Purification and characterization of a tetrameric α -macroglobulin proteinase inhibitor from the gastropod mollusc *Biomphalaria glabrata*

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The α -macroglobulin proteinase inhibitors (α Ms) are a family of proteins with the unique ability to inhibit a broad spectrum of proteinases. Whereas monomeric, dimeric and tetrameric α Ms have been identified in vertebrates, all invertebrate α Ms characterized so far have been dimeric. This paper reports the isolation and characterization of a tetrameric α M from the tropical planorbid snail *Biomphalaria glabrata*. The sequence of 18 amino acids at the N-terminus indicates homology with other α Ms. The subunit mass of approx. 200 kDa was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and SDS/PAGE. The quaternary structure was determined by sedimentation equilibrium centrifugation and native

pore-limit electrophoresis. Evidence for a thioester is provided by the fact that methylamine treatment prevents the autolytic cleavage of the snail α M subunit and results in the release of 4 mol of thiols per mol of snail α M. The snail α M inhibited the serine proteinase trypsin, the cysteine proteinase bromelain and the metalloproteinase thermolysin. The spectrum of proteinases inhibited, together with the demonstration of steric protection of the proteinase active site and a 'slow to fast' conformational change after reacting with trypsin, all suggest that the inhibitory mechanism of the snail α M is similar to the 'trap mechanism' of human α_2 -macroglobulin.

INTRODUCTION

The thioester superfamily of proteins consists of the α -macroglobulin proteinase inhibitors (α Ms) and the complement components C3, C4 and C5 (reviewed in [1,2]). The α Ms, typified by human α_2 -macroglobulin (α_2 M), can be distinguished from other proteinase inhibitors by their ability to inhibit proteinases possessing different catalytic mechanisms [3]. Proteinase inhibition by the α Ms is not due to inactivation of the proteinase active site but is the result of steric shielding of the active site from large substrates. This unique mechanism (the 'trap' hypothesis [4]) is initiated by proteinase cleavage of the so-called bait region, a segment of the α M subunit that is susceptible to cleavage by a variety of proteinases [5]. Bait region cleavage initiates a conformational change in the α M that results in the entrapment of the proteinase. Large protein substrates are denied access to the active site of the trapped proteinase; however, the active site is still free to react with substrates small enough to enter the molecular trap [6]. An additional consequence of bait region cleavage and the subsequent conformational change is the appearance of a receptor recognition site on the surface of the α M, resulting in the clearance of α M–proteinase complexes via receptors on reticuloendothelial cells [7–9]. The most distinctive feature of this superfamily of proteins is the possession of an internal thioester [10,11]. In the α -Ms, proteolytic cleavage of the bait region activates the unique intrachain β -cysteinyl- γ -glutamyl thioester, which then can covalently bind lysine side chains on the attacking proteinase [12]. The complement proteins C3 and C4 use a similar mechanism to bind covalently to the surface of targets such as bacteria or immune complexes (reviewed in [13]). The thioester can be cleaved by small primary amines, and in most α Ms this results in a conformational change similar to that

initiated by bait region cleavage [14,15]. In human α_2 M, the activated thioester can also bind non-proteolytic proteins and peptides. This has led to proposed additional roles for α Ms in cytokine regulation and antigen presentation (reviewed in [2,16]).

The α Ms exist as monomeric, homodimeric and homotetrameric proteins. In higher vertebrates, monomeric [17,18] and dimeric [19] proteins have been identified; however, the tetramer seems to be the predominant α M in the plasma of most mammals, birds, reptiles and amphibians (reviewed in [1]). This is not so in lower vertebrates and the invertebrates, where only the dimeric forms have been found. Dimeric α Ms have been identified in jawless fish [20,21], cartilaginous fish [20] and bony fish [20,22]. Among invertebrates, dimeric α Ms have been purified from plasmas of several crustacean arthropods [23–25], a chelicerate arthropod [26] and a cephalopod mollusc [27]. Here we describe a tetrameric α M from a gastropod mollusc. This demonstrates that tetrameric α Ms evolved much earlier than previously thought. Characterization of this molecule and its homologues from other molluscs could provide insight into the selective pressures that have led to their evolution. This insight could in turn lead to a more complete understanding of the functions of the α Ms in higher animals.

MATERIALS AND METHODS

Materials

Human α_2 M was from Athens Research and Technology (Athens, GA, U.S.A.). Aprotinin was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Recombinant peptide- N^4 -(N -acetyl- β -glucosaminyl)asparagine amidase (PNGase F) was from Genzyme (Cambridge, MA, U.S.A.). Methylamine was from

Abbreviations used: α M, α -macroglobulin proteinase inhibitor; α_2 M, α_2 -macroglobulin; BAPNA, N^{α} -benzoyl-D,L-arginine p -nitroanilide; DFP, diisopropyl fluorophosphate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); E-64, *trans*-epoxysuccinyl-L-leucylamido-3-(4-guanidino)butane; LBTI, lima bean trypsin inhibitor; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PNGase F, peptide- N^4 -(N -acetyl- β -glucosaminyl)asparagine amidase; PZP, pregnancy zone protein; SBTI, soybean trypsin inhibitor.

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Aldrich (Milwaukee, WI, U.S.A.). BSA, bovine chymotrypsinogen A, *N*^ε-benzoyl-D,L-arginine *p*-nitroanilide (BAPNA), Brij 35, di-isopropyl fluorophosphate, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), dithiothreitol, *trans*-epoxysuccinyl-L-leucylamido-3-(4-guanidino)butane (E-64), hide powder azure, lima bean trypsin inhibitor (LBTI) and soybean trypsin inhibitor (SBTI) were from Sigma (St. Louis, MO, U.S.A.). Centricon 100 and Minicon 100 ultrafiltration devices (100 kDa cut-off) were from Amicon (Beverly, MA, U.S.A.). Coomassie Plus protein assay reagent and human IgM (800 kDa) were from Pierce (Rockford, IL, U.S.A.). The following SDS/PAGE standard proteins were from Bio-Rad (Hercules, CA, U.S.A.): myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97.4 kDa) and BSA (66.2 kDa). The following non-denaturing PAGE standard proteins were from Pharmacia LKB (Uppsala, Sweden): thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), BSA (66 kDa).

Animals

The snails used in this study were 1316-R1 and MO (M line Oregon) strains of *Biomphalaria glabrata* [28]. Snails were maintained at 26 °C and fed *ad lib.* on lettuce. Haemolymph was collected by cardiac puncture [29] and cells were removed by centrifugation at 300 *g* for 10 min at 4 °C. Only fresh plasma was used for the purification of snail α M, and because the number of snails available at any given time was limited, the α M was purified in batches. A typical purification consisted of a pool of 12 ml of plasma collected from approx. 200 individuals.

Protein purification

Approximately 4 ml of sterile-filtered plasma was applied to a Mono-Q HR 10/10 (Pharmacia, Uppsala, Sweden) anion-exchange column previously equilibrated with 50 mM Tris/HCl, pH 7.5, containing 10 mM NaCl. Thermolysin inhibitory activity was eluted with a linear gradient from 10 to 100 mM NaCl (50 ml). The remaining protein was eluted by washing with 100 ml of 1 M NaCl and then the column was re-equilibrated with the original buffer in preparation for another 4 ml of plasma. Typically a batch of α M was purified from 12 ml of plasma, requiring three separate anion-exchange runs. The fractions that contained thermolysin inhibitory activity were pooled and concentrated by ultrafiltration to a final volume of 150 μ l (Amicon YM-100 membrane). The final purification step was gel filtration with a tandem arrangement of Superose 6 columns (10 cm $\times$ 30 cm, Pharmacia) equilibrated with 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl. Fractions containing thermolysin inhibitory activity were pooled and purity was determined by SDS/PAGE. The purified α M was stored on ice for biochemical characterization. Protein concentrations were determined with BSA as a standard and the Coomassie Plus reagent in accordance with the manufacturer's protocol.

N-terminal sequence analysis

N-terminal sequencing of the purified protein was performed on an Applied Biosystems 470A gas-phase protein sequencer with an on-line 120A phenylthiohydantoin amino acid analyser.

Sedimentation equilibrium analysis

Sedimentation equilibrium measurements were performed with a Beckman XL-A analytical ultracentrifuge at a rotor speed of

4000 rev./min. The cell was loaded with 70 μ g of snail protein in 175 μ l of snail physiological saline (0.6 mM NaHCO₃, 0.5 mM Na₂HPO₄, 2 mM KCl, 48 mM NaCl, pH 7.4). The rotor temperature was maintained at 4 °C. Protein sedimentation was monitored by measuring absorbance at 280 nm until superimposable scans indicated that equilibrium had been established. After the final scans had been taken the rotor was accelerated to sediment the protein and yield a cell baseline. The data were analysed with UltraScan 2.5 software (B. Demeler, University of Texas Health Science Center, San Antonio, TX, U.S.A.). The partial specific volume of octopus haemocyanin ($v = 0.728$), another molluscan plasma glycoprotein, was used as an assumed value for molecular mass determinations [30].

Mass spectrometry

The reduced subunits of the snail α M and human α_2 M were analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) with a custom-built mass spectrometer equipped with a frequency-tripled (355 nm) Nd:YAG laser [31]. A 0.5 % solution of 4-hydroxy- α -cyanocinnamic acid in H₂O/acetonitrile (2:1, v/v) was used as the matrix [32]. The purified proteins were dialysed against 25 mM Tris buffer, pH 7.5, containing 30 mM dithiothreitol and concentrated to 5 μ g/ μ l with a Microcon 100 (Amicon). Each protein solution was mixed with the matrix solution (1:4 or 1:5, v/v) and 1 μ l of this mixture was deposited on the mass spectrometric probe, which was dried at ambient temperature and introduced into the mass spectrometer. An acceleration potential of 24 kV was applied to the ion source and mass spectra were generated by summing the ion signature produced from 50 consecutive laser pulses. The instrument was calibrated with BSA (66.43 kDa) or bovine chymotrypsinogen A (25.656 kDa).

Endoglycosidase release of N-linked oligosaccharides

Snail α M and human α_2 M (20 μ g in 10 μ l of 50 mM Tris/HCl, pH 7.7, containing 0.5 % SDS and 50 mM 2-mercaptoethanol) were denatured by incubating for 5 min at 70 °C. To the denatured protein 20 μ l of 3 % (v/v) NP40 was added, followed by 3 μ l (0.75 unit) of recombinant PNGase F. The PNGase F-treated and untreated α M were incubated for 34 h at 37 °C and analysed by SDS/PAGE.

Proteinase assays

The inhibition of thermolysin, trypsin and bromelain activity by column fractions or purified protein was measured with the insoluble substrate hide powder azure essentially as described by Barrett [3]. The assay buffer used in determining trypsin and thermolysin activities was 125 mM Tris, pH 7.5, containing 15 mM CaCl₂, 0.05 % Brij 35 and 0.025 % Na₂S₂O₃. For bromelain the buffer was 11 mM citric acid, 63 mM sodium citrate, 5 mM cysteine, 0.05 % Brij 35 and 0.025 % Na₂S₂O₃, pH 5.5. The final concentration of hide powder was 10 mg/ml.

Stoichiometry of trypsin inhibition

For stoichiometric determinations, trypsin activity was determined by active site titration with *p*-nitrophenyl *p*-guanidinobenzoate as substrate [33]. The stoichiometry of trypsin inhibition by the snail α M was measured by reacting increasing concentrations of α M (0, 1.0, 0.75, 0.6, 0.5, 0.25 and 0.125 mol of α M per mol of trypsin) with a fixed amount of trypsin (final concentration 6, 60 or 600 nM) in 125 mM Tris,

15 mM CaCl_2 , 0.03% Brij 35, pH 7.5. After 10 min the residual trypsin activity was determined with the substrate hide powder azure as described above. The results for each trypsin concentration were plotted separately and a linear regression line was fitted by the least-squares method. Stoichiometries were calculated from the point of 100% inhibition (i.e. 0.0 relative activity).

Methylamine incubation and heat denaturation

Snail αM (760 nM) was incubated with either 100 mM Tris/HCl buffer, pH 8.5, or the same buffer containing 250 mM methylamine. After 6 h (15 °C) the ability of the snail αM to inhibit thermolysin activity was determined with hide powder azure as described above.

Titration of thiol groups

The release of SH groups by methylamine treatment was measured by reaction with DTNB. Snail αM (340 μg) in 100 μl of 50 mM Tris/HCl, pH 8.5, containing 100 mM NaCl was added to 100 μl of 50 mM Tris/HCl, pH 8.5, or the same buffer with 500 mM methylamine. After 6 h at 15 °C, 160 μl of each αM solution was mixed into cuvettes containing 840 μl of 630 μM DTNB in 50 mM Tris/HCl, pH 8.5. Absorbance was measured at 412 nm on a dual-beam spectrophotometer with DTNB and buffer in the reference cuvette. The number of thiol groups was calculated from a molar absorption coefficient of 13600 $\text{M}^{-1}\cdot\text{cm}^{-1}$ [34].

Protection assay

The ability of snail αM to protect trypsin from active-site inhibitors was measured with a protocol adapted from Thøgersen et al. [27]. Assay buffer (50 μl ; 25 mM Tris/HCl, pH 7.5, containing 100 mM NaCl) containing snail αM (17 pmol) was added to wells of a 96-well microtitre plate and allowed to react with pig trypsin (6.5 pmol) for 30 min at 25 °C. Each well then received 50 μl of assay buffer containing 0, 5, 20, 40 or 60 pmol of one of the following active site inhibitors: aprotinin (6.5 kDa), LBTI (9.0 kDa) or SBTI (20.1 kDa). After 30 min, 50 μl of assay buffer containing the low-molecular-mass substrate BAPNA (final concentration 0.17 mM) was added to each well. The residual trypsin activity was measured after 4 h by measuring A_{405} . To determine whether all the trypsin was in complex with the snail αM , a proteinase inhibition assay was run in parallel, using the substrate hide powder azure with trypsin (6.5 pmol) and snail αM (17 pmol).

PAGE

SDS/PAGE separations were run at 4 °C with the buffer system of Laemmli [35]. To prevent autocatalytic cleavage of αM subunits, the samples were not heated after addition of sample buffer. Non-reducing analyses were run with 6 M urea in both the gel and sample buffer. All non-denaturing PAGE separations were run with the discontinuous buffer system of Ornstein [36] and Davis [37]. Gels were stained with Coomassie Brilliant Blue.

RESULTS

Protein purification and N-terminal sequence

Snail αM was purified by anion-exchange chromatography followed by gel filtration. The purified protein was homogeneous as determined by SDS/PAGE (Figure 1), non-denaturing pore-

Figure 1 Comparison of human $\alpha_2\text{M}$ and snail αM by SDS/PAGE

(a) SDS/PAGE was performed on a 6% (w/v) separating gel in the presence of 5% (v/v) 2-mercaptoethanol. Lane 1, molecular mass (M) standards; lane 2, 1.5 μg of human $\alpha_2\text{M}$; lane 3, 1.5 μg of purified snail αM . (b) Non-reducing SDS/PAGE was performed on a 4–9% (w/v) gel gradient with 6 M urea in the gel and sample buffer. Lane 1, 1 μg of human $\alpha_2\text{M}$; lane 2, 1 μg of snail αM ; lane 3, 1 μg of IgM (800 kDa); lane 4, molecular mass standards [with 5% (w/v) 2-mercaptoethanol].

Figure 2 Comparison of snail αM and human $\alpha_2\text{M}$ by non-denaturing pore-limit PAGE

Non-denaturing pore-limit electrophoresis was performed on a 4–18% gel for 24 h at 4 °C. Lane 1, molecular mass (M) standards; lane 2, 3.5 μg of snail αM ; lane 3, 3.0 μg of human $\alpha_2\text{M}$.

limit PAGE (Figure 2) and N-terminal sequence analysis. The average yield was approx. 30 μg per ml of snail plasma. Automated N-terminal Edman degradation identified 18 amino acid residues (Figure 3). The signals were unambiguous, indicating that there is a single subunit in the snail αM .

Subunit structure

Figure 1a shows a SDS/PAGE separation of the subunits of human $\alpha_2\text{M}$ and the snail protein in the presence of 2-mercaptoethanol. The mean ($\pm$ S.D.) of three separate determinations yielded a molecular mass of 183 ± 5 kDa for the snail subunit and 171 ± 6 kDa for the human $\alpha_2\text{M}$ subunit. These values are apparently low because the molecular mass of the

	1									10										20
Human α_2 M	S	V	S	G	K	P	Q	Y	M	V	L	V	P	S	L	L	H	T	E	T
Snail α M								G	K	Y	F	I	S	A	P	R	N	V	V	G
Octopus α M	D	E	I	D	E	G	H	Y	M	I	L	V	P	R	N	F	V	H	G	K
Astacus α M								S	Y	V	I	T	T	P	K	Q	W	V	A	G
Pacifastacus α M								S	Y	V	I	T	T	P	K	M	W	V		
Homarus α M								S	Y	I	I	T	T	P	R	M	W	V	A	G
Limulus α M								K	S	G	F	I	L	T	A	P	K	S	L	T

Figure 3 Comparison of N-terminal amino acid sequence of snail α M with human α_2 M and invertebrate α Ms

The sequence obtained from Edman degradation of snail α M is aligned with the sequences of human α_2 M [39] and the α Ms from octopus [27], crayfish (*Astacus*) [25], crayfish (*Pacifastacus*) [24], lobster (*Homarus*) [23] and horseshoe crab (*Limulus*) [38]. The boxes identify Tyr⁸ and Pro¹³, which are highly conserved in all α Ms. The numbering of human α_2 M is indicated.

Figure 4 Positive-ion MALDI-TOF-MS of snail α M and human α_2 M subunits

Positive-ion MALDI-TOF-MS was performed on (a) snail α M and (b) human α_2 M in the presence of 30 mM dithiothreitol as described in the Materials and methods section. The labels above each peak indicate the charge state of the molecular ion. The molecular mass of each subunit was calculated as an average from the m/z values (M/Z) corresponding to the 2+ and 3+ charge states.

human α_2 M subunit has been determined as 179 kDa by amino acid sequencing and carbohydrate analysis [39,40]. This led us to use MALDI-TOF-MS as a means to determine the subunit mass. The spectra from positive ion MALDI-TOF-MS analyses of the reduced subunits of human α_2 M and the snail protein each show a broad molecular ion peak corresponding to the singly charged molecule and three more well-defined peaks corresponding to the doubly, triply and quadruply charged molecular ions (Figure 4). An average mass was calculated from m/z values of the doubly and triply charged molecules. Four separate determinations

Figure 5 Bait region cleavage of snail α M

Snail α M (1 μ M) was reacted with approx. 2 μ M bromelain, thermolysin and trypsin. After 10 min at 24 °C, the inhibitors E-64 (50 μ M), EDTA (25 mM) and di-isopropyl fluorophosphate (1 mM) were added to the appropriate proteinases. The cleavage fragments were analysed by SDS/PAGE on a 6% (w/v) gel in the presence of 5% (v/v) 2-mercaptoethanol. Lane 1, molecular mass (M) standards; lane 2, untreated snail α M; lane 3, bromelain-treated α M; lane 4, thermolysin-treated α M; lane 5, trypsin-treated α M. All lanes received approx. 3 μ g of protein. All three proteinases produced a major heterogeneous band in the range of 100–105 kDa.

yielded molecular masses of 176.3 ± 0.6 kDa for human α_2 M and 197.6 ± 0.9 kDa for the snail protein.

Native pore-limit electrophoresis (Figure 2) demonstrated that the snail α M is considerably larger than the 716 kDa human α_2 M, suggesting a tetrameric structure for the snail α M. The native snail α M was also subjected to sedimentation equilibrium centrifugation and the data were analysed by one- and two-component models. A two-component model provided the best fit of the observed data, yielding a mass of 848 kDa for the major component, which comprised 80% of the total protein. The use of an assumed value for the partial specific volume probably introduced some error into the molecular mass calculations and we therefore view the calculated mass determination of 848 kDa to be an approximation to the native molecular mass. The non-reduced molecular mass of the snail α M was approx. 370 kDa (Figure 1b). We conclude that the snail α M is similar to human α_2 M in that the denatured molecule is composed of two subunits that form a disulphide-bonded dimer and that the native molecule consists of four subunits, each with a molecular mass of approx. 200 kDa.

Bait region cleavage of the snail α M by thermolysin, bromelain and trypsin resulted in fragments with molecular masses of 100–105 kDa (Figure 5). Thus it appears that snail α M resembles human α_2 M in that the bait region is located near the middle of the subunit [39].

Release of N-linked oligosaccharides

Human α_2 M is approx. 10% carbohydrate by weight and the glycans are all N-linked [40,41]. To determine whether the snail protein was similarly glycosylated, both proteins were incubated with PNGase F, which specifically hydrolyses the β -aspartylglycosylamine bond between asparagine and the innermost *N*-acetylglucosamine of the glycan. SDS/PAGE analysis of untreated and glycosidase-treated proteins (Figure 6) yielded the following molecular masses: untreated human α_2 M, 169 kDa; PNGase F-treated human α_2 M, 150 kDa; untreated snail α M, 185 kDa; PNGase F-treated snail α M, 177 kDa. The 11% decrease in mass of the human α_2 M closely corresponds to the total carbohydrate content of human α_2 M and demonstrates that

Figure 6 SDS/PAGE demonstrating the cleavage of N-linked oligosaccharides from human α_2 M and snail α M

Human α_2 M and snail α M were denatured and incubated with PNGase F as described in the Materials and methods section. SDS/PAGE was performed on a 6% (w/v) gel in the presence of 5% (v/v) 2-mercaptoethanol. Lane 1, molecular mass (M) standards; lane 2, 6 μ g of human α_2 M; lane 3, 6 μ g of PNGase F-treated human α_2 M; lane 4, 4 μ g of snail α M; lane 5, 4 μ g of PNGase F-treated snail α M.

Figure 7 Inhibition of proteinases by snail α M

Separate proteolytic assays were performed with the insoluble macromolecular substrate hide powder azure and three enzymes with different proteolytic mechanisms. Increasing amounts of snail α M (0–11 μ g) were incubated with a fixed amount (0.2 μ g) of trypsin (●), bromelain (■) or thermolysin (▽) before addition of the substrate.

the PNGase F released all N-linked glycans. In contrast, the glycosidase treatment of the snail α M resulted in only a 4% decrease in molecular mass of the subunit. This suggests that the greater molecular mass of the snail α M is not due to a greater content of N-linked carbohydrate.

Proteinase inhibition assays and the mechanism of inhibition

The snail protein inhibited serine (pig trypsin), cysteine (bromelain) and metallo- (thermolysin) proteinases (Figure 7), suggesting that it uses the 'entrapment' mechanism that distinguishes the α M from those inhibitors that bind the proteinase active site. To confirm this mechanism of inhibition we investigated the ability of snail α M to protect pig trypsin from interacting with the

Figure 8 Protection of trypsin from active site inhibitors by snail α M

Snail α M (17 pmol) was incubated with trypsin (6.5 pmol) and the resulting α M-trypsin complex (a) or trypsin alone (b) was incubated for 30 min with 0, 5, 20, 40 or 60 pmol of the following active site inhibitors: ●, SBTI (20.1 kDa); ▽, LBTI (9.0 kDa); □, aprotinin (6.5 kDa). The substrate BAPNA was added and after 4 h the amount of substrate hydrolysis was determined by measuring A_{405} . The results presented are relative to that of trypsin incubated alone.

following active site inhibitors: aprotinin (6.5 kDa), LBTI (9.0 kDa) and SBTI (20.1 kDa). At the quantities of snail α M (17 pmol) and trypsin (6.5 pmol) used, the proteolytic activity of trypsin against the macromolecular substrate hide powder azure was completely inhibited, indicating that all the trypsin was trapped by the α M. The entrapped trypsin (Figure 8a) was able to hydrolyse the small substrate BAPNA, although its activity was diminished compared with trypsin alone (Figure 8b). Furthermore the large active-site inhibitors LBTI and SBTI did not significantly alter the activity of the entrapped trypsin; however, the smaller inhibitor aprotinin did cause an additional decrease in activity (Figure 8a). We conclude that the active site of trypsin in the α M complex is sterically shielded from substrates larger than approx. 7 kDa.

The conformational change initiated by bait region cleavage can be detected electrophoretically on non-denaturing gels as a shift from a 'slow' form to a 'fast' form [15]. A similar shift in mobility occurs if α_2 M is incubated with small primary amines. Native snail α M undergoes a significant increase in mobility after becoming complexed with trypsin (Figure 9, lane 2), indicating that the α M-trypsin complex has a more compacted structure; however, methylamine treatment does not alter the electrophoretic mobility of the snail α M (Figure 9, lane 3).

Figure 9 Non-denaturing PAGE demonstrating 'slow' and 'fast' forms of snail α M

Non-denaturing PAGE was performed in 5% (w/w) gels. Snail α M (2.5 nM) was incubated with trypsin (5.5 nM) for 10 min at 24 °C and excess trypsin was removed by ultrafiltration (100 kDa cut-off). The methylamine-treated sample was prepared by incubating snail α M (760 nM) in 100 mM Tris/HCl, pH 8.5, containing 250 mM methylamine for 6 h at 15 °C. Lane 1, 5 μ g of snail α M; lane 2, 5 μ g of snail α M-trypsin complex; lane 3, 5 μ g of methylamine-treated snail α M.

Figure 10 SDS/PAGE demonstrating autolytic cleavage of human α_2 M and snail α M

Autolytic cleavage of human α_2 M and methylamine-treated and untreated snail α M was accomplished by incubating the proteins for 2 h at 80 °C before the addition of SDS/2-mercaptoethanol sample buffer. The snail α M (760 nM) was incubated in 100 mM Tris/HCl buffer, pH 8.5, containing 250 mM methylamine for 6 h at 15 °C to prevent autolytic cleavage. SDS/PAGE was performed on a 5–10% (w/v) gradient gel in the presence of 5% (v/v) 2-mercaptoethanol. Lane 1, control human α_2 M; lane 2, control snail α M; lane 3, human α_2 M after heat treatment; lane 4, snail α M after heat treatment; lane 5, methylamine-treated snail α M after heat treatment; lane 6, methylamine-treated snail α M without heat treatment. Each lane contained approx. 4 μ g of protein.

Thioester

The thermolysin inhibitory activity of snail α M incubated for 6 h with 250 mM methylamine decreased by an average of 11% (determined from three separate batches). These results suggested that the snail α M might lack a reactive thioester. To investigate this possibility, heat denaturation experiments were performed with human α_2 M and snail α M. Native human α_2 M (Figure 10, lanes 1 and 3) and native snail α M (Figure 10, lanes 2 and 4) undergo autolytic fragmentation after being incubated at 80 °C for 2 h. This fragmentation is almost completely prevented in the methylamine-treated snail α M (Figure 10, lane 5). Autolytic

fragmentation and its prevention by a nucleophile provide evidence of an intact thioester [42,43]. To determine whether methylamine was cleaving the thioester, the release of thiols from methylamine-treated α M was measured with DTNB. Two separate titrations determined that the 6 h incubation with 250 mM methylamine liberated an average of 4.5 mol of SH per mol of α M, indicating that all thioesters (one per subunit) were cleaved by the methylamine treatment. These results provide evidence that the snail α M does possess an internal thioester even though cleavage of the thioester by methylamine does not greatly limit proteinase inhibition.

Stoichiometry of proteinase inhibition

The stoichiometry of trypsin inhibition was determined by incubating a fixed amount of trypsin with increasing amounts of snail α M and then measuring the residual trypsin activity against the macromolecular substrate hide powder azure. The results of two separate assays for each trypsin concentration yielded the following inhibition ratios (trypsin to α M): 1.1:1, 1.3:1 and 1.5:1 for trypsin concentrations of 6, 60 and 600 nM respectively. The correlation coefficients for the regression lines were (in order of increasing trypsin concentration) 0.9896, 0.9989 and 0.9992.

DISCUSSION

We previously identified α M-like proteinase inhibitory activity in the plasma of the gastropod snail *B. glabrata* [44]. This report describes the isolation of a high-molecular-mass protein from snail plasma that inhibits serine, thiol and metalloproteinases. This proteinase inhibition is the result of steric shielding of the proteinase active site, because small synthetic substrates and small active site inhibitors were able to react with the proteinase complexed with snail α M. This mechanism of inhibition is unique to the family of α -macroglobulin proteinase inhibitors and involves a proteinase-initiated conformational change of the α M and consequent trapping of the proteinase. Accordingly, the snail α M-trypsin complex had a more compacted structure than did untreated α M when analysed by native PAGE, further confirming that snail α M employs this 'non-standard' mechanism of inhibition.

α Ms have been purified from several invertebrate species [23–26]; however, the only other molluscan homologue to have been isolated is that of the cephalopod *Octopus vulgaris* [27]. This α M shares a similar N-terminal amino acid sequence with the snail α M (53% identity; 8 of 15 residues). Although the N-terminal sequences of human α_2 M and snail α M share only 3 of 15 residues (20% identity), two of these, Tyr⁸ and Pro¹³, are highly conserved in the vertebrate α Ms [39,45] and C3 proteins [46,47]. The strict conservation of Tyr⁸ and Pro¹³ extends to all of the characterized invertebrate α Ms, with the exception of *Limulus* α M, where Tyr⁸ has been conservatively replaced by phenylalanine. Such conservation in distantly related species implies an essential role for these amino acids.

The snail α M exhibits the characteristic autolytic fragmentation of thioester-containing proteins, and reaction with methylamine inhibits the fragmentation and releases one mol of thiol per α M subunit. However, methylamine treatment does not cause a conformational change that can be detected by native PAGE and results in only a slight decrease in the proteinase inhibitory activity of the snail α M. In the two human α Ms, pregnancy zone protein (PZP) and α_2 M, there are two different mechanisms for generating conformational changes [48–50]. The

key step for initiating conformational change in α_2M is cleavage of the thioester, whereas in PZP it is cleavage of the bait region [48]. The snail αM is similar to PZP in that reaction with amines (i.e. cleavage of the thioester) does not cause a conformational change resembling the change induced by bait region cleavage. However, PZP differs from the snail αM in that PZP requires an intact thioester at the time of bait region cleavage, because it must covalently bind the proteinase to achieve inhibition. Cleavage of the snail αM thioester is dissociated from a complete loss of inhibitory capacity and the conformational change that occurs with bait region cleavage, and in this regard the snail αM is similar to rat α_2M [51], frog αM [52] and octopus αM [27].

The amount of proteinase inhibited per mol of snail αM varies according to the proteinase concentration. The ratio of mol trypsin inhibited per mol of snail αM decreased from 1.5:1 to 1.1:1 when the trypsin concentration was decreased from 600 to 6 nM. Similarly, the maximal binding ratio for trypsin and human α_2M decreases from 2:1 to 1.3:1 as the concentrations of the reactants decrease from 850 to 0.5 nM [53]. However, unlike human α_2M , the snail αM does not bind the maximum 2 mol of trypsin as the reactants approach micromolar concentrations. Larsson et al. [53] propose a model in which the binding ratio of proteinase to α_2M depends on the proteinase concentration, the association rate of a second proteinase with the initial 1:1 complex, and the rate of conformational alteration. Therefore the differences in stoichiometry observed under similar reaction conditions might reflect differences in association rates or conformational change rates between the various tetrameric αMs .

The molecular mass of the snail αM subunit is approx. 200 kDa and the native protein consists of four subunits. The tetrameric structure of the snail αM is unique among all characterized invertebrate and lower vertebrate αMs , because only dimeric αMs have been isolated from these groups. Until now, tetrameric αMs seemed to be restricted to higher vertebrates, because amphibians were the most primitive chordate group found to possess them. The physiological significance of the different quaternary structures is not clear. Enghild et al. [54] have suggested that the adaptive advantage of multimeric αMs might be increased efficiency in proteinase inhibition. However, a recent study suggests that the coexistence of monomeric and tetrameric αMs might be widespread among vertebrates [18]. Because the monomeric and tetrameric αMs in rat plasma each have a distinct bait region and each is under different transcriptional regulation [55–57], they could have discrete functional roles. Likewise, the dimeric PZP and the tetrameric α_2M of human plasma each possess a distinct bait region [58], and their plasma concentrations are regulated differently [59]. Thus monomeric, dimeric and tetrameric αMs seem to have distinct physiological roles and their unique structural features (e.g. quaternary structure, regulation of conformational change and bait region sequence) might allow each to perform unique functions. Because the αMs might have additional or alternative roles to that of proteinase inhibition, such as functioning as carrier or binding proteins ([57]; reviewed in [2,16]), it could be misleading to view the multimeric αMs as merely more efficient proteinase inhibitors. The identification of a tetrameric αM in a gastropod mollusc demonstrates that the selective pressures that led to the evolution of this tetrameric structure were not exclusive to higher vertebrates, although the nature of these selective pressures is not clear at this time.

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