per mole of α_2M. This exceeds the values obtained by proteolytic incorporation when only 1 mol of lysozyme bound covalently per mole of α_2M (*6*). However, our data and that of Chu et al. (*6*) are in general agreement. During the proteolytic reaction, the proteinase is co-trapped with the ligand in the internal cavity of α_2M, and the sizes of the ligand and the proteinase limit the number of molecules that can be incorporated. Furthermore, the activating proteinase competes with lysozyme for reaction with the thiol esters. Interestingly, when incorporated through a proteolytic mediator, the bond between lysozyme and α_2M was resistant to reduction (*6*), whereas we find that some of the lysozyme incorporated by nucleophile activation is released from the α_2M*−lysozyme complex by reduction. During the proteolytic activation, nucleophiles on the surface of the protein can react with the γ-glutamyl group of the thiol ester (Gln^{952}), but in α_2M*, this group is modified with $-NH_2$. The thiol group from the thiol ester (Cys^{949}) is, however, available for

thiol−disulfide interchange (*31*). It appears that temperature affects the distribution between Gln^{952} and Cys^{949} incorporation. The complexes formed at 37 °C were more resistant to reduction than the complexes formed at 50 °C, indicating an increase in preference for reaction with Cys^{949} as opposed to exchange of nucleophiles at the site of Gln^{952} at the elevated temperature.

Very little protein ligand incorporates into native, slow migrating α_2M. This supports the notion that covalent incorporation of ligand into thiol ester cleaved, fast migrating, α_2M* occurs at the site of either Cys^{949} or Gln^{952}. Consistent with this hypothesis, competition for either Cys^{949} (with DNPSCN or iodoacetamide) or Gln^{952} (with β-aminopropionitrile) reduces the covalent incorporation of ligand significantly.

Insulin is a small, growth factor-like molecule of a size (6 kDa) at the limit of what can diffuse in and out of the closed trap in α_2M*, whereas lysozyme (14 kDa) is too large for diffusion (*32*). Although at room temperature insulin does not form covalent complexes with α_2M* (*33*), as was observed with lysozyme, at elevated temperatures covalent incorporation does occur. Incubation at 50 °C allows ≈3 mol of insulin to covalently incorporate per mole of α_2M*, which is comparable to the proteolytic incorporation of 3−4 mol of insulin per mole of α_2M (*33*).

From a structural point of view, the ability of nucleophile-inactivated α_2M* to entrap and form SDS-stable complexes with diverse, nonproteolytic proteins expands the previously characterized binding mechanisms known for α_2M and α_2M* (as reviewed in refs *1* and *30*). We predict this mechanism will prove useful for directed manipulation of the immune response, especially with regard to vaccine development. In vitro formed complexes are receptor recognized and could represent an effective tool for receptor-mediated enhancement of antigen uptake and processing by macrophages. In previous studies an enhanced immune response has been achieved with nonproteolytic ligands that were incorporated passively during proteolytic activation of α_2M (*6*, *7*). However, the proteolytic incorporation of small, specific, immunogenic peptides is limited by proteolytic degradation of the peptide itself by the activating proteinase, and for many purposes it is not acceptable to have a proteinase present in the immunogenic complex. The mechanism outlined in this paper offers a means by which to incorporate protein and peptide ligands while eliminating the proteolytic step which might, therefore, have significant potential in several applications.

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