Structural and functional analysis of the spontaneous re-formation of the thiol ester bond in human α_2 -macroglobulin, rat α_1 -inhibitor-3 and chemically modified derivatives

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The α -macroglobulins are proteinase inhibitors that form part of a superfamily along with components of the complement system. Internal β -cysteinyl- γ -glutamyl thiol ester bonds are an important structural feature of most α -macroglobulins and several complement components. We have studied the reversibility of thiol ester cleavage caused by NH_3 or CH_3NH_2 in tetrameric human α_2 -macroglobulin ($\alpha_2\text{M}$) and monomeric rat α_1 -inhibitor-3 ($\alpha_1\text{I}_3$). When employing NH_3 as the nucleophile, the thiol ester in $\alpha_1\text{I}_3$ re-formed spontaneously at room temperature after gel filtration to remove excess nucleophile, and an active proteinase inhibitor was regained. When CH_3NH_2 was employed as the nucleophile, thiol ester reversibility was more energy-demanding.

With either nucleophile, $\alpha_2\text{M}$ once inactivated did not regain proteinase-inhibitory capacity at room temperature. At elevated temperatures, however, the reaction between $\alpha_2\text{M}$ and NH_3 or CH_3NH_2 was reversible and the inhibitory capacity could be recovered. Modification of the cysteinyl groups from the thiol ester prevented its re-formation but did not prevent the heat-induced retrieval of inhibitory capacity, suggesting that conformational features rather than the thiol ester are essential for $\alpha_2\text{M}$ to function as an inhibitor. As demonstrated by non-denaturing PAGE, the conformation of native $\alpha_2\text{M}$ is restored when the proteinase-inhibitory capacity is recovered.

INTRODUCTION

The α -macroglobulin superfamily contains both proteinase inhibitors (α -macroglobulins) and complement components. Internal β -cysteinyl- γ -glutamyl thiol esters are present in both C3 and C4 and most α -macroglobulins (reviewed in [1,2]). Complement components, when activated, utilize their thiol esters to form covalent linkages to the surface of bacteria or cells, which are thus targeted for destruction. The function of the thiol ester in the proteinase-inhibiting members of the superfamily is less clearly understood. Proteolytic cleavage of α -macroglobulins at a site distant from the thiol esters (the so-called bait region) induces conformational changes which result in rupture of these thiol ester bonds. With many α -macroglobulins, NH_3 and small primary amines can directly attack the thiol esters, inducing a similar conformational change in the molecule. For most α -macroglobulins, however, the thiol ester is not required for them to function as proteinase inhibitors, and some members of the family have lost this structural feature [3–5]. It has been proposed that thiol esters serve a function of covalently attaching non-proteolytic proteins to α -macroglobulins when they are attacked by proteinases [1,2,6–14]. Many growth factors, for example, bind covalently via these structures to α -macroglobulins during proteolytic activation, and complexes formed between α -macroglobulins and non-proteolytic proteins facilitate antigen presentation from macrophages to T-cells.

Historically, thiol ester cleavage has been viewed as an irreversible event. However, Erickson and colleagues have argued, on theoretical grounds, that these labile thiol esters survive

in the macroglobulins because of a favourable equilibrium in a protected environment [15–17]. These data suggest that cleaved thiol esters may re-form under appropriate conditions. More recently, Pangburn has shown that C3, C4 and α_2 -macroglobulin ($\alpha_2\text{M}$) inactivated by NH_3 treatment can, at least partially, regain their thiol esters after removal of excess NH_3 [18,19]. In the present study we have examined the reversibility of thiol ester bond formation by employing two members of the α -macroglobulin family, namely human tetrameric $\alpha_2\text{M}$ and monomeric rat α_1 -inhibitor-3 ($\alpha_1\text{I}_3$). We find that with both macroglobulins the reaction with nucleophiles is a reversible process, with energy requirements dependent on the nucleophile and the macroglobulin. Furthermore, the proteinase-inhibiting properties of $\alpha_2\text{M}$ are dependent not on the integrity of the thiol ester itself, but rather on the structural conformation characteristic of $\alpha_2\text{M}$ when it has intact thiol esters.

EXPERIMENTAL

Materials

5,5'-Dithiobis-(2-nitrobenzoate) (DTNB), hide powder azure, Triton X-100, NH_4HCO_3 , CH_3NH_2 , *p*-nitrophenyl *p*-guanidinobenzoate and bovine trypsin were from Sigma (St. Louis, MO, U.S.A.). Thiocyanic acid 2,4-dinitrophenyl ester (DNPSN) was obtained from TCI America (Portland, OR, U.S.A.). Electrophoresis reagents were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Rat plasma was purchased from Pel-Freez Biologicals (Rogers, AR, U.S.A.), and frozen platelet-depleted

Abbreviations used: $\alpha_2\text{M}$, α_2 -macroglobulin; $\alpha_1\text{I}_3$, α_1 -inhibitor-3; $\alpha_1\text{I}_3^*(\text{CH}_3\text{NH}_2)$, CH_3NH_2 -inactivated $\alpha_1\text{I}_3$; $\alpha_2\text{M}^*(\text{CH}_3\text{NH}_2)$, CH_3NH_2 -inactivated $\alpha_2\text{M}$; $\alpha_1\text{I}_3^*(\text{NH}_3)$, NH_3 -inactivated $\alpha_1\text{I}_3$; $\alpha_2\text{M}^*(\text{NH}_3)$, NH_3 -inactivated $\alpha_2\text{M}$; DTNB, 5,5'-dithiobis-(2-nitrobenzoate); DNPSN, thiocyanic acid 2,4-dinitrophenyl ester; $R_{50\%}$, molar ratio of macroglobulin derivative to trypsin required to decrease trypsin activity to 50%; RNH_2 , general nucleophile (CH_3NH_2 or NH_3).

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outdated human plasma was from the American Red Cross (Charlotte, NC, U.S.A.).

Characterization of α -macroglobulin preparations

Human α_2 M and rat α_1 I₃ were purified as previously described [20,21]. The concentration of intact thiol esters was determined by titration with DTNB [20,22]. The protein concentration was based on $A_{280,1\text{ cm}}^{1\%} = 8.93$ (molecular mass 718 kDa) for α_2 M [23], and $A_{280,1\text{ cm}}^{1\%} = 7.5$ (molecular mass 200 kDa) for α_1 I₃ [24]. The DTNB titration confirmed that 94% of the thiol esters in the α_2 M preparation were intact, and all α_2 M concentrations were corrected to the active concentration.

PAGE analysis

SDS/PAGE was performed in 5–15% gradient gels (10 cm $\times$ 10 cm $\times$ 1.5 mm) using the glycine/2-amino-2-methyl-1,3-propanediol/HCl system described by Bury [25]. Non-denaturing pore-limit PAGE separates proteins according to their radius of gyration, and was carried out as previously described [20]. When α_2 M is treated with small nucleophiles such as CH_3NH_2 or NH_3 , the thiol ester is cleaved and the associated conformational changes can be monitored by non-denaturing pore-limit PAGE [26–28]. The electrophoretic mobility of native α_2 M is traditionally referred to as ‘slow’ and that of nucleophile-inactivated α_2 M as ‘fast’. In all work presented here the electrophoretic mobility of α_2 M and its derivatives will be referred to as ‘slow’, ‘fast’ or ‘intermediate’ relative to these two standards.

Hide powder azure assay

The proteinase-inhibiting capacities of the α -macroglobulins and their derivatives were determined with trypsin as the model proteinase in a hide powder azure assay, using this macromolecular substrate to titrate the amount of trypsin bound by active α -macroglobulin [29,30]. Trypsin was active-site-titrated with *p*-nitrophenyl *p*-guanidinobenzoate as described by Chase and Shaw [31]. Increasing amounts (0–0.5 nmol) of the purified α -macroglobulins were incubated with 4 pmol of active trypsin for 60 min at room temperature in 600 μ l of 50 mM NaCl, 50 mM Tris, pH 7.5. A 400 μ l sample of a suspension of the substrate hide powder azure (12.5 mg/ml) in 0.6 M sucrose/0.05% Triton X-100 was added to each tube and incubated for 30 min at room temperature with agitation to keep the substrate in suspension. The reaction was stopped by the addition of 300 μ l of 3 M glycine, pH 3. After centrifugation, the proteolytic activity was determined by measuring the absorbance of the supernatant at 595 nm. For the purpose of comparing the inhibitory capacities of different macroglobulin preparations, we defined a parameter, $R_{50\%}$, as the molar ratio of α_2 M to trypsin whereby the proteolytic activity of trypsin is decreased to 50% (see Figure 1A).

Preparation of macroglobulin derivatives

Unless otherwise stated, the thiol ester-cleaved derivatives [NH_3 -inactivated α_2 M [$\alpha_2\text{M}^*(\text{NH}_3)$], CH_3NH_2 -inactivated α_2 M [$\alpha_2\text{M}^*(\text{CH}_3\text{NH}_2)$], NH_3 -inactivated α_1 I₃ [$\alpha_1\text{I}_3^*(\text{NH}_3)$] and CH_3NH_2 -inactivated α_1 I₃ [$\alpha_1\text{I}_3^*(\text{CH}_3\text{NH}_2)$]] were prepared by incubating the macroglobulins (0.5–5.5 mg/ml) with 0.2 M NH_4HCO_3 (pH adjusted to 8.5 with NH_4OH) or with 0.5 M CH_3NH_2 (0.2 M Tris, pH 8.5) for 60 min at room temperature. More than 95% of the thiol esters were cleaved by this treatment, as judged by thiol ester titration with DTNB, electrophoretic mobility and the hide powder azure assay. Thiol ester-cleaved

α_1 I₃ was in some cases carboxamidomethylated by incubation with 10 mM iodoacetamide for 15 min at room temperature.

To generate a thiocyanylated derivative, α_2 M (6.7 μ M) was made 0.2 M in CH_3NH_2 and 1 mM in DNPSCN (from a 25 mM stock solution in DMSO), pH 8.5, and left at room temperature for 16 h. To obtain a carboxamidomethylated α_2 M derivative, α_2 M (6.7 μ M) was incubated for 16 h at room temperature with 0.2 M CH_3NH_2 and 10 mM iodoacetamide at pH 8.5.

Excess modifying reagent was removed by gel filtration on a PD-10 Sephadex G-25 column (Pharmacia, Piscataway, NJ, U.S.A.) equilibrated in 50 mM Tris, 50 mM NaCl, pH 7.5. Native macroglobulins were exposed to identical treatment and analysed using the assays described above. Certain derivatives of α_2 M have a lower solubility than native α_2 M, and precipitation was observed. Before incubation at fixed temperatures, all α_2 M derivatives were filtered through a Millex-GV 0.22 μ m filter (Millipore, Bedford, MA, U.S.A.) and the protein concentration determined from the absorbance at 280 nm and the percentage of active thiol esters in the native α_2 M preparation. At each time point the samples were spun for 5 min in an Eppendorf Microfuge and the absorbance at 280 nm was corrected.

RESULTS

Cleavage of thiol esters in α_2 M

With native α_2 M, the proteolytic activity of trypsin was reduced to 50% at a molar ratio of α_2 M/trypsin ($R_{50\%}$) of 0.33 (Figure 1A). As expected, thiol-ester-cleaved α_2 M, $\alpha_2\text{M}^*(\text{CH}_3\text{NH}_2)$ and $\alpha_2\text{M}^*(\text{NH}_3)$ had no inhibitory capacity (Figure 1A). Excess nucleophile (CH_3NH_2 or NH_3) was removed by gel filtration, and at room temperature the desalted derivatives remained inactive. However, when placed at elevated temperatures the inhibitory capacity of the macroglobulins was restored (Figures 1B and 1C). With both derivatives the $R_{50\%}$ approached 0.6 after 24 h incubation at 50 °C. Since native α_2 M lost about 40% of its inhibitory activity after 24 h at 50 °C (Table 1), all the expected activity was recovered.

The recovery of proteinase-inhibiting capacity was paralleled by a conformational rearrangement which was monitored by non-denaturing PAGE as described in the Experimental section. $\alpha_2\text{M}^*(\text{NH}_3)$ migrated with the characteristic ‘fast’ electrophoretic mobility (Figure 2A, lane 2). After 5 h at 50 °C most of the α_2 M preparation migrated at a ‘slow’ migratory position, characteristic of native α_2 M (Figure 2A, lanes 1 and 7). The recovery of slow migration proceeded via a partial change in conformation identified by a band migrating at an intermediate position (Figure 2A, lane 3). A similar intermediate band was previously described for α_2 M derivatives in which only half of each molecule reacted with proteinase [32–35]. The status of the thiol esters in the samples which recovered their inhibitory capacity was examined by SDS/PAGE (5–15% gels). Before electrophoresis the samples were boiled for 10 min in 8 mM dithiothreitol/1% SDS. The fully inactivated desalted derivatives produced no heat-induced autolytic cleavage fragments (Figure 2B, lane 2), indicating that the thiol esters were fully cleaved [36,37]. After 5 min at 50 °C some thiol ester was regenerated, and heat-induced fragments appeared on the gel (Figure 2B, lane 3). With longer incubation times the intensity of the heat fragment bands increased, but never to the level of native α_2 M. A thiol ester titration of $\alpha_2\text{M}^*(\text{NH}_3)$, desalted and incubated at 50 °C for 5 h, demonstrated that for each 1 mol of α_2 M nearly 2 mol of thiol groups remained free. When treated with trypsin an additional 2 mol of thiol groups was released. Thus, despite recovery of inhibitory capacity and a slow-migrating conformation, not all thiol esters had been regenerated.

Figure 1 Proteinase-inhibitory capacity of $\alpha_2\text{M}$, $\alpha_2\text{M}^*(\text{CH}_3\text{NH}_2)$ and $\alpha_2\text{M}^*(\text{NH}_3)$, as determined with trypsin in a hide powder azure assay

The $\alpha_2\text{M}$ derivatives were prepared as described in the Experimental section. Trypsin was incubated for 60 min with increasing amounts of $\alpha_2\text{M}$. The macromolecular substrate hide powder azure was added and the incubation proceeded with agitation for 30 min. The decrease in absorbance (A_{595}) of the hydrolysed macromolecular substrate was used to titrate the amount of proteinase inhibited by the macroglobulin. The absorbance change with no $\alpha_2\text{M}$ added was set to 100% trypsin activity. $[\alpha_2\text{M}]/[\text{trypsin}]$ is the molar ratio of macroglobulin to trypsin. (A) Native $\alpha_2\text{M}$ ($\square$), $\alpha_2\text{M}^*(\text{CH}_3\text{NH}_2)$ ($\circ$) and $\alpha_2\text{M}^*(\text{NH}_3)$ ($\oplus$). The $R_{50\%}$ is indicated. (B) Native $\alpha_2\text{M}$ ($\square$), $\alpha_2\text{M}^*(\text{NH}_3)$ ($\oplus$) and $\alpha_2\text{M}^*(\text{NH}_3)$ desalted and placed at 50 °C for the indicated times: 0 min ($\triangle$), 5 min ($\star$), 30 min ($\diamond$), 2 h ($\blacklozenge$), 5 h ($\triangleright$) and 24 h ($\times$). (C) Native $\alpha_2\text{M}$ ($\square$), $\alpha_2\text{M}^*(\text{CH}_3\text{NH}_2)$ ($\circ$) and $\alpha_2\text{M}^*(\text{CH}_3\text{NH}_2)$ desalted and left at 50 °C for the indicated times: 0 min ($\oplus$), 5 min ($\triangle$), 30 min ($\star$), 2 h ($\blacklozenge$), 5 h ($\triangleright$) and 24 h ($\times$).

Table 1 Stability of native $\alpha_2\text{M}$ kept at room temperature (RT), 37 °C or 50 °C, given by the $R_{50\%}$ values

$\alpha_2\text{M}$ was incubated at the three temperatures, and at the indicated times the samples were tested in a hide powder azure assay with trypsin and the $R_{50\%}$ values determined as described in the Experimental section.

Incubation time (h)	$R_{50\%}$		
	RT	37 °C	50 °C
0	0.32	0.32	0.36
24	0.40	0.38	0.62
48	0.36	0.36	0.76
72	0.36	0.44	1.84

With both nucleophiles, the recovery of proteinase-inhibitory activity was much slower at 37 °C than at 50 °C. After 24 h less than 20% of the trypsin-inhibitory activity (given by $R_{50\%}$) of $\alpha_2\text{M}^*(\text{NH}_3)$ had been regenerated (Figure 3). At room temperature no activity was regained (results not shown). Regeneration of the inhibitory activity was independent of the conditions under which the thiol ester was cleaved and the choice of nucleophile, NH_3 or CH_3NH_2 . No differences were observed with NH_3 derived from NH_4HCO_3 or NH_4Cl , or when incubation times were prolonged (up to 21 h at room temperature). When $\alpha_2\text{M}$ was modified under more gentle conditions, as suggested by Pangburn [18], by long incubations (12–15 h) at low temperature (0 °C) and/or lower nucleophile concentrations, the derivatives behaved identically to the observations presented above, with respect to inhibitory activity, migration on a non-denaturing gel and re-formation of the thiol ester as visualized by SDS/PAGE.

Cleavage of thiol esters in $\alpha_1\text{I}_3$

The proteinase-inhibitory capacity of native $\alpha_1\text{I}_3$ is smaller than that of $\alpha_2\text{M}$ [21]. The activity of 1 pmol of trypsin could be

Figure 2 Electrophoretic analysis of desalted $\alpha_2\text{M}^*(\text{NH}_3)$ incubated at 50 °C

$\alpha_2\text{M}^*(\text{NH}_3)$ was prepared as described in the Experimental section. The sample was desalted and left at 50 °C. At the indicated times, aliquots were placed on ice to be analysed for electrophoretic mobility by non-denaturing pore-limit PAGE (A), and for the presence of intact thiol esters by SDS/PAGE (B). The sample concentrations were not corrected for precipitation after prolonged exposure at 50 °C (lanes 7 and 8). (A) Non-denaturing pore-limit PAGE (5–15% gels). The lanes are as follows: 1, native $\alpha_2\text{M}$; 2–7, $\alpha_2\text{M}^*(\text{NH}_3)$ left at 50 °C for 0 min, 5 min, 30 min, 1 h, 2 h and 5 h respectively. (B) SDS/PAGE of $\alpha_2\text{M}^*(\text{NH}_3)$. Lanes 1–7 are as described for (A). In lane 8, the sample was incubated at 50 °C for 24 h. Lane 9 contains fast form $\alpha_2\text{M}^*(\text{CH}_3\text{NH}_2)$, and lane 10 contains the molecular mass markers.

reduced to 50% by approx. 6 pmol of native $\alpha_1\text{I}_3$ (i.e. $R_{50\%} = 6$). Like $\alpha_2\text{M}$, $\alpha_1\text{I}_3$ lost its inhibitory activity when incubated with NH_3 or CH_3NH_2 (Figures 4A and 4B). Removal of excess NH_3 from $\alpha_1\text{I}_3^*(\text{NH}_3)$ demonstrated that the thiol ester in $\alpha_1\text{I}_3$ re-

Figure 3 Restoration of the inhibitory capacity of $\alpha_2\text{M}$ after treatment with and removal of NH_3

$\alpha_2\text{M}^*(\text{NH}_3)$ was prepared as described in the Experimental section. The sample was desalted and left at 37 °C (□) or 50 °C (■). At the indicated times, aliquots were analysed for inhibitory capacity with trypsin in the hide powder azure assay. For each time point and each temperature the $R_{50\%}$ value was determined. The percentage of inhibitory capacity restored was determined relative to fully active native $\alpha_2\text{M}$: $100 \times R_{50\%}(\text{native})/R_{50\%}(\text{derivative})$.

formed spontaneously at room temperature and the inhibitory activity was fully recovered (Figure 4A). With $\alpha_1\text{I}_3^*(\text{CH}_3\text{NH}_2)$ the same treatment only partially restored the proteinase-inhibiting capacity, with an $R_{50\%}$ value of approx. 60 (Figure 4B). However, after 5 h at room temperature the $R_{50\%}$ had decreased to 26, and at 37 °C the inhibitory capacity was restored in less than 5 h (Figure 4B). Inhibition of proteinases by $\alpha_1\text{I}_3$ is fully dependent on covalent cross-linking to the thiol ester [21], which suggests that the thiol ester is restored in the derivatives that regain full activity. In support of this hypothesis, no inhibitory activity was recovered when $\alpha_1\text{I}_3^*(\text{NH}_3)$ was carboxamidomethylated at Cys⁹⁴⁹ before being passed over the desalting column (results not shown).

Chemical modification of $\alpha_2\text{M}$

The importance of the thiol ester in $\alpha_2\text{M}$ and the conformational rearrangements caused by changes in its microenvironment were further investigated using chemically modified derivatives. Thiocyanylated $\alpha_2\text{M}$ is modified at Gln⁹⁵² with CH_3NH_2 and at Cys⁹⁴⁹ with CN, and migrated as slow, native $\alpha_2\text{M}$ (Figure 5, lane 6). The migration profile has previously been described [38–42], and this $\alpha_2\text{M}$ derivative is receptor-recognized. Although retained in a slow-migrating conformation characteristic of native $\alpha_2\text{M}$, the inhibitory capacity of this derivative was decreased ($R_{50\%} = 3.1$) (Figure 6). Removal of excess reagents by gel filtration at room temperature improved the inhibitory activity, and the $R_{50\%}$ decreased to 1. At room temperature the inhibitory capacity did not improve further. When incubated at 37 °C for 1 h the inhibitory capacity approached that of native $\alpha_2\text{M}$ ($R_{50\%}$ approx. 0.6). Longer incubation times (5 h) or higher temperatures (50 °C) did not improve the efficiency of inhibition significantly. It has been suggested that the thiocyanylated $\alpha_2\text{M}$ derivative is labile and can revert to a fast-migrating conformation [42]. During the time course of this experiment no such reversal was observed by non-denaturing PAGE. SDS/PAGE confirmed the absence of intact thiol esters. No heat-induced autolytic fragments were generated when the samples were boiled with dithiothreitol and SDS (results not shown).

$\alpha_2\text{M}$ incubated with iodoacetamide/ CH_3NH_2 is modified at Gln⁹⁵² with CH_3NH_2 and carboxamidomethylated at Cys⁹⁴⁹.

Figure 4 Proteinase-inhibitory capacity of $\alpha_1\text{I}_3$, $\alpha_1\text{I}_3^*(\text{CH}_3\text{NH}_2)$ and $\alpha_1\text{I}_3^*(\text{NH}_3)$, as determined with trypsin in a hide powder azure assay

The $\alpha_1\text{I}_3$ derivatives were prepared as described in the Experimental section. Trypsin was incubated for 60 min with increasing amounts of $\alpha_1\text{I}_3$. The macromolecular substrate hide powder azure was added and the incubation proceeded with agitation for 30 min. The decrease in absorbance (A_{595}) of the hydrolysed macromolecular substrate was used to titrate the amount of proteinase inhibited by the macroglobulin. The absorbance change with no $\alpha_1\text{I}_3$ added was set to 100% trypsin activity. $[\alpha_1\text{I}_3]/[\text{trypsin}]$ is the molar ratio of macroglobulin to trypsin. (A) Native $\alpha_1\text{I}_3$ (○), $\alpha_1\text{I}_3^*(\text{NH}_3)$ (●) and $\alpha_1\text{I}_3^*(\text{NH}_3)$ desalted (■). (B) $\alpha_1\text{I}_3$ (○), $\alpha_1\text{I}_3^*(\text{CH}_3\text{NH}_2)$ (*) and $\alpha_1\text{I}_3^*(\text{CH}_3\text{NH}_2)$ desalted and kept on ice (●), or placed at room temperature (■), 37 °C (◆) or 50 °C (△) for 5 h.

Figure 5 Electrophoretic analysis of $\alpha_2\text{M}$, $\alpha_2\text{M}^*(\text{CH}_3\text{NH}_2)$, thiocyanylated $\alpha_2\text{M}$ and carboxamidomethylated $\alpha_2\text{M}$

The $\alpha_2\text{M}$ derivatives were prepared as described in the Experimental section, and the samples were analysed for electrophoretic mobility by non-denaturing pore-limit PAGE (5–15% gels). The sample concentrations were not corrected for precipitation after prolonged exposure to 50 °C (lane 5). The gel lanes are as follows: 1, molecular mass markers; 2, native $\alpha_2\text{M}$; 3, $\alpha_2\text{M}^*(\text{CH}_3\text{NH}_2)$; 4, carboxamidomethylated $\alpha_2\text{M}$; 5, carboxamidomethylated $\alpha_2\text{M}$ after 5 h at 50 °C; 6, thiocyanylated $\alpha_2\text{M}$.

Non-denaturing PAGE of carboxamidomethylated $\alpha_2\text{M}$ revealed both slow- and fast-migrating bands (Figure 5, lane 4), as has previously been described [38]. This derivative did not inhibit

Figure 6 Proteinase-inhibitory capacity of α_2 M and thiocyanated α_2 M, as determined with trypsin in a hide powder azure assay

The derivatives were prepared as described in the Experimental section. The samples were desalted and left for 5 h at room temperature, 37 °C or 50 °C. Trypsin was incubated for 60 min with increasing amounts of α_2 M derivatives as indicated below. The macromolecular substrate hide powder azure was added and the incubation proceeded with agitation for 30 min. The decrease in absorbance (A_{595}) of the hydrolysed macromolecular substrate was used to titrate the amount of proteinase inhibited by the macroglobulin. The absorbance change with no α_2 M was set to 100% trypsin activity. $[\alpha_2\text{M}]/[\text{trypsin}]$ is the molar ratio of macroglobulin to trypsin. Samples: native α_2 M ($\square$), thiocyanated α_2 M ($\triangle$), thiocyanated α_2 M desalted and kept on ice (*), or incubated for 5 h at room temperature ($\triangle$), 37 °C ($\bullet$) or 50 °C ($\circ$) prior to analysis.

trypsin (results not shown). Removal of excess reagents by gel filtration at room temperature improved the inhibitory activity very little ($R_{50\%} = 12$). However, when the derivative was placed at 50 °C for 1 h the $R_{50\%}$ value was reduced to 2.5. Full recovery of the inhibitory activity was not achieved, and after 5 h at 50 °C the $R_{50\%}$ remained at 2.

α_2 M incubated with DNPSCN or iodoacetamide in the absence of NH_3 or CH_3NH_2 behaved like native α_2 M in thiol ester titration, non-denaturing PAGE and the hide powder azure assay.

DISCUSSION

The thiol ester in macroglobulins is accessible to cleavage by NH_3 and small primary amines, and this causes most macroglobulins to lose their activity. This event is generally considered to be irreversible, and in many experimental procedures nucleophile-treated α_2 M is used to simulate proteinase-inactivated α_2 M. However, it has been reported that cleavage of the thiol esters in macroglobulins of the complement factor family and α_2 M is not necessarily an irreversible process [18,19]. Pangburn [18,19] reported that C3 treated gently with NH_3 regained about 70 % of its thiol ester activity when excess nucleophile was removed, although with α_2 M and C4 the recovery of thiol ester was very poor under the same reaction conditions. In the present work we report that fully cleaved thiol esters can re-form in human α_2 M and in rat $\alpha_1\text{I}_3$.

It is well established that with some macroglobulins, including the monomeric $\alpha_1\text{I}_3$, the ability to inhibit proteinases is dependent on an intact thiol ester [21,43,44]. We demonstrate here that the thiol ester in $\alpha_1\text{I}_3$ readily re-forms under mild conditions when excess nucleophile is removed. When Cys⁹⁴⁹ of $\alpha_1\text{I}_3$ is carboxamidomethylated prior to removal of excess nucleophile, the

cysteinyll group cannot undergo a nucleophile attack on the C- γ of Gln⁹⁵² and thiol ester re-formation is prevented. Interestingly, the energy requirements for thiol ester re-formation are dependent on the type of nucleophile employed to cleave the thiol ester. During the re-formation of the thiol ester, the modifying nucleophile is released from Gln⁹⁵², and CH_3NH_2 is not as good a leaving group as NH_3 ; thus higher temperatures are required to revert the process of thiol ester cleavage in the former case. Recent studies of the biosynthesis of $\alpha_1\text{I}_3$ [45] suggest that the thiol ester is formed only after the protein has undergone a post-translational conformational readjustment in which a compact structure is stabilized by disulphide bridges. Our results suggest that the rigid structure of $\alpha_1\text{I}_3$ restrains the constituents of the thiol ester in close vicinity to each other such that the equilibrium between cleaved and intact thiol ester can be readily driven in either direction (Figure 7A). In support of this, $\alpha_1\text{I}_3^*(\text{CH}_3\text{NH}_2)$ migrates like native $\alpha_1\text{I}_3$ in pore-limited PAGE, whereas trypsin-inactivated $\alpha_1\text{I}_3$ has a slower-migrating, less compact conformation (gel not shown).

With tetrameric α -macroglobulins the situation is more complex. An intact thiol ester is not a prerequisite for inhibition of proteinases, and the conformational changes associated with thiol ester cleavage are not well understood [3,4,38–42,46–48]. In most assays proteinase-inactivated α_2 M behaves identically to nucleophile-inactivated α_2 M, despite the presence of an intact bait region in the latter. However, differential scanning calorimetry has demonstrated that proteinase-inactivated α_2 M exhibits two thermal transitions, one considerably higher than that of native α_2 M, whereas nucleophile-treated α_2 M is almost identical to native α_2 M with only one thermal transition temperature [39,49]. This indicates that the energies associated with the conformational changes induced by nucleophile treatment are considerably lower than those induced by proteinase inactivation. In the present study we describe how the conformational and inhibitory characteristics of intact thiol ester can be restored in nucleophile-inactivated α_2 M. The changes proceed via at least three conformations that can be distinguished on non-denaturing PAGE. However, not all of the thiol esters are regenerated within the time course of this experiment. Thus a derivative of α_2 M having none or only some of the four thiol esters intact is inhibitory towards proteinases and has a slow electrophoretic mobility. Each of the thiol esters in the four subunits of α_2 M can interact with nucleophiles in a reversible process. This induces a conformational response, and energy is required for the structural rearrangements necessary for thiol ester regeneration. From our data it is not possible to distinguish whether the α_2 M derivatives with intermediate electrophoretic mobilities have any intact thiol esters present. Most probably in these forms only one, two or three of the four thiol esters are intact, and their presence might indicate a mechanism by which non-proteolytic proteins can access activated thiol esters and thus play a role in the non-inhibiting properties of α_2 M.

The simplest reaction scheme that can describe the reaction between α_2 M and nucleophile (RNH_2) includes three steps, as outlined in Figure 7(B). Native α_2 M is slow-migrating, inhibits proteinases and has four intact thiol ester groups. Slow-migrating, partially reacted, α_2 M [$\alpha_2\text{M}^{\text{slow}}(\text{RNH}_2)$] inhibits proteinases. It contains both cleaved and intact thiol esters, with Gln⁹⁵² and Cys⁹⁴⁹ constrained in similar conformational orientations, thereby preserving the overall native structure. Most probably, intermediate-migrating α_2 M [$\alpha_2\text{M}^{\text{int}}(\text{RNH}_2)$] represents derivatives of the tetrameric α_2 M in which only some of the four subunits have undergone conformational readjustment in response to nucleophilic cleavage of the thiol ester. Fast-migrating α_2 M [$\alpha_2\text{M}^*(\text{RNH}_2)$] will not inhibit proteinases, and all

A: **B:**

Figure 7 Reaction schemes for nucleophile (RNH₂) interacting with $\alpha_1 I_3$ (A) or $\alpha_2 M$ (B)

(A) $\alpha_1 I_3$ reacts with ammonia or primary amines (RNH₂) in a one-step mechanism; $\alpha_1 I_3^*(\text{RNH}_2)$ is the resultant covalent adduct. (B) $\alpha_2 M$ reacts with ammonia or primary amines (RNH₂) in a mechanism with at least three steps. The electrophoretic mobility of native, proteinase-inhibiting $\alpha_2 M$ is slow, and all four thiol groups are locked in a thiol ester with Gln⁹⁵². $\alpha_2 M^{\text{slow}}(\text{RNH}_2)$ migrates as for slow $\alpha_2 M$, contains both intact thiol esters and free thiol groups, and will inhibit proteinases. $\alpha_2 M^{\text{int}}(\text{RNH}_2)$ migrates at an intermediate position and has not been isolated. The electrophoretic mobility of $\alpha_2 M^*(\text{RNH}_2)$ is fast; this derivative will not inhibit proteinases, and all thiol esters are cleaved, with each of the four glutamyl groups modified with RNH₂.

four thiol esters are cleaved. Thus our data do not support the two-step irreversible reaction scheme suggested by Pangburn [18,19]. According to that scheme, thiol ester reversibility is only observed when an intermediate state is trapped by performing the thiol ester cleavage at low temperatures. In our hands $\alpha_2 M$ treated gently with NH₃ according to the procedures of Pangburn [18] did not behave differently from $\alpha_2 M$ treated more harshly as in the present study.

The native conformation of thiol-ester-cleaved $\alpha_2 M$ is stabilized when both Cys⁹⁴⁹ and Gln⁹⁵² are modified, as demonstrated here and in previous reports [35,38–42]. We have extended these studies to demonstrate that thiol ester regeneration is not required for $\alpha_2 M$ to inhibit proteinases. We prevented $\alpha_2 M^*(\text{CH}_3\text{NH}_2)$ from reverting to $\alpha_2 M$ by cyanylating Cys⁹⁴⁹. This proteinase-inhibiting form migrates like slow native $\alpha_2 M$ although it is receptor-recognized [38]. Incubation at 50 °C did not alter the electrophoretic mobility of this derivative, but it improved the inhibitory capacity to the level of native $\alpha_2 M$. This indicates the occurrence of a favourable, heat-induced, readjustment of the structure, similar to the repositioning of Cys⁹⁴⁹ and Gln⁹⁵² that we observed at high temperatures with the NH₃- or CH₃NH₂-inactivated, desalted, $\alpha_2 M$ derivatives. We speculate that the heat-treated thiocyanylated derivative is not receptor-recognized, which implies that the reaction scheme in Figure 7(B) should be extended with a receptor-recognized, partially proteinase-inhibiting, slow-migrating, $\alpha_2 M$ derivative between $\alpha_2 M^{\text{slow}}(\text{RNH}_2)$ and $\alpha_2 M^{\text{int}}(\text{RNH}_2)$.

In summary, our data demonstrate that the cleavage of thiol esters is a reversible process. The structures of native and thiol-ester-cleaved $\alpha_1 I_3$ are kept in a similar rigid conformation, and the equilibrium between cleaved and intact thiol ester is readily driven in either direction. Reversal of thiol ester bond cleavage is not energetically favoured in tetrameric $\alpha_2 M$, and it occurs at a reasonable rate only at elevated temperatures. However, an intact thiol ester is not a prerequisite for $\alpha_2 M$ to function as an inhibitor. Rather, the conformational features of the protein appear to determine its proteinase-inhibitory capacity.

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REFERENCES

- Chu, C. T. and Pizzo, S. V. (1994) *Lab. Invest.* **71**, 792–812
- Sottrup-Jensen, L. (1987) in *The Plasma Proteins: Structure, Function, and Genetic Control* (Putman, F. W., ed.), pp. 191–291, Academic Press, Orlando, FL
- Nagase, H., Harris, E. D. J., Woessner, J. F. J. and Brew, K. (1983) *J. Biol. Chem.* **258**, 7481–7489
- Nagase, H. and Harris, Jr., E. D. (1983) *J. Biol. Chem.* **258**, 7490–7498
- Nielsen, K. L. and Sottrup-Jensen, L. (1993) *Biochim. Biophys. Acta* **1162**, 230–232
- Quigley, J. P. and Armstrong, P. B. (1994) *Ann. N. Y. Acad. Sci.* **712**, 131–145
- Borth, W. (1992) *FASEB J.* **6**, 3345–3353
- Chu, C. T. and Pizzo, S. V. (1993) *J. Immunol.* **150**, 48–58
- Crookston, K. P., Webb, D. J., LaMarre, J. and Gonias, S. L. (1993) *Biochem. J.* **293**, 443–450
- LaMarre, J., Wollenberg, G. K., Gonias, S. L. and Hayes, M. A. (1991) *Lab. Invest.* **65**, 3–14
- LaMarre, J., Hayes, M. A., Wollenberg, G. K., Hussaini, I., Hall, S. W. and Gonias, S. L. (1991) *J. Clin. Invest.* **87**, 39–44
- Osada, T., Noro, N., Kuroda, Y. and Ikai, A. (1988) *Biochem. Biophys. Res. Commun.* **150**, 883–889
- Wolf, B. B. and Gonias, S. L. (1994) *Biochemistry* **33**, 11270–11277
- Misra, U.K., Chu, C. T., Gawdi, G. and Pizzo, S. V. (1994) *J. Biol. Chem.* **269**, 18303–18306
- Khan, S. A. and Erickson, B. W. (1982) *J. Biol. Chem.* **257**, 11864–11867
- Erickson, B. W. and Khan, S. A. (1983) *Ann. N. Y. Acad. Sci.* **421**, 167–177
- Khan, S. A., Sekulski, J. M. and Erickson, B. W. (1986) *Biochemistry* **25**, 5165–5171
- Pangburn, M. K. (1992) *FEBS Lett.* **308**, 280–282
- Pangburn, M. K. (1992) *J. Biol. Chem.* **267**, 8584–8590
- Salvesen, G. and Enghild, J. J. (1993) *Methods Enzymol.* **223**, 121–141
- Enghild, J. J., Salvesen, G., Thgersen, I. B. and Pizzo, S. V. (1989) *J. Biol. Chem.* **264**, 11428–11435
- Habeeb, A. F. S. A. (1972) *Methods Enzymol.* **25**, 457–464
- Hall, P. K. and Roberts, R. C. (1978) *Biochem. J.* **173**, 27–38
- Esnard, F. and Gauthier, F. (1980) *Biochim. Biophys. Acta* **614**, 553–563
- Bury, A. F. (1981) *J. Chromatogr.* **213**, 491–500
- Barrett, A. J., Brown, M. A. and Sayers, C. A. (1979) *Biochem. J.* **181**, 401–418
- Imber, M. J. and Pizzo, S. V. (1981) *J. Biol. Chem.* **256**, 8134–8139
- Van Leuven, F., Cassiman, J.-J. and Van den Berghe, H. (1981) *J. Biol. Chem.* **256**, 9016–9022
- Salvesen, G. and Nagase, H. (1989) in *Proteolytic Enzymes: A Practical Approach* (Beyton, R. J. and Bond, J. S., eds.), pp. 83–104, IRL Press at Oxford University Press, New York
- Enghild, J. J., Thgersen, I. B., Salvesen, G., Fey, G. H., Figler, N. L., Gonias, S. L. and Pizzo, S. V. (1990) *Biochemistry* **29**, 10070–10080
- Chase, Jr., T. and Shaw, E. (1967) *Biochem. Biophys. Res. Commun.* **29**, 508–514
- Gettins, P., Crews, B. C. and Cunningham, L. W. (1989) *Biochemistry* **28**, 5613–5618
- Roche, P. A. and Pizzo, S. V. (1988) *Arch. Biochem. Biophys.* **267**, 285–293

-
- 34 Gettins, P. G. W., Crews, B., Beth, A. H. and Hideg, K. (1995) *FEBS Lett.* **367**, 137–140
- 35 Gettins, P. G. W. (1995) *Biochemistry* **34**, 12233–12240
- 36 Howard, J. B., Vermeulen, M. and Swenson, R. P. (1980) *J. Biol. Chem.* **255**, 3820–3823
- 37 Harpel, P. C., Hayes, M. B. and Hugli, T. E. (1979) *J. Biol. Chem.* **254**, 8669–8678
- 38 Van Leuven, F., Marynen, P., Cassiman, J.-J. and Van den Berghe, H. (1982) *Biochem. J.* **203**, 405–411
- 39 Cunningham, L. W., Crews, B. C. and Gettins, P. (1990) *Biochemistry* **29**, 1638–1643
- 40 Björk, I. (1985) *Biochem. J.* **231**, 451–457
- 41 Jensen, P. E. H., Shanbhag, V. P. and Stigbrand, T. (1995) *Eur. J. Biochem.* **227**, 612–616
- 42 Jensen, P. E. H. and Stigbrand, T. (1992) *Eur. J. Biochem.* **210**, 1071–1077
- 43 Rubenstein, D. S., Thgersen, I. B., Pizzo, S. V. and Enghild, J. J. (1993) *Biochem. J.* **290**, 85–95
- 44 Christensen, U., Simonsen, M., Harrit, N. and Sottrup-Jensen, L. (1989) *Biochemistry* **28**, 9324–9331
- 45 Wassler, M., Esnard, F. and Fries, E. (1995) *J. Biol. Chem.* **270**, 24598–24603
- 46 Gettins, P. G. W., Boel, E. and Crews, B. C. (1994) *FEBS Lett.* **339**, 276–280
- 47 Feldman, S. R. and Pizzo, S. V. (1984) *Arch. Biochem. Biophys.* **235**, 267–275
- 48 Feldman, S. R. and Pizzo, S. V. (1984) *Biochem. Biophys. Res. Commun.* **123**, 771–777
- 49 Cummings, H. S., Pizzo, S. V., Strickland, D. K. and Castellino, F. J. (1984) *Biophys. J.* **45**, 721–724
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