

# Proteinase Binding and Inhibition by the Monomeric $\alpha$ -Macroglobulin Rat $\alpha_1$ -Inhibitor-3\*

(Received for publication, January 23, 1989)

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The inhibitory capacity of the  $\alpha$ -macroglobulins resides in their ability to entrap proteinase molecules and thereby hinder the access of high molecular weight substrates to the proteinase active site. This ability is thought to require at least two  $\alpha$ -macroglobulin subunits, yet the monomeric  $\alpha$ -macroglobulin rat  $\alpha_1$ -inhibitor-3 ( $\alpha_1I_3$ ) also inhibits proteinases. We have compared the inhibitory activity of  $\alpha_1I_3$  with the tetrameric human homolog  $\alpha_2$ -macroglobulin ( $\alpha_2M$ ), the best known  $\alpha$ -macroglobulin, in order to determine whether these inhibitors share a common mechanism.  $\alpha_1I_3$ , like human  $\alpha_2M$ , prevented a wide variety of proteinases from hydrolyzing a high molecular weight substrate but allowed hydrolysis of small substrates. In contrast to human  $\alpha_2M$ , however, the binding and inhibition of proteinases was dependent on the ability of  $\alpha_1I_3$  to form covalent cross-links to proteinase lysine residues. Low concentrations of proteinase caused a small amount of dimerization of  $\alpha_1I_3$ , but no difference in inhibition or receptor binding was detected between purified dimers or monomers. Kininogen domains of 22 and 64 kDa were allowed to react with  $\alpha_1I_3$ - or  $\alpha_2M$ -bound papain to probe the accessibility of the active site of this proteinase.  $\alpha_2M$ -bound papain was completely protected from reaction with these domains, whereas  $\alpha_1I_3$ -bound papain reacted with them but with affinities several times weaker than uncomplexed papain. Cathepsin G and papain antisera reacted very poorly with the enzymes when they were bound by  $\alpha_1I_3$ , but the protection provided by human  $\alpha_2M$  was slightly better than the protection offered by the monomeric rat  $\alpha_1I_3$ . Our data indicate that the inhibitory unit of  $\alpha_1I_3$  is a monomer and that this protein, like the multimeric  $\alpha$ -macroglobulins, inhibits proteinases by steric hindrance. However, binding of proteinases by  $\alpha_1I_3$  is dependent on covalent cross-links, and bound proteinases are more accessible, and therefore less well inhibited, than when bound by the tetrameric homolog  $\alpha_2M$ . Oligomerization of  $\alpha$ -macroglobulin subunits during the evolution of this protein family has seemingly resulted in a more efficient inhibitor, and we speculate that  $\alpha_1I_3$  is analogous to an evolutionary precursor of the tetrameric members of the family exemplified by human  $\alpha_2M$ .

Many animals, including limulus, reptiles, birds, and mammals, possess one or more of a family of homologous proteins

\* This work was supported by National Heart, Lung, and Blood Institute Grant HL-24066 and National Cancer Institute Grant CA-29589. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

known as  $\alpha$ -macroglobulins that share the ability to inhibit a vast array of proteinases irrespective of catalytic mechanism or substrate specificity (1–3). The broad specificity of the  $\alpha$ -macroglobulins is due to a mechanism that distinguishes them from the other active site-directed protein inhibitors of proteinases (4, 5). This mechanism has been analyzed in greatest detail using human  $\alpha_2$ -macroglobulin ( $\alpha_2M$ )<sup>1</sup> and was first described by Barrett and Starkey (6) who attempted to relate the unusual properties of human  $\alpha_2M$  to its inhibitory activity. It seems that the large size of human  $\alpha_2M$  enables proteins to become entrapped within the molecule as it undergoes a change in shape (6). This conformational change is the result of proteolysis of a single peptide bond within a region of 20 or so amino acids near the middle of one of the four identical subunits that comprise the molecule, the actual bond cleaved depending on the specificity of the attacking proteinase (2, 3, 7). Only endopeptidases can cause the conformational change leading to inhibition and, by analogy with the waiting "trap," the proteinase-susceptible region has been called the "bait region" (8) and the mechanism of action referred to as the "trap hypothesis" (6). Entrapped proteinase molecules, because of physical constraints, are unable to hydrolyze large substrates but retain the ability to hydrolyze small ones (9). A very approximate limit of 10–20 kDa can be placed on the size of a protein that may enter the sprung "trap" in  $\alpha_2M$  and gain access to the proteinase (1, 10).

One of the key inhibitory abilities of human  $\alpha_2M$  is thus, by necessity, its large size (720 kDa, composed of four 180-kDa identical subunits), and we have previously documented the dimensions of the proteinase-reacted molecule and proposed a model that predicts the minimal inhibitory unit of human  $\alpha_2M$  (and, by inference, other  $\alpha$ -macroglobulins) to be a dimer of the 180-kDa subunits (11). Normally, the subunits of the native tetramer cooperate to inhibit a proteinase (11–13). Several animals possess homologues of the human  $\alpha_2M$  in which the inhibitory unit is dimeric. Mice (14, 15) and rats, moreover (16, 17), possess monomeric  $\alpha$ -macroglobulins composed of single subunits, yet there is disagreement concerning their inhibitory capacities (16, 21). Indeed a recent publication has suggested that the rat monomeric  $\alpha_1I_3$  functions as a general binding protein rather than a proteinase inhibitor (22).

Based on several models of human  $\alpha_2M$ , it might be predicted that  $\alpha_1I_3$  should not inhibit proteinases, except by association of monomers to multimeric forms. We have tested this hypothesis and found it to be incorrect, and we present

<sup>1</sup> The abbreviations used are:  $\alpha_2M$ ,  $\alpha_2$ -macroglobulin;  $\alpha_1I_3$ ,  $\alpha_1$ -inhibitor-3; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; E-64, N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-4-aminobutyrguanidine; DCI, 3,4-dichloroisocoumarin; L-kgn, low molecular weight kininogen; AMC, 7-amino,4-methyl coumarylamide; pNA, 4-nitroanilide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RIA, radioimmunoassay.

details of the methods we have used to refute this prediction. We have compared  $\alpha_2M$  and  $\alpha_1I_3$  with respect to the following properties: (i) inhibitory selectivity against a range of proteinases of different primary specificities and from three catalytic classes; (ii) protection of bound proteinases from reaction with antisera or protein inhibitors of various sizes; (iii) chain composition and molecular dimension of the proteinase inhibitory unit; (iv) relationship between covalent linking and inhibition of proteinases, thus enabling us to better understand the requirements for inhibition of proteinases by  $\alpha$ -macroglobulins in general.

## EXPERIMENTAL PROCEDURES

**Materials**—Pig pancreatic elastase, papain, thermolysin, and 1,10-phenanthroline were from Sigma. 1-Chloro-3-tosylamido-7-amino-2-heptanone-treated bovine chymotrypsin, the general serine proteinase inhibitor 3,4-dichloroisocoumarin (DCI), and the general cysteine proteinase inhibitor E-64 were from Boehringer Mannheim. Human plasma was from Duke University Medical Center Blood Bank. Rat plasma was purchased from Pel-Freez Biologicals. Human neutrophil elastase and cathepsin G were kind gifts of Wieslaw Watorek and James Travis, University of Georgia. Papain antiserum was generously provided by Alan Barrett, Strangeways Laboratory, Cambridge, United Kingdom, and the cathepsin G antiserum has previously been described (23).

**Protein Purification**— $\alpha_1I_3$  was purified by dialysis of rat plasma against 200 volumes of deionized water overnight at 4 °C. Following removal of the precipitate the supernatant was made 50 mM in Tris-HCl and 50 mM NaCl, pH 7.4, and batch adsorbed to 2 volumes of packed Cibacron blue Sepharose CL-4B. After 30 min of gentle stirring at 4 °C, the beads were washed on a sintered glass funnel, and the batch adsorption was repeated on regenerated Cibacron blue Sepharose CL-4B. Unadsorbed material was applied to a 2.5 × 10-cm Q Sepharose fast flow column in 50 mM Tris-HCl, 50 mM NaCl, pH 7.4, and eluted with a linear gradient from 0.1 to 0.4 M NaCl (1.33 mM ml<sup>-1</sup>) using a Pharmacia FPLC system. The fractions containing  $\alpha_1I_3$  were concentrated by ultrafiltration using a SepettaPor 100-kDa cutoff membrane. The final purification step was gel filtration on Sephadryl S300 or Superose 6 in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4. Human  $\alpha_2M$  was purified as described by Kurecki *et al.* (24). Low molecular weight kininogen (L-kgn) was purified as described by Johnson *et al.* (25). Kininogen domain 3 (22 kDa) was prepared and purified as described by Salvesen *et al.* (26).  $\alpha_1$ -Proteinase inhibitor was purified essentially as described by Pannell *et al.* (27).

**Active Site Standardization of Proteins**—Trypsin was standardized by titration with *p*-nitrophenyl *p*-guanidinobenzoate as described by Chase and Shaw (28). All subsequent assays were performed in 50 mM Tris-HCl, 0.1 M NaCl, pH 8.0. The active concentration of  $\alpha_1$ -proteinase inhibitor and human  $\alpha_2M$  was determined using standardized trypsin under conditions that result in an equimolar complex (29), using hide powder azure as the substrate. The active concentrations of pancreatic elastase, neutrophil elastase, cathepsin G, and chymotrypsin were determined by titration with active site-standardized  $\alpha_1$ -proteinase inhibitor, using hide powder azure as a substrate (30). Papain was activated by incubation in assay buffer containing 1 mM cysteine and 0.5 mM EDTA for 15 min at 37 °C and titrated immediately by using compound E-64 (31) and D-Pro-Phe-Arg-AMC as substrate. The active concentration of L-kgn and the 22-kDa tryptic fragment thereof was determined by titration with standardized papain. All active site-standardized preparations were kept on ice and used within 3 h unless otherwise indicated in the text. Thermolysin was not active site-titrated and assumed to be 100% active by weight.

**Proteinase Bait Region Cleavage Sites in  $\alpha_1I_3$** —1 nmol of  $\alpha_1I_3$  in 50 mM Tris-Cl, pH 7.4, 100 mM NaCl was reacted with 0.5 nmol of thermolysin or active site-standardized pig pancreatic elastase, chymotrypsin, cathepsin G, neutrophil elastase, or papain. The reaction was terminated after 1 min by making the mixtures 50  $\mu$ M DCI for the serine proteinases, 10  $\mu$ M E-64 for papain, and 1 mM 1,10-phenanthroline for thermolysin. The sample was made 1% in recrystallized SDS (32), 10 mM in dithiothreitol, and incubated at 80 °C for 5 min. The resulting fragments were resolved by SDS-7% polyacrylamide gel electrophoresis (PAGE). The protein material was transferred to polyvinylidene difluoride membranes (Millipore Immobilon transfer membranes) according to the procedure of Matsudaira (33).

The bands were detected by Coomassie Blue, and both of the two characteristic bait region cleavage bands (85 and 75 kDa) and the 110-kDa enzyme: $\alpha_1I_3$  cross-linked band was excised. The bands were separately placed onto Polybrene-treated precycled glass fiber filters and sequenced by automated Edman degradation.

**Amino Acid Sequence Analysis**—Automated Edman degradation was carried out in an Applied Biosystem 477 A pulsed-liquid phase sequence with on-line phenylthiohydantoin analysis using an Applied Biosystem 120 A HPLC system. Both instruments were operated as recommended in the user bulletins and manuals distributed by the manufacturer.

**Radiolabeling of Proteins**—Proteins were labeled with <sup>125</sup>I by the solid-state lactoperoxidase method (34), using 0.5 mg of protein/0.1 mCi of <sup>125</sup>I, followed by desalting on Sephadex G-25.

**Plasma Clearance Studies**—<sup>125</sup>I-Labeled  $\alpha_1I_3$ -chymotrypsin complexes were injected into the lateral tail vein of female CD-1 mice. Blood, 25  $\mu$ l, was collected from the retroorbital venous plexus at various times and counted for  $\gamma$  radioactivity. The initial ligand concentration was determined as the radioactivity in an aliquot removed 5 to 10 s after injection, and the radioactivity remaining in circulation was expressed as a fraction of this initial value (35).

**Polyacrylamide Gel Electrophoresis**—SDS-PAGE was performed in 7% gels or 5–15% gradient gels (10 cm × 10 cm × 1.5 mm) using the glycine/2-amino-2-methyl-1,3-propanediol/HCl system described by Bury (36). Nondenaturing pore limit gel electrophoresis was performed in 4–20% or 4–15% gradient gels (10 cm × 10 cm × 1.5 mm) in a Tris/EDTA/boric acid buffer system according to Manwell (37) with continuous circulation of upper and lower reservoir buffers. Some samples were run in the nondenaturing pore-limit gel system as the first dimension and the denaturing SDS-PAGE system as second dimension. After brief staining and destaining of the pore-limit gels the bands of interest were excised and equilibrated for 30 min at 37 °C in 0.5 ml of the SDS-containing upper reservoir buffer with or without 50 mM dithiothreitol. The gel pieces were pushed to the bottom of the wells in the second dimension 5–15% gradient gel system described above, and electrophoresis was carried out in the normal way.

**Hydrodynamic Studies**—The molecular weight of rat  $\alpha_1I_3$  was determined in a Spinco model E ultracentrifuge as previously described (39).

**Purification of  $\alpha_1I_3$  Dimers**—To generate dimers,  $\alpha_1I_3$  was purified by gel filtration on Superose 6 to remove any dimers that might already be present.  $\alpha_1I_3$  was reacted with a 0.04 molar ratio of chymotrypsin in 50 mM Tris-HCl, 100 mM NaCl, pH 7.4. After 15 min the reaction was terminated by adding 50  $\mu$ M DCI, and the reaction mixture was applied to a Superose 6 gel filtration column to separate monomeric from dimeric  $\alpha_1I_3$  products.

**Inhibitory Assays**—Human  $\alpha_2M$  and rat  $\alpha_1I_3$  were compared for their ability to protect the various proteinases from digesting the high molecular weight substrate hide powder azure. Increasing amounts, from 0 to 0.1 nmol of human  $\alpha_2M$  and from 0 to 2 nmol of rat  $\alpha_1I_3$ , were reacted with 0.04 nmol of active site-standardized proteinase in 0.6 ml of 50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, pH 8 (or 5 mM CaCl<sub>2</sub> substituting 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% 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 blue hide powder was pelleted by centrifugation, and residual proteolytic activity was determined by measuring the  $A_{595}$  of the supernatant.

**Kinetic Assays**— $K_i$  values for the reaction of  $\alpha_1I_3$ -bound papain and L-kgn or the 22-kDa tryptic fragment thereof were determined in a continuous assay by the method of progress curves (38). An  $\alpha_1I_3$ -papain complex, 0.5 nM with respect to papain, was incubated in a 2-ml assay buffer containing 0.1  $\mu$ M D-Pro-Phe-Arg-AMC to establish the rate of substrate hydrolysis ( $v_0$ , Fig. 4) which was determined by fluorescence emission at 450 nm following excitation at 380 nm using a Shimadzu RF540 recording spectrophotometer. 0.3 nmol of L-kgn or 0.12 nmol of the 22-kDa fragment (in a volume of 20  $\mu$ l) was added, with rapid mixing, and the substrate hydrolysis followed until the rate had relaxed to a new value ( $v_i$ ). Since substrate concentrations were well below  $K_m$ , the apparent  $K_i$  of the reaction of L-kgn or the 22-kDa fragment was determined according to the following equation (38).

$$v_0/v_i = 1 + [I]/K_i$$

Attempts to determine the  $K_i$  for the reaction of  $\alpha_2M$ -bound papain with L-kgn or the 22-kDa fragment were carried out at higher enzyme and inhibitor concentrations, necessitating the use of the less sensitive substrate D-Pro-Phe-Arg-pNA. 40 nM of free or  $\alpha_2M$ -bound papain was incubated in the presence of 0.1 mM D-Pro-Phe-Arg-pNA to establish  $v_0$ , followed by the addition of L-kgn or the 22-kDa fragment to a concentration of 20  $\mu$ M, and substrate hydrolysis was monitored by following absorbance at 405 nm using a Shimadzu UV160 recording spectrophotometer.

**Radioimmunoassays Using Antisera against Cathepsin G and Papain**—Microtiter plates, 96-well, were coated with 100  $\mu$ l of antisera diluted 50 times and incubated for 24 h at 4 °C. The wells were washed once with 200  $\mu$ l of 25 mM HEPES, 100 mM NaCl, pH 7.4, and twice with the same buffer containing 1% bovine serum albumin (RIA buffer). Residual binding sites were blocked by leaving the wells in the last RIA buffer wash for 2 h before the wells were washed again with RIA buffer.

Increasing concentrations of  $^{125}$ I-labeled cathepsin G inactivated with DCI were added to the wells. For comparison the same amount of cathepsin G was reacted for 15 min at 37 °C with a 5-fold molar excess of rat  $\alpha_1I_3$ , 2-fold excess human  $\alpha_2M$ , or a 5-fold excess of  $\alpha_1$ -proteinase inhibitor before the reaction was terminated by making the reaction mixture 50  $\mu$ M in DCI. All samples were added to the wells in RIA buffer and incubated overnight. The wells were washed three times with 200  $\mu$ l of RIA buffer, removed, and counted for radioactivity in a  $\gamma$  counter.

A similar experiment was performed using  $^{125}$ I-labeled papain and papain antiserum. Papain was reacted with excess  $\alpha_1I_3$ ,  $\alpha_2M$ , or L-kgn for 15 min before the papain was inactivated with 50  $\mu$ M E-64. Binding of free and inhibitor-complexed papain to the antiserum was monitored as described above.

## RESULTS

**Bait Region Cleavage Sites**—Our preparation of rat  $\alpha_1I_3$  resolved into two peaks on ion-exchange chromatography on mono Q, as has been previously noted (17). Representative fractions from each peak were pooled and reacted with 0.5 molar ratio of active site-standardized proteinase. Bovine chymotrypsin, porcine pancreatic elastase, human cathepsin G, human neutrophil elastase, and papain all cleaved  $\alpha_1I_3$  to generate equivalent derivatives of 85 and 75 kDa (Fig. 1). In each case the lower of these two bands contained the amino-terminal sequence of intact  $\alpha_1I_3$  (22) whereas protein sequence analysis of the upper band revealed cleavage at the sites shown in Table I. The observed cleavage sites were within a region that aligns with the proteinase-susceptible "bait" region of  $\alpha_2M$ . Bait region sequence analysis allowed us to assign the majority of material in the first mono Q peak as representing

the product of the predicted  $\alpha_1I_3$  mRNA designated 27J (22, 40). Similarly, the majority of material in the second mono Q peak represents the product of the predicted  $\alpha_1I_3$  mRNA designated 2J (22). Consequently, our results prove that both transcripts are expressed by the rat.

Thermolysin, even at low enzyme: $\alpha_1I_3$  molar ratios, degraded the protein to several fragments in the range 25–70 kDa, and we were unable to determine the bait region cleavage site for this enzyme.

**Inhibition of Proteinases**—The inhibition of a variety of proteinases by  $\alpha_2M$  and  $\alpha_1I_3$  was tested using the high molecular weight (particulate) substrate hide powder azure as described under "Experimental Procedures," and the results are presented in Table II. Human neutrophil elastase and thermolysin were not inhibited by  $\alpha_1I_3$ , although both were efficiently inhibited by human  $\alpha_2M$ . The other proteinases were inhibited by  $\alpha_1I_3$  (Table II), but the inhibition was apparently nonstoichiometric such that more than 1 mol of  $\alpha_1I_3$  was required to inhibit 1 mol of proteinase. Inhibition against low molecular weight substrates was variable. The ability of papain to hydrolyze D-Pro-Phe-Arg-AMC was not decreased by reaction with  $\alpha_1I_3$ , but the ability of pig pancreatic elastase to hydrolyze methoxysuccinyl-Ala-Ala-Pro-Val-AMC and of chymotrypsin to hydrolyze methoxysuccinyl-Arg-Pro-Tyr-pNA was decreased by 25% (data not shown). We conclude that  $\alpha_1I_3$  inhibits proteinases by steric hindrance as does human  $\alpha_2M$ , although more  $\alpha_1I_3$  is needed for complete inhibition. Our interpretation of this is discussed later.

**Size of the Inhibitory Unit of  $\alpha_1I_3$** —Although proteinases react with artificially produced monomers of human  $\alpha_2M$  (12, 41) it is thought that the inhibitory unit of this  $\alpha$ -macroglobulin is a dimer (11, 41) and that inhibition results from dimer or tetramer formation (12, 41, 42). Rat  $\alpha_1I_3$  also forms dimers when treated with proteinases (Fig. 2, band 5, and Fig. 6). The proportion of dimers varies according to the  $\alpha_1I_3$ :proteinase molar ratio, but the amount of proteinase associated with the dimer was never more than 15%, based on radioactive enzyme associated with the monomer and dimer. This contrasts with human  $\alpha_2M$  where monomers generated by partial reduction assemble to tetramers or dimers following proteinase reaction irrespective of the proteinase used (12, 41). Sedimentation ultracentrifugation of four separate preparations resulted in a measured molecular mass of 174,000 Da for the monomer and 189,000 Da for the complex with chymotrypsin. We conclude that the inhibitory unit of rat  $\alpha_1I_3$  is the 174,000-Da monomer and that the small amount of dimerization of the protein, whatever its cause, is not responsible for the observed inhibition.

Reaction of  $\alpha_1I_3$  with various concentrations of  $^{125}$ I-labeled proteinase (chymotrypsin or pancreatic elastase) resulted in five distinct protein bands when analyzed by pore limit gel electrophoresis (Fig. 2). The bands were cut out of the pore limit gel and analyzed by SDS-PAGE as described under "Experimental Procedures." Band 1 was native material, it was not radioactive, and no bait region cleavage was detected. The bait region was cleaved in all the protein material migrating in band 2, but no radioactivity was associated with the band. This suggests that a proteinase is able to cleave the bait region of  $\alpha_1I_3$  molecules without becoming bound. Bands 3, 4, and 5 all had bait regions cleaved and were radioactive due to covalent binding of proteinase. These results suggest that  $\alpha_1I_3$  undergoes bait region cleavage with (bands 3–5) or without (band 2) concomitant covalent cross-linking of the proteinase. In the latter case, the proteinase is neither inhibited nor remains bound to the  $\alpha_1I_3$ .

**Covalent Linking Is Required for Inhibition**—Of the pro-

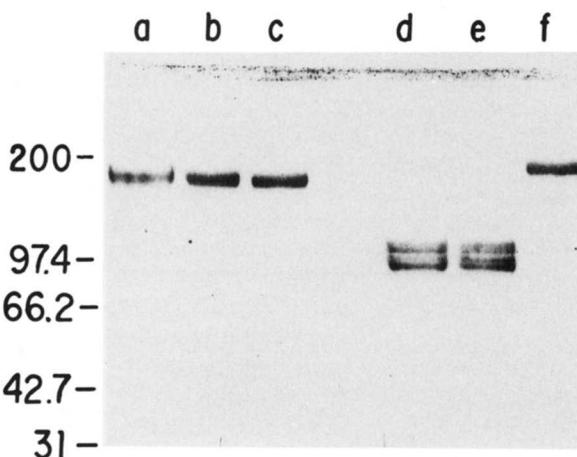


FIG. 1. SDS-PAGE of  $\alpha_1I_3$  reacted with neutrophil elastase. Neutrophil elastase was allowed to react with  $\alpha_1I_3$  at molar ratios of 0.8 (a, d), 0.1 (b, e), or 0.01 (c, f) for 10 min at 37 °C in 0.1 M Tris/HCl buffer, pH 8, 0.1 M NaCl. DCI was added to 50  $\mu$ M, and the samples were run in SDS-PAGE without (a–c) or with (d–f) reduction.

TABLE I  
Bait region cleavage sites in the two rat  $\alpha_1I_3$  isoforms

The bait region sequences of  $\alpha_1I_3$  isoforms is shown. Separation of rat  $\alpha_1I_3$  on Mono Q results in two major peaks. 1 nM mol of material from each peak was reacted with bovine chymotrypsin (CHYMO), porcine pancreatic elastase (PPE), human cathepsin G (CATG), human neutrophil elastase (HNE), and papain for 1 min at a molar ratio at 0.5–1. The samples were inactivated with DCI or E-64, made 1% in SDS, reduced with dithiothreitol, run in 7% SDS-PAGE, and the bait region cleavage sites were determined as described under "Experimental Procedures." The two sequences correspond to the two  $\alpha_1I_3$  cDNA clones recently identified (22, 38, 40). Cys-654 is numbered according to Braciak *et al.* (22).

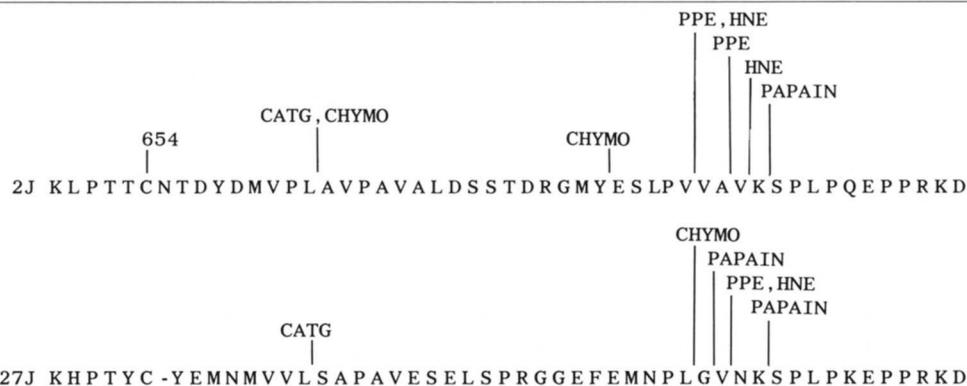


TABLE II

Comparative proteinase binding ratios of human  $\alpha_2M$  and rat  $\alpha_1I_3$ 

Proteinases were titrated with increasing amounts of  $\alpha_2M$  or  $\alpha_1I_3$  and allowed to react for 30 min at 25°C before quantitation of residual activity against blue hide powder. With the exception of neutrophil elastase and thermolysin, which are not inhibited by  $\alpha_1I_3$  at 100-fold molar excess of inhibitor, all titration plots were linear and showed >95% inhibition at the binding ratios noted below. Binding ratios for pig pancreatic elastase were determined in the absence (a) or presence (b) of 10 mM 3-aminopropionitrile.

Inhibitor	Proteinase/inhibitor molar binding ratio					
	Pig pancreatic elastase	Chymotrypsin	Cathepsin G	Neutrophil elastase	Papain	Thermolysin
(a)	(b)					
$\alpha_2M$	1.8	1.7	1.2	1.8	1.8	1.0
$\alpha_1I_3$	0.3	0.06	0.36	0.55	<0.01	0.42
						<0.01

teinases tested that were not inhibited by  $\alpha_1I_3$ , thermolysin degraded the protein to a variety of distinct polypeptides whereas human neutrophil elastase caused complete bait region cleavage, even at low proteinase: $\alpha_1I_3$  molar ratios (Fig. 1), but did not further degrade the protein. Neutrophil elastase contains no lysine residues (43), and we reason, therefore, that it is unable to form the 6-amino-3-carbonyl cross-link that results from reaction of most  $\alpha$ -macroglobulins with other proteinases (1, 3, 44). We explored the relevance of cross-linking to inhibition by reacting  $\alpha_1I_3$  with pancreatic elastase in the presence of a nucleophile (3-aminopropionitrile) that is known to compete with proteinase side chains during the covalent linking reaction in human  $\alpha_2M$  (45). When titrated in the presence of 10 mM 3-aminopropionitrile much more  $\alpha_1I_3$  was required to inhibit pancreatic elastase (see Table II). Moreover, increasing amounts of 3-aminopropionitrile resulted in excessive bait region cleavage by a small amount of pancreatic elastase (Fig. 3). 3-Aminopropionitrile at a concentration of 0.1 M caused less than 0.1 mol of thiol release per mol of  $\alpha_1I_3$  in 30 min at 25 °C, and we conclude that the decreased binding ratio in the presence of 3-aminopropionitrile is due to competition for covalent linking and not to inactivation of the inhibitor by the amine. These data imply that the inhibitory potential of  $\alpha_1I_3$  is dependent on its

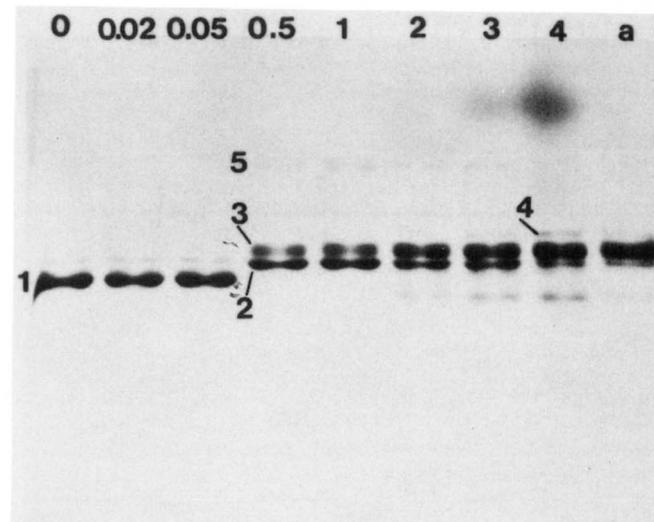


FIG. 2. Pore limit gel electrophoresis of  $\alpha_1I_3$  proteinase complexes.  $\alpha_1I_3$  was reacted with the molar equivalents of  $^{125}I$ -labeled chymotrypsin indicated in the figure for 20 min at 37 °C. DCI was added to 50  $\mu$ M, and samples were run overnight in non-denaturing pore-limit PAGE as described under "Experimental Procedures." The stained gel, shown above, was autoradiographed, and the numbered bands were cut from an equivalent gel, equilibrated with SDS, and run in SDS-PAGE as described under "Experimental Procedures," stained, and autoradiographed. Material in the last lane (a) was purified by Superose 6 fast protein liquid chromatography gel filtration and run in a model E ultracentrifuge for accurate  $M_r$  determination as described under "Experimental Procedures."

ability to form covalent cross-links to proteinases.

**Protection of Bound Papain from Kininogen Domains—** Human low-molecular-weight kininogen (L-kgn) contains three homologous tandem repeats that can be released from the parent molecule by digestion with trypsin to generate papain-inhibitory fragments of 22 and 42 kDa (26). We have used the 22-kDa fragment and intact L-kgn (64 kDa) to probe the accessibility of proteins to the active site of  $\alpha_2M$ - and  $\alpha_1I_3$ -bound papain (Fig. 4). Measurement of the apparent affinities of the 22-kDa fragment and L-kgn with  $\alpha_1I_3$  bound papain is presented in Table III. No inhibition of  $\alpha_2M$ -bound

papain was detected within the 20-min span of the assay. The concentrations of L-kgn and the 22-kDa fragment employed in these assays were  $2 \times 10^{-5}$  M. Assuming that it is possible to detect a  $v_0/v_i$  ratio of at least 2.0, we conclude (based on the equation) that the inhibitors react with  $\alpha_2M$ -bound papain with a  $K_i$  of more than  $10^{-5}$  M or that the reaction takes much longer to reach equilibrium than we are currently able to detect. Nevertheless,  $\alpha_2M$ -bound papain is much more efficiently shielded from the L-kgn domains than is the  $\alpha_1I_3$ -bound enzyme. We detected inhibition of  $\alpha_1I_3$ -bound papain (Fig. 4), but the affinities were several times weaker than for free papain (Table III). Moreover, the ratio of the binding constants was lower for the 22-kDa fragment than for L-kgn. Since the 22-kDa fragment and L-kgn share the same high affinity site (26) we infer that the lower ratio indicates more ready access to the bound papain by this smaller fragment.

**Protection of  $\alpha_2M$ - and  $\alpha_1I_3$ -bound Cathepsin G and Papain from Their Respective Antibodies**— $^{125}I$ -Labeled cathepsin G was complexed with human  $\alpha_2M$ , rat  $\alpha_1I_3$ , and  $\alpha_1$ -proteinase inhibitor. The ability of the cathepsin G-inhibitor complexes to bind to immobilized cathepsin G antisera was compared with that of the free enzyme as a measure of steric hindrance (Fig. 5). When cathepsin G was complexed with human  $\alpha_2M$ , most of the complexed enzyme failed to react with the antibody. Similarly, when cathepsin G was reacted with  $\alpha_1I_3$ , most of the binding was prohibited, although slightly more binding

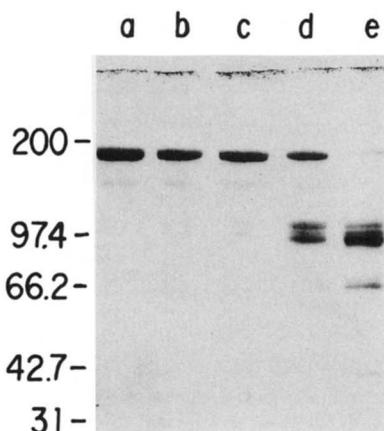
was observed than with the  $\alpha_2M$  complex. The ability of the  $\alpha_1$ -proteinase inhibitor-cathepsin G complex to bind to the immobilized antisera was comparable to the binding of free cathepsin G. Thus the decreased reactivity of the antiserum is not simply a result of inhibition of the proteinase. A similar result was obtained using papain and papain antisera (data not shown). We conclude that  $\alpha_2M$  and  $\alpha_1I_3$  both hinder access of antibodies to bound proteinases, though  $\alpha_1I_3$ -bound ones are significantly more accessible.

**Plasma Elimination of  $\alpha_1I_3$** —Proteinase complexes of both monomeric and dimeric  $\alpha_1I_3$  (Fig. 6) were injected into mice and the clearance rates compared to unreacted  $\alpha_1I_3$  (Fig. 7). As can be seen, the clearance rate of both reacted forms is identical and much more rapid than the rate of clearance of native protein. Within about 2 min, half the total injected proteinase complex of either dimer or monomer had cleared from the circulation while the half-life of unreacted  $\alpha_1I_3$  exceeds 4 h.

## DISCUSSION

The majority of protein proteinase inhibitors combine rapidly in a substrate-like manner with proteinases to give complexes that dissociate very slowly (4). The reactive site region of the "active site-directed" inhibitors is held in a rigid conformation that allows binding to but discourages hydrolysis by a proteinase (5). Inhibition, therefore, results from a tight complementarity between the substrate binding sites of the proteinase and the reactive site region of the inhibitor (5), a process that has been called the "standard mechanism" (4).

The  $\alpha$ -macroglobulins, whose mode of inhibition enables them to bind and thereby inhibit most proteinases irrespective of catalytic mechanism or substrate specificity (1-3), violate the standard mechanism. The best studied  $\alpha$ -macroglobulin, human  $\alpha_2M$ , allows rapid hydrolysis of its "bait region" by a proteinase, much as if it were a good substrate (7, 46). Following hydrolysis of a peptide bond in the bait region, the molecule undergoes a large scale conformational change that results in the entrapment of the reacting proteinase molecule (12). The entrapped proteinase is sterically hindered, and its ability to react with large substrates is drastically decreased, though it retains almost full activity against small substrates (1-3). The properties of the monomeric  $\alpha_1I_3$  reflect those of  $\alpha_2M$  to some extent. There are, however, differences in the degree of steric hindrance placed upon proteinases following reaction with  $\alpha_1I_3$ , as  $\alpha_2M$ -bound papain did not interact with L-kgn or the 22-kDa kininogen fragment, even after prolonged incubation. These findings are consistent with the well established high degree of steric hindrance of  $\alpha_2M$ -bound protein-



**FIG. 3. SDS-PAGE of  $\alpha_1I_3$  and pancreatic elastase reacted in the presence of 3-aminopropionitrile.**  $\alpha_1I_3$  was incubated without (a) or in the following concentrations of 3-aminopropionitrile with a 0.1 molar ratio of pig pancreatic elastase for 10 min at 25 °C: 0.1 mM (b), 1 mM (c), 10 mM (d), 50 mM (e). DCI was added to 50  $\mu$ M, and samples were run in SDS-PAGE with reduction.

**FIG. 4. Reaction of L-kgn and the 22-kDa L-kgn fragment with free or  $\alpha$ -macroglobulin-bound papain.** The hydrolysis of D-Pro-Phe-Arg-AMC by a preformed  $\alpha_1I_3$ -papain complex (left panel) and D-Pro-Phe-Arg-pNA by free papain or a preformed  $\alpha_2M$ -papain complex (right panel) was observed in continuous assays. L-kgn or the 22-kDa fragment thereof was added at times indicated by the arrows, and the reaction was observed until hydrolysis had relaxed to a new rate, as described under "Experimental Procedures."  $K_i$  values were calculated from substrate hydrolysis rates before ( $v_0$ ) and after ( $v_i$ ) kininogen addition.

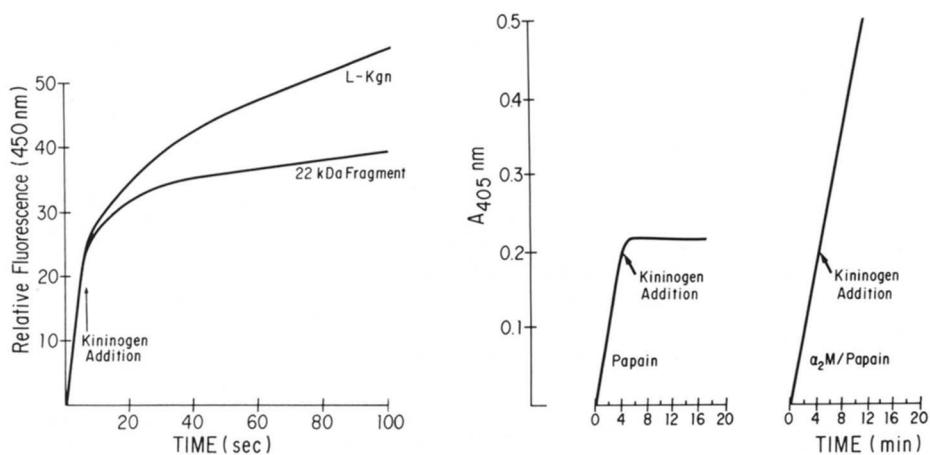


TABLE III

Inhibition of free and  $\alpha_1I_3$ -macroglobulin-bound papain by L-kgn and the 22-kDa L-kgn fragment

Papain- $\alpha_1I_3$  complexes were allowed to react with L-kgn (64 kDa) or the 22-kDa fragment thereof in a continuous assay.  $K_i$  values for inhibition were calculated from the ratio of the substrate hydrolysis rates in the presence and absence of the inhibitors (see "Experimental Procedures").

	$K_i$ value	
	L-kgn	22-kDa fragment
	M	
$\alpha_2M$ -papain	$>10^{-5}$	$>10^{-5}$
$\alpha_1I_3$ -papain	$8.5 \times 10^{-9}$	$1.1 \times 10^{-9}$
Papain <sup>a</sup>	$1.5 \times 10^{-11}$	$3.0 \times 10^{-11}$
$K_i$ ratio <sup>b</sup>	567	37

<sup>a</sup> Data from Ref. 26.

<sup>b</sup> Calculated from  $\alpha_1I_3$ -papain  $K_i$ /papain  $K_i$ .

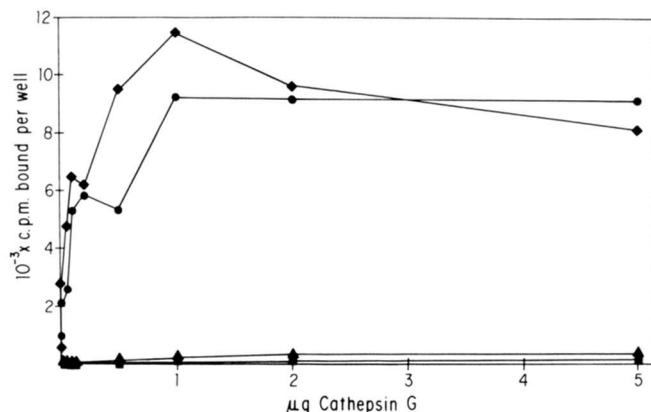


FIG. 5. Reaction of cathepsin G complexes with anti(cathepsin G) antiserum. Wells of a microtiter plate were coated with a cathepsin G antiserum as described under "Experimental Procedures." The indicated amounts of  $^{125}I$ -labeled cathepsin G (●) or complexes of the enzyme with  $\alpha_1$ -proteinase inhibitor (◆),  $\alpha_2M$  (■), or  $\alpha_1I_3$  (▲) were added to determine the reactivity of each form with the antiserum. Each form contained an equivalent amount of cathepsin G.

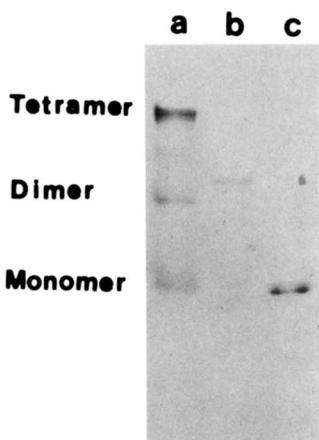


FIG. 6. Purification of  $\alpha_1I_3$ -chymotrypsin monomer and dimer.  $\alpha_1I_3$  was reacted with a 0.04 molar ratio of chymotrypsin for 20 min at 25 °C. Monomer (c) was separated from dimer (b) by Superose 6 fast protein liquid chromatography gel filtration. A partially reduced  $\alpha_2M$  preparation (a) containing monomers, dimers, and the native tetramer is included for comparative purposes on this 4–20% non-denaturing pregradient gel.

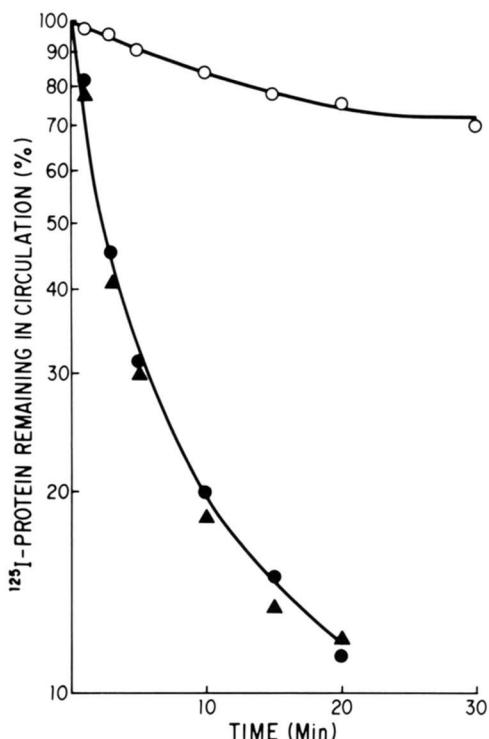


FIG. 7. Plasma elimination of  $\alpha_1I_3$  monomer and dimer.  $\alpha_1I_3$  was reacted with chymotrypsin as described under "Experimental Procedures." The dimer was separated from the monomer by gel filtration and injected into a mouse. The clearance of the radioactive monomeric complex (●) from circulation was monitored by removing 25- $\mu$ l aliquots of blood and compared with that of the dimer (▲) and unreacted  $\alpha_1I_3$  (○).

ases. When bound by  $\alpha_1I_3$ , however, these proteinases were still able to react with the inhibitors, although the affinities were several times worse than those obtained with unbound proteinases. We have thus been able to determine, to a limited extent, the degree of hindrance placed on these proteinases following reaction with  $\alpha_1I_3$ . These data are reminiscent of the reaction of soybean trypsin inhibitor with  $\alpha_2M$ -bound trypsin. Bieth and colleagues (10) found that soybean trypsin inhibitor (21 kDa) reacts with  $\alpha_2M$ -bound trypsin, although the reaction is extremely slow, with a  $K_i$  about 1000-fold higher than for free trypsin (10). They concluded that the alteration in  $K_i$  was due almost exclusively to a decrease in the rate of association with the sterically-shielded trypsin to produce a relatively weak ternary complex. We examined the reaction of  $\alpha_1I_3$ -bound papain with L-kgn and the 22-kDa fragment thereof, since this allowed us to investigate the effect of size on the accessibility of proteins to the bound papain. The 22-kDa fragment of L-kgn reacts 37 times more weakly with  $\alpha_1I_3$ -bound papain whereas L-kgn (64 kDa) reacts 567 times more weakly with  $\alpha_1I_3$ -bound papain than with the free enzyme. Both L-kgn fragments react so poorly with  $\alpha_2M$ -bound papain that we were unable to detect any inhibition. The reaction of these protein inhibitors with  $\alpha_1I_3$ -bound papain presumably results in the formation of ternary complexes, and the slow rate of their formation likely results from a decreased productive collision rate, the rate being significantly slower for L-kgn than for the smaller 22-kDa fragment since both have comparable  $K_i$ s values (26) and association rates (47) with free papain. Since  $\alpha_1I_3$  can (i) decrease the productive collision rate of L-kgn and the 22-kDa fragment, (ii) completely inhibit the hydrolysis of hide powder azure by proteinases, and (iii) prevent antibodies raised against cathepsin G and papain from reacting with these  $\alpha_1I_3$  complexes,

it must be classified as an  $\alpha_2M$ -like proteinase inhibitor and not as a "standard mechanism" one.

Much of the disagreement in the literature regarding the inhibitory capacity of  $\alpha_1I_3$  (17–21) revolves around the choice of substrates. Lonberg-Holm *et al.* (17, 21) in their comprehensive study of the reaction of trypsin with rat  $\alpha$ -macroglobulins defined the ability of the proteins to decrease activity against hide powder azure as "inhibition." At the same time they defined the ability of  $\alpha$ -macroglobulins to prevent trypsin from reacting with soybean trypsin inhibitor as "protection," and this division is common to the literature since the work of Ganrot (9). These capacities are, almost certainly, part of the same phenomenon which is the ability of  $\alpha$ -macroglobulins to prevent access of proteins to the bound sterically hindered proteinase. The larger the protein the more shielded it is from access. Thus our data and those of others (17–21) may simply be interpreted as the degree of shielding. In this case,  $\alpha_1I_3$  is an  $\alpha$ -macroglobulin proteinase inhibitor that shields less well than  $\alpha_2M$ , probably because it is a monomer whereas  $\alpha_2M$  is a tetramer. The final part of our study was aimed at determining whether these two inhibitors share a common mechanism.

Like  $\alpha_2M$ ,  $\alpha_1I_3$  contains a thiol ester that is able to covalently link reacting proteinases (21). However, unlike  $\alpha_2M$  whose covalent linking is coincidental to inhibition (8, 43),  $\alpha_1I_3$  seems to absolutely require linking in order to bind and thereby inhibit proteinases. Neutrophil elastase, which contains no lysine residues (43) is not bound by  $\alpha_1I_3$  although it is efficiently inhibited by  $\alpha_2M$ . Moreover, a 75% decrease of the covalent linking of pancreatic elastase is observed when this enzyme is allowed to react with  $\alpha_2M$  in the presence of 10 mM 3-aminopropionitrile, with no decrease in inhibition (45). Under the same conditions  $\alpha_1I_3$  loses a large amount of its inhibitory capacity, indicating that it is not able to "trap" proteinases in the manner postulated by Barrett and Starkey (6). Several authors (17–21) have previously noted that more than one molecule of  $\alpha_1I_3$  is required to inhibit one molecule of trypsin or chymotrypsin. The present study demonstrates that this nonstoichiometric reaction applies to all proteinases tested that are inhibited by  $\alpha_1I_3$ . The following is offered as a mechanism. Inhibition by  $\alpha$ -macroglobulins is initiated by cleavage of the bait region by a proteinase. This cleavage causes the putative thiol ester of  $\alpha_2M$  to become more reactive with proteinase lysine residues (45, 48), and the same is likely for  $\alpha_1I_3$ . Following bait region cleavage, the subunits of  $\alpha_2M$  compact to entrap the proteinase, and covalent linking is largely incidental (8, 45). A similar compacting of the  $\alpha_1I_3$  monomer may occur. However, this is not sufficient to entrap the proteinase, and binding leading to inhibition can only occur if covalent linking takes place. If the proteinase molecule is not positioned correctly or closely enough to the putative thiol ester, then it can escape linking and react with another  $\alpha_1I_3$  molecule. If the proteinase contains no lysine residues (for example, neutrophil elastase) it cannot be inhibited and will, therefore, catalytically inactivate a large amount of  $\alpha_1I_3$ . This may explain why the reaction of trypsin with  $\alpha_1I_3$  leads to excessive thiol ester cleavage (20, 21). The binding ratio of  $\alpha_1I_3$ , therefore, is explained by the probability of a proteinase molecule adopting the correct orientation(s) to enable covalent linking before it diffuses away. The alternative explanation for the nonstoichiometric binding ratio is that an  $\alpha_1I_3$ -bound proteinase may catalytically inactivate other  $\alpha_1I_3$  molecules. This is ruled out by our finding that a preformed pancreatic elastase- $\alpha_1I_3$  complex is unable to cleave additional  $\alpha_1I_3$  bait regions (data not shown). Our data, therefore, confirm that  $\alpha_1I_3$  is an effective inhibitor of several

proteinases, irrespective of catalytic class or primary specificity, against large substrates such as hide powder azure but that it is less effective against smaller soluble substrates. It is likely, therefore, that trapping of proteinases, resulting in extremely efficient inhibition, is a property of the multimeric  $\alpha$ -macroglobulins such as  $\alpha_2M$  but that the monomeric ones such as  $\alpha_1I_3$  share the inhibitory strategy of steric hindrance.

Since  $\alpha_1I_3$ -proteinase complexes are cleared rapidly from circulation, as are other  $\alpha$ -macroglobulin-proteinase complexes (49), and since the bound proteinase is reasonably well inhibited, the question arises as to the origin of an evolutionary driving pressure for the development of tetrameric  $\alpha$ -macroglobulins. If, as seems probable,  $\alpha_1I_3$  is analogous to the progenitor of the tetrameric  $\alpha$ -macroglobulins, it is possible to offer a hypothesis that tetrameric homologs are able to inhibit proteinases devoid of lysines and better able to protect in situations where clearance of complexes may be slow, as for example, in ascites or synovial fluid.

**Acknowledgments**—We thank Pat Burks and Andrea Tillotson for expert editorial help, Lars Sottrup-Jensen for communicating results before publication, Alan Barrett for helpful discussions, and Alan Mast for purifying  $\alpha_1$ -proteinase inhibitor.

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