Purification and characterization of an α -macroglobulin proteinase inhibitor from the mollusc *Octopus vulgaris*

Ida B. THØGERSEN, Guy SALVESEN, Frederic H. BRUCATO, Salvatore V. PIZZO and Jan J. ENGHILD*

Duke University Medical Center, Durham, NC 27710, U.S.A.

The cell-free haemolymph of the mollusc *Octopus vulgaris* inhibited the proteolytic activity of the thermolysin against the high-molecular-mass substrate hide powder azure. The purified inhibitor was a glycoprotein composed of two identical 180 kDa disulphide-linked subunits. In addition to the inhibition of the metalloproteinase thermolysin, the protein inhibited the serine proteinases human neutrophil elastase, pig pancreatic elastase, bovine chymotrypsin, bovine trypsin and the cysteine proteinase papain. A fraction of the proteinase-inhibitor complex resisted dissociation after denaturation indicating that some of the proteinase molecules became covalently bound. The nucleophile β -aminopropionitrile decreased the covalent binding of proteinases to the *Octopus vulgaris* protein, suggesting that this interaction is mediated by an internal thiol ester; the reactivity and the amino acid sequence flanking the reactive residues of the putative thiol ester were consistent with this hypothesis. Bound trypsin remained active against the low-molecular-mass chromatogenic substrate H-D-Pro-Phe-Arg *p*-nitroanilide and was protected from inhibition by active-site-directed protein inhibitors of trypsin; however, the bound trypsin was readily inhibited by small synthetic inhibitors. This indicates that the inhibition of proteinases is accomplished by steric hindrance. The proteinase-inhibitory activity of this protein is characteristic of inhibition by mammalian α -macroglobulins and the presence of a putative thiol ester suggests that the *Octopus vulgaris* proteinase inhibitor is a homologue of human α_2 -macroglobulin.

INTRODUCTION

Proteinases are ancient enzymes and have most likely been present throughout molecular evolution (Zwilling & Neurath, 1981). Since these enzymes by their nature pose a potential threat to the host organism, it is essential that their activity be strictly regulated. It is likely that proteinase inhibitors have co-evolved with proteinases, and a large number of inhibitors have indeed been identified. These can be divided into several distinct families on the basis of structural and functional similarities (Laskowski & Kato, 1980; Read & James, 1986). Some proteinase-inhibitor families, for example serpins (Carrell & Boswell, 1986) and cystatins (Barrett *et al.*, 1986), are present in plants and animals, suggesting that these families developed early in evolution. Proteinase-binding α -macroglobulins (α Ms) have been detected only in animals (Sottrup-Jensen, 1987, 1989) and this family is presumably a more recent addition to the proteinase-inhibitor families.

Most inhibitors combine with the active site of proteinase forming a tight but non-covalent inactive complex (Laskowski & Kato, 1980; Read & James, 1986). Because they specifically interact with the active site of the proteinase, such inhibitors will bind and inhibit only proteinases from one mechanistic class. On the other hand, the proteinase inhibitory mechanism of α Ms is unique and is recognized by several distinct properties including: (1) similar subunit structure resulting in large proteins composed of identical 180 kDa subunits, (2) the presence of an internal reactive β -cysteinyl- γ -glutamyl thiol ester in each subunit of most α Ms, (3) proteinase-inhibitory activity against endopeptidases from all four catalytic classes (Barrett, 1982; Travis & Salvesen, 1983; Roberts, 1986), (4) an inhibitory mechanism that is initiated by a proteolytic attack on an exposed peptide stretch, the 'bait region,' followed by a conformational

change that results in entrapment of the active proteinase by the α M. Consequently, the inhibitory activity of α Ms resides in their ability to physically constrain and sterically hinder the access of the substrate to the bound proteinase (Barrett & Starkey, 1973). A portion of the proteinase is often covalently bound via interaction with an activated thiol ester, resulting in the formation of ϵ -lysyl- γ -glutamyl cross-links [for reviews see Travis & Salvesen (1983) and Sottrup-Jensen (1987, 1989)].

α Ms have been detected in vertebrates including mammals [see Sottrup-Jensen (1987) for review], birds (Nagase *et al.*, 1983; Nagase & Harris, 1983), reptiles (Starkey & Barrett, 1982a), amphibians (Ikai *et al.*, 1983; Feldman & Pizzo, 1985), fishes and sharks (Starkey & Barrett, 1982a,b; Starkey *et al.*, 1982). The α Ms have also been identified and characterized in invertebrate species belonging to the phylum Arthropoda, including crayfish (Hergenroth *et al.*, 1988; Stöcker *et al.*, 1991), lobster (Spycher *et al.*, 1987) and horseshoe crab (Quigley & Armstrong, 1983). In an attempt to elucidate the evolution of α M proteinase inhibitors further, we have identified a high molecular-mass proteinase-binding protein in the haemolymph of a mollusc, *Octopus vulgaris*. Because this protein shares so many properties with human α_2 -macroglobulin (α_2 M), we suggest that it is an α M homologue. α Ms have not been previously characterized from molluscs. As such, this protein represents the most primitive α M described to date.

MATERIALS AND METHODS

Materials

Pig pancreatic elastase, papain, thermolysin, bovine trypsin, bovine chymotrypsin, soya-bean trypsin inhibitor (SBTI), bovine pancreatic secretory trypsin inhibitor (PSTI), bovine pancreatic trypsin inhibitor (BPTI), 1,10-phenanthroline, hide powder azure

Abbreviations used: α M, α -macroglobulin; DCI, 3,4-dichloroisocoumarin; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; SBTI, soya-bean trypsin inhibitor; PSTI, pancreatic secretory trypsin inhibitor; BPTI, bovine pancreatic trypsin inhibitor; α_1^{PI} , α_1 -proteinase inhibitor; CMTI I, *Cucurbita maxima* trypsin inhibitor I.

* To whom correspondence should be addressed.

and β -aminopropionitrile were from Sigma. The general serine-proteinase inhibitor 3,4-dichloroisocoumarin (DCI) and the general cysteine-proteinase inhibitor *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) were from Boehringer Mannheim. Iodo[14 C]acetic acid (55 mCi/mmol) was from Amersham. The substrate H-D-Pro-Phe-Arg *p*-nitroanilide was obtained from Helena Laboratories (Beaumont, TX, U.S.A.). SDS/PAGE standard proteins were: rat α_1 -inhibitor 3 (190 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), hen's-egg-white albumin (42.7 kDa), bovine carbonic anhydrase (31.0 kDa), soya-bean trypsin inhibitor (21.5 kDa) and hen's-egg-white lysozyme (14.4 kDa). The standard proteins used in pore-limit gel electrophoresis were: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and BSA (67 kDa). Human α_2 M was purified as described by Kurecki *et al.* (1979). Rat α_1 -inhibitor 3 was purified as described by Enghild *et al.* (1989a). Limac, the α M from *Limulus polyphemus*, was purified as described by Enghild *et al.* (1990). Recombinant human α_1 -proteinase inhibitor (α_1 PI) was a gift from Dr. Hans-Peter Schnebli, Ciba-Geigy, Basel, Switzerland. *Curcubita maxima* trypsin inhibitor I (CMTI I) (Wieczorek *et al.*, 1985; Otlewski, 1990) was a gift from Dr. Jacek Otlewski, Max-Planck-Institute für Biochemie, Martinsried, Germany. Human neutrophil elastase was a gift from Dr. Wieslaw Watorek and Dr. James Travis, University of Georgia, Atlanta, GA, U.S.A.

Animals

Specimens of *Octopus vulgaris* were collected in Atlantic coastal waters and maintained at the Duke University Marine Laboratory, Beaufort, NC, U.S.A. Haemolymph was taken from the aorta, centrifuged for 5 min at 1200 rev./min (380 g) and the supernatant frozen at -70°C until used.

Protein purification

All steps were performed at 4°C . *Octopus vulgaris* α -macroglobulin (octopus α M) was purified by diluting cell-free haemolymph (20 ml) 5-fold with 25 mM-Tris/HCl/25 mM-NaCl, pH 7.4. The diluted haemolymph was filtered through a 0.22 μm -pore-size Sterivex-gs filter (Millipore) and applied to a DEAE-Sephacel column (2.5 cm $\times$ 20 cm) (Pharmacia) in 25 mM-Tris/HCl/25 mM-NaCl. The column was eluted with a linear gradient from 0.025 to 0.5 M-NaCl (0.475 mM $\cdot$ ml $^{-1}$). The fractions containing inhibitory activity against thermolysin and bovine trypsin were pooled and concentrated by ultrafiltration using a SpectraPor 100 kDa cut-off membrane. The final purification step was gel filtration on a Sephacryl S-300 column (2.5 cm $\times$ 150 cm) in 50 mM-Tris/HCl/150 mM-NaCl, pH 7.4. The absorption coefficient was determined by amino acid analysis with the use of norleucine to correct for loss during transfer and hydrolysis (Kupke & Dorrier, 1978).

Labelling of octopus α M with iodo[14 C]acetic acid

Purified octopus α M (10 mg) was denatured in 6 M-guanidinium chloride/200 mM-NH $_4$ HCO $_3$, pH 8, followed by addition of 50 mCi of iodo[14 C]acetic acid. This procedure would be expected to result in cleavage of a thiol ester by NH $_3$ with generation of a free thiolate ion reactive with iodo[14 C]acetic acid. The reaction was continued for 45 min before it was quenched with 20 mM-iodoacetic acid. The reaction mixture was dialysed extensively against 100 mM-Tris/HCl/100 mM-NaCl, pH 8.3. Solid guanidinium chloride was added to a final concentration of 6 M and the disulphides were reduced and alkylated by using 5 mM-dithiothreitol and 15 mM-iodoacetic acid. The

iodo[14 C]acetic acid-labelled protein was dialysed extensively into 50 mM-NH $_4$ HCO $_3$, pH 7.8, and digested with trypsin (1:50, w/w) at 37°C . The reaction was stopped after 4 h with 200 μM -DCI.

Peptide purification

The tryptic peptides were purified by reversed-phase h.p.l.c. on columns supplied by Applied Biosystems (Aquapore RP-300; 4.6 mm $\times$ 220 mm), Waters (Nova-pak C $_{18}$; 3.9 mm $\times$ 150 mm) and Beckman (Ultrasphere; 2 mm $\times$ 250 mm). The 2.0 mm column was connected to an Applied Biosystems 130A h.p.l.c. system. The other columns were used in the LKB h.p.l.c. system described previously (Enghild *et al.*, 1989b). Portions from each peak were counted for ^{14}C radioactivity in a Beckman LS 5000 TD liquid-scintillation system to locate the radioactive peptides.

Amino acid composition and sequence analysis

Protein or peptide samples (500 pmol) were hydrolysed for 24 h at 110°C in 6 M-HCl containing 0.1 % (w/v) phenol. The tubes were evacuated and flushed with nitrogen several times before they were sealed under vacuum. The hydrolysates were analysed in a Beckman 6300 amino acid analyser using sodium citrate buffers provided by the manufacturer. Automated Edman degradation was carried out in an Applied Biosystems 477A sequencer with on-line phenylthiohydantoin analysis using an Applied Biosystems 120A h.p.l.c. system. The samples were applied to Porton peptide or protein sample support discs employing the modified cycles PI-BGN and PI-1 recommended by Porton instruments.

PAGE

SDS/PAGE was performed in 5–15 % (w/v) gradient gels (10 cm $\times$ 10 cm $\times$ 1.5 mm) by using the glycine/2-amino-2-methylpropane-1,3-diol/HCl system described by Bury (1981). Non-denaturing pore-limit gel electrophoresis with continuous circulation of upper and lower reservoir buffers was performed in 5–15 % and 4–20 % gradient gels (10 cm $\times$ 10 cm $\times$ 1.5 mm) by using the Tris/EDTA/boric acid buffer system described by Manwell (1977).

Active-site standardization of proteins

The active concentrations of human neutrophil elastase, pig pancreatic elastase, bovine chymotrypsin and bovine trypsin were determined by titration with active-site-standardized human α_1 PI as described by Salvesen & Nagase (1989). Papain was activated by incubation with 1 mM-cysteine and 0.5 mM-EDTA for 15 min at 37°C (Anastasi *et al.*, 1983). The active concentration of papain was determined by titration with E-64 with hide powder azure as substrate. Thermolysin was assumed to be 80 % active by weight. The active concentrations of octopus α M and human α_2 M were determined by thiol ester titration essentially as described by Sottrup-Jensen *et al.* (1980).

Inhibitory assays

Octopus α M was examined for its ability to prevent various proteinases from digesting the insoluble high-molecular-mass substrate hide powder azure. Increasing amounts, from 0 to 0.1 nmol of octopus α M, were allowed to react with active-site-standardized proteinases: 0.03 nmol of human neutrophil elastase, 0.03 nmol of pig pancreatic elastase, 0.04 nmol of bovine chymotrypsin, 0.004 nmol of bovine trypsin and 0.03 nmol of thermolysin in 0.6 ml of 50 mM-Tris/HCl/100 mM-NaCl/0.1 mM-EDTA, pH 8 (5 mM-CaCl $_2$ was substituted for EDTA when thermolysin was used). Reactants were preincubated for 30 min at room temperature before 0.4 ml of a blue hide powder suspension, 12.5 mg/ml in 0.6 M-sucrose/0.05 % (v/v)

Triton X-100, was added. The tubes were incubated with sufficient agitation to keep the particulate substrate in suspension. The reaction was terminated by adding 0.3 ml of 3 M-glycine/HCl, pH 3. The hide powder azure was pelleted by centrifugation (13000 *g*), and the residual proteolytic activity was determined by measuring the A_{595} of the supernatant.

Protection assays

Octopus α M (4.2 pmol) was added to wells of a 96-well microtitre plate and allowed to react with bovine trypsin (0.42 pmol) in 50 mM-Hepes/100 mM-NaCl/0.05 % Nonidet P40, pH 7.4, at 25 °C for 15 min. Five separate assays were performed by adding increasing amounts (0–42 pmol) of the active-site-directed inhibitors: α_1 PI (45 kDa), SBTI (21.5 kDa), PSTI (10.9 kDa), BPTI (6.5 kDa) and CMTI I (3.7 kDa). The volume of each well was adjusted to 160 μ l and the reactants were incubated for 15 min before 40 μ l (final concentration 0.4 mM) of the chromogenic substrate H-D-Pro-Phe-Arg *p*-nitroanilide was added. The residual trypsin activity was measured by determining continuous rates of substrate hydrolysis over 15 min using a micro-plate reader (Thermomax, Molecular Devices, Menlo Park, CA, U.S.A.).

Radiolabelling of proteins

Proteins were labelled with 125 I by the solid-state lactoperoxidase method (David & Reisfeld, 1974), by using 0.5 mg of protein/0.1 mCi of 125 I followed by desalting on a PD-10 column (Pharmacia).

Covalent binding of proteinases

Octopus α M was incubated with 125 I-labelled pig pancreatic elastase, bovine chymotrypsin, bovine trypsin or thermolysin (1:0.8 mol/mol) in 50 mM-Tris/HCl/100 mM-NaCl, pH 7.5. After 4 min the reactions were terminated with an inhibitor cocktail consisting of 200 μ M-DCI, 40 μ M-E-64 and 4 mM-1,10-phenanthroline (Salvesen & Nagase, 1989). The proteinase-inhibitor complexes were purified by Superose-6 gel filtration and subjected to reduced SDS/PAGE. After autoradiography the radioactive bands were excised and counted in a γ counter (LKB, model 1275).

RESULTS AND DISCUSSION

Purification and chain composition

Octopus α M was purified by a combination of anion-exchange chromatography and gel filtration. Fractions were assayed for inhibitory activity against thermolysin and bovine trypsin by using hide powder azure as a substrate. Approx. 70 mg of protein was purified from 20 ml of haemolymph. The purified protein was homogeneous when analysed by *N*-terminal Edman degradation, Superose-6 gel filtration, unreduced SDS/PAGE (Fig. 1, lane g) and pore-limit gel electrophoresis (Fig. 2, lane f). The following 20 amino acids were identified by automated *N*-terminal Edman degradation and the gross yields are indicated in pmol: D₍₁₁₆₎, E₍₈₃₎, I₍₉₃₎, D₍₁₀₄₎, E₍₉₅₎, G₍₈₆₎, H₍₆₀₎, Y₍₇₁₎, M₍₅₇₎, I₍₅₂₎, L₍₄₆₎, V₍₅₂₎, P₍₃₀₎, R₍₄₀₎, N₍₂₄₎, F₍₂₃₎, V₍₂₈₎, H₍₂₀₎, G₍₂₄₎, K₍₁₅₎. No significant sequence similarity to available protein sequences was detected (National Biomedical Research Foundation Protein Identification Resources, release number 26). The absorption coefficient ($A_{1\text{ cm}, 280}^{1\%}$) was 14.0.

SDS/PAGE, under reducing conditions, yielded a heterogeneous band, whose leading edge corresponded to a subunit molecular mass of approx. 180 kDa (Fig. 1, lane d). This value is similar to that obtained for other α Ms. Under non-reducing conditions the molecular mass was 360 kDa (Fig. 1, lane g), also

similar to that of other α Ms. This suggests that the denatured molecule is composed of two 180 kDa subunits forming a 360 kDa disulphide-bonded dimer. The apparent molecular mass of the native protein, determined by pore-limit electrophoresis, was estimated to be 600 kDa (Fig. 2, lane f). The apparent molecular mass of the native molecule is lower than expected for a tetramer (720 kDa), but seems high for a dimer (360 kDa). If the native octopus α M molecule is a tetramer like human α_2 M composed of two dimers, we would expect the dimers to dissociate after a 4 h incubation in 4 M-urea, as determined by pore-limit gel electrophoresis (Barrett *et al.*, 1979). When octopus α M was analysed under these conditions, it did not dissociate (Fig. 3, lane e), suggesting that the native protein is a dimer. We believe that the large apparent molecular mass determined by pore-limit gel electrophoresis is due to glycosylation since a high content of glucosamine was detected after amino acid hydrolysis. Fur-

Fig. 1. Comparison of α M by SDS/PAGE

Electrophoresis was performed on 5–15 % gradient gels as described in the Materials and methods section. The gel lanes are as follows: molecular-mass (*M*) standards (lane a), human α_2 M, limac and octopus α M with reduction (lanes b, c and d respectively) and without reduction (lanes e, f and g respectively).

Fig. 2. Comparison of α Ms by non-denaturing pore-limit gel electrophoresis

Electrophoresis was performed on 4–20 % gradient gel as described in the Materials and methods section. The gel lanes are as follows: the protein molecular-mass (*M*) standards thyroglobulin, ferritin, catalase, lactate dehydrogenase (lane a); human α_2 M (lane b), rat α_1 -inhibitor 3 (lane c), limac (lane d), limac-chymotrypsin, 1:0.8 mol/mol (lane e), octopus α M (lane f), octopus α M-chymotrypsin, 1:0.8 mol/mol (lane g).

Fig. 3. Failure of octopus α M to dissociate in urea

The samples run on a 4–20% pore-limit gel as described in the Materials and methods section were the protein molecular-mass (M) standards thyroglobulin, ferritin, catalase, lactate dehydrogenase (lane a), human α_2 M (lane b), human α_2 M incubated in 4 M-urea for 4 h at 25 °C before the run (lane c), octopus α M (lane d), octopus α M incubated in 4 M-urea for 4 h at 25 °C before the run (lane e). Octopus α M did not dissociate in 4 M-urea, suggesting that the protein is not composed of two non-covalently associated dimers.

thermore, after treatment with endo- β -*N*-acetylglucosaminidase F, the protein band became less diffuse on reducing SDS/PAGE as would be expected after deglycosylation (not shown). This supports the hypothesis that the anomalous behaviour observed, diffuse and broad bands on polyacrylamide gels, is due to glycosylation (Fig. 1, lane d). From these observations we conclude that octopus α M is a dimer composed of two disulphide-bonded 180 kDa subunits.

All mammalian species appear to contain a tetrameric proteinase-binding α M, although dimeric (Sottrup-Jensen *et al.*, 1984a) and monomeric α Ms (Esnard & Gauthier, 1980; Enghild *et al.* 1989a) have been identified as well. Fishes, however, apparently contain α Ms composed of two subunits (Starkey & Barrett, 1982a,b; Starkey *et al.*, 1982), and this appears to be true of invertebrate species as well. Previously, all α Ms purified from invertebrates had been from organisms belonging to the phylum Arthropoda. These proteins have all been shown to be dimeric (Spycher *et al.*, 1987; Hergenroth *et al.*, 1988; Enghild *et al.*, 1990; Stöcker *et al.*, 1991). Similarly, *Octopus vulgaris*, which belongs to the more primitive phylum Mollusca, appears to be a dimeric protein.

Inhibition of proteinases

The inhibition of proteinases by octopus α M was tested by using the high-molecular-mass (particulate) substrate hide powder azure. Five proteinases from three catalytic classes, serine proteinases (human neutrophil elastase, pig pancreatic elastase, bovine chymotrypsin and bovine trypsin), metalloproteinases

Fig. 4. Reaction of active-site-directed proteinase inhibitors with α M–trypsin complexes

The ability of active-site-directed proteinase inhibitors of decreasing size to react with trypsin and trypsin bound to human α_2 M and octopus α M was compared. The trypsin and the trypsin– α M complex were incubated with increasing amounts of the inhibitors for 15 min. After this, continuous rates of substrate hydrolysis were measured to determine the residual trypsin activity (results given relative to that of trypsin incubated alone): recombinant human α_1 PI, 45 kDa (Δ); SBTI, 21.5 kDa ($\circ$); PSTI, 10.9 kDa ($\square$); BPTI, 6.5 kDa ($\bullet$); CMTI I, 3.7 kDa ($\blacksquare$). (a) Trypsin alone; (b) trypsin bound to human α_2 M; (c) trypsin bound to octopus α M.

Table 1. Proteinase-binding ratios of octopus α M

Proteinases were titrated with increasing amounts of octopus α M and allowed to react for 30 min at 25 °C before residual proteinase activity against hide powder azure was determined. The inclusion of 100 mM- β -aminopropionitrile in the reaction buffer did not affect the binding ratios (not shown).

Proteinase/inhibitor molar binding ratio					
Human neutrophil elastase	Porcine pancreatic elastase	Bovine chymotrypsin	Bovine trypsin	Thermolysin	Papain
1.0	1.2	1.3	0.8	1.1	1.1

Fig. 5. Non-denaturing pore-limit gel electrophoresis of octopus α M-chymotrypsin complexes

Electrophoresis was performed on a 4–15% gradient gel as described in the Materials and methods section. Octopus α M was incubated with 125 I-labelled chymotrypsin 1:0 (lane B), 1:0.08 (lane C), 1:0.2 (lane D), 1:0.4 (lane E), 1:0.8 (lane F) mol/mol. (a) Coomassie Blue-stained gel. It is evident from the autoradiography of the same gel (b) that the proteinase is associated with the Coomassie-stained band and no faster migrating band is apparent after proteinase binding. Lane A shows molecular-mass (M) standards.

(thermolysin) and cysteine proteinases (papain), were investigated. When allowed to react with the octopus α M, all were inhibited against the high-molecular-mass substrate hide powder azure and most of them formed close to equimolar complexes with octopus α M (Table 1). We conclude that the octopus α M shares with other α Ms the ability to inhibit proteinases of more than one catalytic class.

Inhibitory mechanism of octopus α M

The proteinase-inhibitory capacity of α Ms resides in their ability to hinder physically the access of substrate to the bound proteinase (Barrett & Starkey, 1973). Different α Ms possess various degrees of inhibitory capacity; for example, the monomeric α M rat α_1 -inhibitor 3 does not offer the same degree of shielding as the tetrameric human α_2 M (Enghild *et al.*, 1989a). In general, the inhibitory capacity of α Ms appears to be related to their size and therefore to their ability to shield (Enghild *et al.*, 1989a). The degree of shielding can be investigated by probing the accessibility of active-site-directed proteinase inhibitors of distinct sizes to the active site of the α M-bound proteinase. We compared the ability of octopus α M and human α_2 M to protect bound bovine trypsin from interacting with the following active-site-directed inhibitors: α_1 PI (45 kDa), SBTI (21.5 kDa), bovine PSTI (10.9 kDa), BPTI (6.5 kDa) and CMTI I (3.7 kDa) (Fig. 4). Human α_2 M and octopus α M decreased the activity of the bound trypsin against H-D-Pro-Phe-Arg *p*-nitroanilide by 35% and 55% respectively (Fig. 4b and 4c). The presence of contaminating active-site-directed inhibitors was ruled out since a 10-fold molar excess of human α_2 M and octopus α M was unable to decrease the trypsin activity further. This suggests that the α Ms alter the local environment of the proteinase to the extent that its activity is decreased. As expected, in the absence of α Ms all the inhibitors readily inactivated trypsin (Fig. 4a). When trypsin was in complex with the α Ms, the affinities were several times less and little inhibition was detected (Fig. 4b and 4c). The reaction of the

inhibitors with the bound trypsin presumably results in the formation of ternary complexes, and the weaker binding by the larger inhibitors probably results from a decreased productive collision rate. Within the concentration range used, human α_1 PI was unable to decrease the tryptic activity. The smaller inhibitors BPTI and CMTI I were able to decrease the activity significantly, suggesting that their smaller sizes permitted more access to the trapped proteinase. The small synthetic inhibitor DCI (0.215 kDa) completely inhibited the bound trypsin at a concentration of 100 μ M. We conclude that the active site of the trypsin in both α M complexes is sterically shielded, and that the degree of shielding offered by the octopus dimeric α M is comparable with, or better than, that of the tetrameric human α_2 M (Fig. 4b and 4c).

When human α_2 M binds a proteinase, a characteristic change in shape is observed on analysis by pore-limit gel electrophoresis (Barrett & Starkey, 1973). This increase in mobility is thought to be a consequence of subunit compaction of the tetrameric human α_2 M, trapping the bound proteinase. This key observation played a central role in the elucidation of the inhibitory mechanism of α Ms (Barrett & Starkey, 1973). The compaction of the molecule is not seen in all proteinase-binding α Ms (Starkey *et al.*, 1982; Christensen *et al.*, 1989; Enghild *et al.*, 1989a). The reaction of octopus α M with 125 I-labelled chymotrypsin did not result in any conformational change detectable by pore-limit gel electrophoresis (Fig. 5). This may be explained by an inhibitory mechanism in which a conformational change occurs that encages the proteinase without altering the apparent Stokes' radius.

Covalent binding of proteinases

Human neutrophil elastase contains no lysine (Sinha *et al.*, 1987), and is therefore unable to form the ϵ -amino- γ -glutamyl cross-link that results from reaction of most α Ms with other proteinases (Howard, 1981; Barrett, 1982; Sottrup-Jensen, 1987). Nevertheless the octopus α M was able to inhibit human

Table 2. Ability of the nucleophile β -aminopropionitrile to prevent the covalent binding of proteinases

Octopus α M was allowed to react with 125 I-labelled proteinase (1:0.8 mol/mol) for 5 min in the presence and absence of 50 mM- β -aminopropionitrile before the complex was purified by gel filtration and submitted to reducing SDS/PAGE. The autoradiogram was aligned with the gel and the radioactive bands were excised to quantify the covalent binding of the proteinase relative to as described in the Materials and methods section.

Presence of β -aminopropio- nitrile	Relative covalent binding of proteinases by octopus α M (%)			
	Pig pancreatic elastase	Bovine chymotrypsin	Bovine trypsin	Thermolysin
Without	52	44	44	46
With	17	16	12	17

neutrophil elastase (Table 1), suggesting that the inhibitory capacity of octopus α M was not dependent on the covalent linking reaction. To explore the relevance of cross-linking to inhibition further, octopus α M was allowed to react with 125 I-labelled proteinases in the presence or absence of β -aminopropionitrile, a nucleophile that is known to compete with proteinase side chains during the covalent linking reaction in human α_2 M and rat α_1 -inhibitor 3 (Salvesen *et al.*, 1981; Enghild *et al.*, 1989a). The complex was purified by gel filtration and examined by SDS/PAGE followed by autoradiography. The radioactive bands were counted in a γ counter and the results are presented in Table 2. The covalent binding of octopus α M is comparable with human α_2 M (Salvesen & Barrett, 1980; Sottrup-Jensen, 1987), but the ability of the nucleophile to abolish the covalent binding is diminished relative to human α_2 M (Salvesen *et al.*, 1981) and rat α_1 -inhibitor 3 (Enghild *et al.*, 1989a). The decrease in the covalent binding did not affect the proteinase-inhibitory capacity, suggesting that octopus α M is capable of restraining the proteinase in a manner independent of the covalent interaction. This is in contrast with the monomeric rat α_1 -inhibitor 3 (Enghild *et al.*, 1989a) and the dimeric human pregnancy zone protein (Christensen *et al.*, 1989) which depend on the covalent binding reaction for inhibition. The function of the covalent binding, as for most proteinase-binding α Ms, remains unclear.

Putative internal thiol ester

Human α_2 M (Harpel & Hayes, 1979) and octopus α M have the ability to bind proteinases covalently (Table 2). Furthermore, they both undergo autolytic cleavage of the polypeptide chain, suggesting the presence of an internal thiol ester. To investigate this possibility further, octopus α M was denatured in the presence

of 200 mM-NH₄HCO₃, pH 8, and iodo[14 C]acetic acid. This procedure would be expected to result in a nucleophilic substitution of the thiol ester by NH₃, resulting in the release of a glutamine and a cysteine. The thiol of the cysteine is subsequently carboxymethylated by the iodo[14 C]acetic acid. After reduction and alkylation, the protein was digested with trypsin and the resulting peptides were separated by h.p.l.c. The major radioactive peptide was analysed by Edman degradation and amino acid analysis. The phenylthiohydantoin amino acids detected and gross yield in pmol were: K₍₄₇₂₎, P₍₃₄₂₎, S₍₁₆₆₎, G₍₅₀₅₎, X_(not quantified), G₍₃₃₀₎, E₍₂₅₅₎, Q₍₂₈₁₎, N₍₂₅₅₎, M₍₂₃₃₎, I₍₂₄₇₎, N₍₈₁₎, F₍₁₇₃₎, Y₍₁₅₈₎, P₍₉₉₎, N₍₈₇₎, V₍₄₂₎, L₍₂₃₎. The amino acid composition in pmol of the peptide, CM-Cys₍₄₁₀₎, B₍₉₈₈₎, S₍₃₃₅₎, Z₍₇₁₆₎, P₍₈₁₀₎, G₍₇₈₂₎, V₍₄₈₆₎, Met₍₁₆₁₎, I₍₃₆₇₎, L₍₃₈₀₎, Y₍₃₃₃₎, F₍₃₅₆₎, K₍₃₆₁₎, was in good agreement with the protein sequence. During Edman degradation, radioactivity was released as indicated: -[14 C]Xaa-Gly-Glu-Gln- and the sequence, amino acid composition and reaction products are consistent with the predicted reactivity of a β -cysteinyl- γ -glutamyl thiol ester. We note that the major radioactive peptide is apparently a cleavage variant since the C-terminal amino acid is leucine. This may be due to residual chymotrypsin activity in the trypsin preparation used. The sequence of the octopus α M thiol ester shows high identity with other known invertebrate thiol ester sequences and human α_2 M (Fig. 6).

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Octopus α M	Lys-Pro-Ser-Gly-Cys-Gly-Glu-Gln-Asn-Met-Ile-Asn-Phe-Tyr-Pro-Asn-Val-Leu
(1) Limac	Leu-Pro-Thr-Gly-Cys-Gly-Glu-Gln-Asn-Met-Ile-Lys-Phe-Val-Pro-Asn-Ile-Phe
(2) Crayfish	Met-Pro-Tyr-Gly-Cys-Gly-Glu-Gln-Asn-Met-Val-Asn-Phe-Ala-Pro-Asn-Ile-Phe
(3) Lobster	Met-Pro-Tyr-Gly-Cys-Gly-Glu-Gln-Asn-Met-Val-Asn-Phe-Ala-Pro-Asn
(4) Human α_2 M	Met-Pro-Tyr-Gly-Cys-Gly-Glu-Gln-Asn-Met-Val-Leu-Phe-Ala-Pro-Asn-Ile-Phe

Fig. 6. Thiol ester sequence of octopus α M

The sequence of the 18-amino-acid-residue peptide derived from octopus α M is compared with the corresponding region in human α_2 M and with available invertebrate thiol-ester-containing proteins. It is apparent that the putative thiol ester sequence is present in the octopus α M; furthermore, the reactivity of the residues is consistent with that of a β -cysteinyl- γ -glutamyl-thiol. The bold-typed residues indicate the components of the putative thiol ester. (1) Enghild *et al.* (1990); (2) Hall *et al.* (1989); (3) Spycher *et al.* (1987); (4) Sottrup-Jensen *et al.* (1984b).

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