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Mechanism of Action of Inter-α-trypsin Inhibitor†

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ABSTRACT: Inter-α-trypsin inhibitor (IαI) is a unique proteinase inhibitor that can be proteolyzed by the same enzymes that are inhibited, to generate smaller inhibitors. This study examines the reactions of IαI with trypsin, chymotrypsin, plasmin, and leukocyte elastase. Complexes of IαI and proteinase were demonstrated by gel filtration chromatography. Complete digestion of IαI by each proteinase was not accompanied by a comparable loss of inhibition of that enzyme or a different enzyme. Following proteolysis, inhibitory activity was identified in IαI fragments of molecular weight 50 000–100 000 and less than 40 000. Addition of a second proteinase inhibitor prevented proteolysis. Both IαI and its complex with proteinase were susceptible to degradation. Kinetic parameters for both the inhibition and proteolysis reactions of IαI with four proteinases were measured under physiological conditions. On the basis of these results, a model for the mechanism of action of IαI is proposed: Proteinase can react with either of two independent sites on IαI to form an inhibitory complex or a complex that leads to proteolysis. Both reactions occur simultaneously, but the inhibitory capacity of IαI is not significantly affected by proteolysis since the product of proteolysis is also an inhibitor. For a given proteinase, the inhibition equilibrium constant and the Michaelis constant for proteolysis describe the relative stability of the inhibition and proteolysis complexes; the second-order rate constants for inhibition and proteolysis indicate the likelihood of either reaction. The incidence of inhibition or proteolysis reactions involving IαI in vivo cannot be assessed without knowledge of the exact concentrations of inhibitor and proteinases; however, analysis of inhibition rate constants suggests that IαI might be involved in plasmin inhibition.

Plasma proteinase inhibitors are implicated in the regulation of proteolytic processes such as coagulation, fibrinolysis, and inflammation. Among the best-studied inhibitors are the serine proteinase inhibitors of the α_1-proteinase inhibitor class which share a common mechanism of action [for a review, see Travis and Salvesen (1983)]. The classification of a number of other proteinase inhibitors is incomplete owing to insufficient knowledge of their inhibitory mechanisms. One such protein is inter-α-trypsin inhibitor (IαI).[1]

IαI consists of a single glycopeptide chain of molecular weight 160 000–200 000 (Steinbuch, 1976), and it appears in

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[1] Abbreviations: IαI, inter-α-trypsin inhibitor; I′, proteolyzed form of inter-α-trypsin inhibitor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Bz, benzoyl; Boc, *tert*-butoxycarbonyl; pNA, *p*-nitroanilide; 7AMC, 7-(aminomethyl)coumarin; $NaDodSO_4$, sodium dodecyl sulfate.

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a number of mammalian species (Albrecht et al., 1983a). IαI inhibits the activity of trypsin, chymotrypsin, acrosin, plasmin, leukocyte elastase, and cathepsin G (Balduyck et al., 1985; Jochum & Bittner, 1983; Steinbuch, 1976), although in some cases inhibition is weak. Recent evidence (Pratt & Pizzo, 1986) suggests that IαI may function as a proteinase shuttle by virtue of its ability to transfer proteinase to other plasma proteinase inhibitors for subsequent removal from the circulation.

IαI is believed to be the precursor of urinary proteinase inhibitors (Proksch et al., 1973; Steinbuch, 1976) on the basis of immunological cross-reactivity and at least partial sequence identity of the proteins (Morrii & Travis, 1985; Reisinger et al., 1985). Active inhibitor species have been derived from purified IαI by treatment with concentrated acid or excess proteinase (Lambin et al., 1978; Hochstrasser et al., 1976; Albrecht et al., 1983a); however, the reactions have not been characterized under physiological conditions. Interestingly, the same proteinases that are inhibited by IαI are capable of cleaving it. This feature of IαI prompted a study to describe the function of IαI in terms of two reactions involving the same proteinases: inhibition and proteolysis. This report presents a model for the mechanism of action of IαI, consisting of equations describing the inhibition of proteinases and the degradation of the inhibitor by free proteinase to produce smaller inhibitors. In addition to experiments designed to test the model, the kinetics of the reactions of IαI and trypsin, chymotrypsin, plasmin, and leukocyte elastase under physiological conditions are presented.

Materials and Methods

Fresh frozen human plasma was obtained from the Duke University Medical Center Transfusion Service. Chromatography supplies were purchased from Pharmacia; electrophoresis reagents were from Bio-Rad. Triton X-100 was purchased from Research Products International; lactoperoxidase–Sepharose was obtained from P-L Biochemicals, and $Na^{125}I$ was from New England Nuclear. Human urokinase was purchased from Calbiochem; human leukocyte elastase was from Elastin Products, and porcine pancreatic elastase was from Worthington Biochemicals. The following were purchased from Sigma: bovine trypsin, bovine chymotrypsin, soybean trypsin inhibitor, bovine serum albumin, molecular weight standards, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), *p*-nitrophenyl *p*-guanidinobenzoate, Bz-Arg-pNA, MeOSuc-Ala-Pro-Val-pNA, Suc-Ala-Phe-Lys-7AMC, and Boc-Ile-Glx-Gly-Arg-7AMC. Val-Leu-Lys-pNA (S-2251) and MeOSuc-Arg-Pro-Tyr-pNA (S-2586) were obtained from KabiVitrum. MeOSuc-Ala-Ala-Pro-Val-7AMC was purchased from Vega Biotechnologies.

Protein Purification. IαI was purified from fresh frozen human plasma by the method of Salier et al. (1980) as modified by Morrii and Travis (1985). IαI was stored in 20 mM HEPES/150 mM NaCl, pH 7.4 at −20 °C. The preparation was 95% pure as determined by $NaDodSO_4$ electrophoresis and scanning densitometry.

The proteolyzed form of IαI, I′, was prepared by incubation of IαI with chymotrypsin–Sepharose, prepared by the method of Porath et al. (1973), followed by gel filtration chromatography. The fragment was 90% pure by gel electrophoresis and had a molecular weight of 68 000.

Human plasminogen type II was purified by the method of Deutsch and Mertz (1970) as modified by Brockway and Castellino (1972). Plasminogen was converted to plasmin by incubation with 0.2 unit of urokinase/μg of plasminogen for 30 min at 25 °C, and was used immediately. When stock solutions were diluted to concentrations below 1 μM, 0.01% Triton X-100 was added to increase the stability of plasmin. IαI did not react with urokinase.

Human α_1-proteinase inhibitor was purified as described by Pannell et al. (1974).

Proteinases. Trypsin and chymotrypsin were stored in 20 mM $CaCl_2$, pH 3.0; plasmin was stored in phosphate-buffered saline, pH 7.4, and leukocyte and pancreatic elastase were stored in 200 mM sodium acetate, pH 5.0. Trypsin, chymotrypsin, and plasmin were active site titrated with *p*-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1969). The inhibitory activities of IαI, I′, α_1-proteinase inhibitor, and soybean trypsin inhibitor were determined by adding a known amount of trypsin to increasing amounts of inhibitor and assaying for residual trypsin activity with Bz-Arg-pNA. Titrated α_1-proteinase inhibitor was used to calculate the activity of leukocyte elastase in the presence of the substrate MeOSuc-Ala-Ala-Pro-Val-pNA.

Gel Filtration. Proteinases were labeled with ^{125}I by the solid phase lactoperoxidase method (David & Reisfeld, 1974) and incubated with IαI for a period sufficient to reach equilibrium. Reaction mixtures of 100 μL were applied to a 28 × 0.66 cm column of Sephacryl S-300 equilibrated with 20 mM HEPES/150 mM NaCl, pH 7.4, at 4 mL/h; 0.3-mL fractions were collected and analyzed by an LKB Model 1272 γ counter.

Electrophoresis. $NaDodSO_4$ gel electrophoresis was performed according to Weber and Osborn (1969) using 6% polyacrylamide slabs containing 4 M urea. Proteins were stained with Coomassie Brilliant Blue. Quantification of protein was made by use of a Gelman ACD-15 scanning densitometer or by cutting out radioactive protein bands for analysis by the γ counter. For experiments requiring recovery of proteolytic or inhibitory activity from gels, samples were denatured at 37 °C. Following electrophoresis, lanes were cut into 15 8-mm slices and incubated in 600 μL of HEPES buffer for 24 h at 4 °C. Proteinase inhibitor activity was measured by assaying the effect of 100 μL of gel/buffer sample on 1 nM trypsin or chymotrypsin using 80 mM Val-Leu-Lys-pNA as a substrate for trypsin (after correction for the activity of endogenous trypsin and plasmin) and 80 μM MeOSuc-Arg-Pro-Tyr-pNA for chymotrypsin (after correction for endogenous chymotrypsin activity). The presence of proteinase in gel slices was determined by using Val-Leu-Lys-pNA for trypsin and plasmin, MeOSuc-Arg-Pro-Tyr-pNA for chymotrypsin, and MeOSuc-Ala-Pro-Val-pNA for pancreatic elastase. The activity of leukocyte elastase was not recovered following electrophoresis.

Inhibition Equilibrium Constants. All kinetic experiments were performed in 20 mM HEPES/150 mM NaCl, pH 7.4, at 37 °C. Bovine serum albumin, 0.5 mg/mL, was added to minimize surface denaturation of proteins. Samples containing elastase included 0.01% Triton X-100. Measurements of proteinase inhibition by IαI were based on the reaction:

$$\begin{array}{ccccc} E + S & \overset{K_m}{\rightleftharpoons} & ES & \longrightarrow & E + P \\ + & & & & \\ I & & & & \\ {\scriptstyle K_i}\downarrow\uparrow & & & & \\ EI & & & & \end{array}$$

where E, S, and I represent proteinase, substrate, and inhibitor, respectively; K_m is the Michaelis constant for the enzyme–substrate reaction, and K_i is the inhibition equilibrium constant. K_i was determined by the method of Henderson (1972) as

Table I: Concentrations of Inhibitor, Proteinase, and Synthetic Substrate for Kinetic Studies

proteinase	trypsin	chymotrypsin	plasmin	elastase
K_i				
[IαI]	0–25 nM	0–12 nM	0–600 nM	0–1 μM
[proteinase]	10 nM	3 nM	8 nM	10 nM
[substrate]	1 mM[a]	40 μM[b]	200 μM[c]	200 μM[d]
K_i'				
[I′]	0–3 nM	0–3 nM		
[proteinase]	1 nM	1 nM		
[substrate]	2 mM[a]	40 μM[b]		
k_i				
[IαI]	0.6 nM	3 nM	0–20 nM	0.25 nM
[proteinase]	0.5 nM	3 nM	0.2 nM	0.5 nM
[substrate]	2 μM[e]	50 μM[b]	3 μM[f]	5 μM[g]
k_i'				
[I′]	0.5 nM	1.5 nM		
[proteinase]	0.5 nM	0.75 nM		
[substrate]	2 μM[e]	150 μM[b]		
K_p				
[IαI]	0–1.5 μM	20–500 nM	0–2.8 μM	0–1 μM
[proteinase]	5 nM	0.5 nM	100 nM	5 nM

[a] Bz-Arg-pNA; K_m = 1.7 mM. [b] MeOSuc-Arg-Pro-Tyr-pNA; K_m = 43 μM. [c] Val-Leu-Lys-pNA; K_m = 0.34 mM. [d] MeOSuc-Ala-Pro-Val-pNA; K_m = 0.26 mM. [e] Boc-Ile-Glx-Gly-Arg-7AMC. [f] Suc-Ala-Phe-Lys-7AMC. [g] MeOSuc-Ala-Pro-Val-7AMC.

simplified by Bieth (1980), which involved adding increasing amounts of inhibitor to a constant amount of proteinase and assaying for residual proteinase activity. Graphical analysis yielded an apparent equilibrium constant, using the equation:

$$[I]_0/(1 - a) = K_{i,app}(1/a) + [E]_0$$

where $[I]_0$ and $[E]_0$ are the initial concentrations of inhibitor and proteinase and a is the fractional proteinase activity. In order to correct for the effect of substrate on the equilibrium between inhibitor and proteinase, the K_m for the enzyme–substrate reaction was determined independently. The true equilibrium constant for inhibition was calculated as

$$K_i = \frac{K_{i,app}}{1 + [S]_0/K_m}$$

K_i', the inhibition constant for I′, was determined by the same technique. Measurements of proteinase activity were made by incubating proteinase and inhibitor for a period sufficient to reach equilibrium and then adding substrate and following its hydrolysis at 405 nm in a temperature-controlled Shimadzu UV-240 recording spectrophotometer. The concentrations of inhibitor, proteinase, and substrate, as well as the K_m of the enzyme–substrate reaction, are given in Table I.

Inhibition Rate Constants. The rate constants for the association of inhibitor with proteinase (k_i) in the equation

$$E + I \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EI$$

were measured by assaying proteinase activity at intervals following the addition of inhibitor. Activity assays were performed in a temperature-controlled recording spectrophotometer for *p*-nitroanilide substrates or in a Shimadzu RF-540 recording spectrofluorophotometer for fluorescent substrates (excitation at 370 nm and emission at 460 nm; Castillo et al., 1979). The rates of association of IαI and I′ with trypsin and chymotrypsin, which are strongly inhibited, were measured under second-order reaction conditions. The rates of association of IαI with plasmin and leukocyte elastase were measured under pseudo-first-order reaction conditions in order to maximize inhibition. The concentrations of proteins and substrates are given in Table I. The rate constant for the dissociation of inhibitor and proteinase (k_{-i}) was calculated as the product of K_i and k_i.

Michaelis Constant for Proteolysis. Proteolysis of IαI was measured by the Eadie–Hofstee method (Hofstee et al., 1959) for the reaction:

$$E + I \underset{}{\overset{K_p}{\rightleftharpoons}} EI \xrightarrow{k_p} E + I'$$

where EI represents an enzyme–substrate, rather than enzyme–inhibitor, complex and I′ is the proteolyzed inhibitor. K_p is the Michaelis constant for proteolysis. The initial concentrations of proteinase and IαI were chosen such that the concentration of uninhibited proteinase, calculated from K_i, was constant for a given inhibitor concentration. Without this correction, linear plots could not be obtained. The velocity of the reaction was monitored for 20–30 min and was measured as the rate of disappearance of the IαI band (by scanning densitometry or radiometric analysis of the excised band) in $NaDodSO_4$ gel electrophoresis. Protein concentrations are givein in Table I.

Hydrolysis Constants. The hydrolysis constants (k_p) for proteolysis reactions were estimated as $k_p = V_{max}/[E]_0$, using V_{max} determined by Eadie–Hofstee analysis. Lower limits for the second-order rate constant governing the forward reaction of proteolysis were calculated as the quotient of k_p and K_p.

Physiological Half-Time of Inhibition. The half-time of inhibition was calculated according to Bieth (1980) as

$$t_{1/2} = 1/k_i[I]$$

where k_i is the association rate constant and [I] is the physiological concentration of the inhibitor.

Results

Proteinase Inhibition. The ability of IαI to inhibit four serine proteinases of different specificity was examined by colorimetric assay and by gel filtration. A number of proteinases tested were not inhibited by IαI; these included α-thrombin, factor Xa, plasma or glandular kallikrein, urokinase, tissue plasminogen activator, and pancreatic elastase. A report that IαI inhibitied bromelain (Steinbuch et al., 1966) prompted an investigation of sulfhydryl proteinases. Inhibition of papain hydrolysis of Bz-Arg-pNA was demonstrated; however, graphical analysis revealed that inhibition was not competitive, as is the case for other proteinases (data not shown). Four proteinases were chosen for detailed study: trypsin, chymotrypsin, plasmin, and leukocyte elastase. Measurements of the relative stability of inhibition are presented below.

The ability of IαI to form stable complexes with proteinases as evidence for inhibition was examined by gel filtration. The association of proteinase with inhibitor was assessed by the presence of radioiodinated proteinase in the IαI peak. Trypsin, chymotrypsin, plasmin, and leukocyte elastase formed a complex with IαI, while pancreatic elastase, which is not inhibited by IαI, did not form a complex (Figure 1). Complex formation was demonstrated to some extent in nondenaturing polyacrylamide gel electrophoresis (not shown); in all cases, inhibitor–proteinase complexes were not stable in $NaDodSO_4$ electrophoresis. The reversibility of complex formation was further proved by kinetic analysis (see Table II).

Effect of Proteolysis on Inhibition. IαI was degraded by a variety of enzymes, including those that were inhibited by IαI. The products of proteolysis differed depending on the proteinase (see Figure 4) and the length of the reaction. The effect of proteolysis on the inhibitory activity of IαI was examined in two studies. In the first, the inhibition of a given

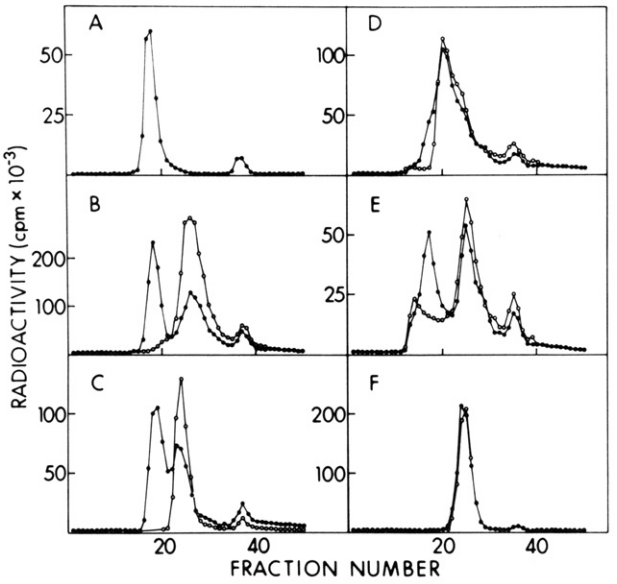


FIGURE 1: Demonstration of inhibitor–proteinase complex formation by gel filtration. Radioiodinated proteinase was reacted with IαI and subjected to gel filtration as described under Materials and Methods. (A) Radioiodinated IαI control; (B) 1 μM IαI reacted with 0.6 μM trypsin for 10 min at 37 °C; (C) 0.28 μM IαI reacted with 0.06 μM chymotrypsin for 40 min at 37 °C; (D) 3.8 μM IαI reacted with 0.08 μM plasmin for 10 min at 37 °C; (E) 3.2 μM IαI reacted with 0.1 μM leukocyte elastase for 10 min at 37 °C; (F) 3.8 μM IαI reacted with 0.2 μM pancreatic elastase for 10 min at 37 °C. (Open circles) Proteinase alone; (closed circles) proteinase following reaction with IαI. Radioactivity at fractions 35–37 represents free ^{125}I.

Table II: Kinetic Constants for Inhibition and Proteolysis Reactions of IαI

proteinase	trypsin	chymotrypsin	plasmin	leukocyte elastase
IαI inhibition reaction				
K_i (nM)	0.078	1.1	190	61
k_i (M^{-1} s^{-1})	7.3×10^6	3.9×10^5	6.2×10^5	8.0×10^5
k_{-i} (s^{-1})	5.7×10^{-4}	4.3×10^{-4}	1.2×10^{-1}	4.9×10^{-2}
I′ inhibition reaction				
K_i' (nM)	0.097	0.80		
k_i' (M^{-1} s^{-1})	4.0×10^6	5.7×10^5		
k_{-i} (s^{-1})	3.9×10^{-4}	4.6×10^{-4}		
proteolysis reaction				
K_p (μM)	2.1	0.60	4.4	0.90
k_p (s^{-1})	0.078	0.34	0.022	0.12
k_p/K_p (M^{-1} s^{-1})	3.7×10^4	5.6×10^5	5.0×10^3	1.3×10^5
K_p/K_i	27000	550	23	15
$K_i/(k_p/K_p)$	200	0.68	120	6.2

proteinase was measured during an interval in which that proteinase degraded IαI almost completely, as judged by $NaDodSO_4$ electrophoresis and densitometry. As shown in Figure 2, loss of intact IαI did not correlate exactly with loss of inhibition. When 50% of the initial IαI was degraded by trypsin, chymotrypsin, plasmin, or leukocyte elastase, inhibition decreased by less than 15% of the initial value.

In a second experiment, IαI was incubated with proteinase under conditions for complete degradation of IαI, and then the inhibition of trypsin and chymotrypsin was measured colorimetrically. Control experiments were performed in order to correct for the effect of endogenous proteinase on substrate hydrolysis. Inhibition of trypsin or chymotrypsin by samples containing only proteinase was not observed. Following proteolysis of IαI by trypsin, plasmin, leukocyte elastase, and pancreatic elastase, the inhibition of trypsin was virtually unchanged (Figure 3A). Chymotrypsin inhibition (Figure 3B) was affected only slightly following proteolysis by chymotrypsin, plasmin, leukocyte elastase, and pancreatic elastase. Inhibition of trypsin by samples containing chymotrypsin, or inhibition of chymotrypsin by samples containing trypsin, did not reach expected levels due to the stability of inhibition of these two proteinases (see Table II) which resulted in only partial displacement of the endogenous proteinase by the second enzyme. Separate experiments verified that trypsin or chymotrypsin initially bound to IαI could be displaced to a limited extent by the other enzyme.

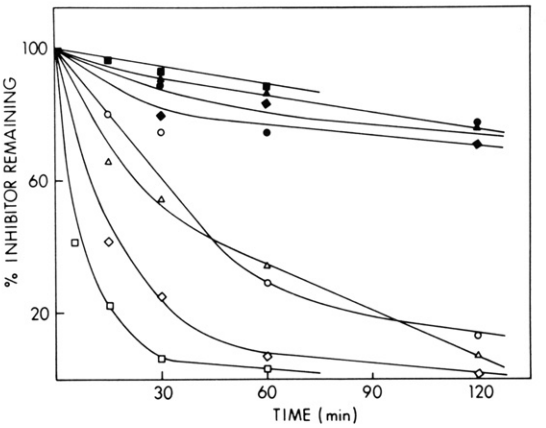


FIGURE 2: Inhibition during proteolysis. IαI was digested by proteinase at 37 °C. At intervals, samples were subjected to $NaDodSO_4$ electrophoresis or diluted 50-fold for analysis of proteinase activity. (○) 2 μM IαI digested with 2 μM trypsin; activity assayed with 0.8 mM Bz-Arg-pNA. (□) 2 μM IαI digested with 1 μM chymotrypsin; activity assayed with 40 μM MeOSuc-Arg-Pro-Tyr-pNA. (△) 2 μM IαI digested with 2 μM plasmin; activity assayed with 80 μM Val-Leu-Lys-pNA. (◇) 2 μM IαI digested with 0.4 μM leukocyte elastase; activity assayed with 0.4 mM MeOSuc-Ala-Pro-Val-pNA. (Open symbols) IαI remaining as determined by densitometry following electrophoresis; (closed symbols) inhibition remaining, expressed as a percentage of initial inhibition.

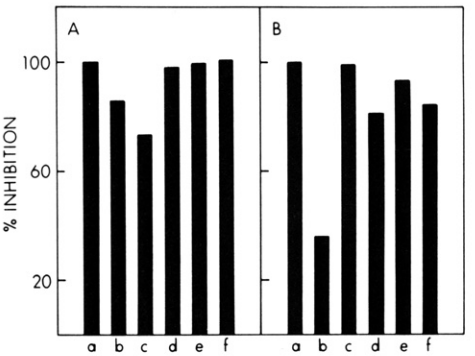


FIGURE 3: Inhibition of trypsin and chymotrypsin following proteolysis. 2 μM IαI was digested completely by various proteinases at 37 °C, then diluted to 40 nM, and assayed for inhibition of 55 nM trypsin by using 1 mM Bz-Arg-pNA (A) or for inhibition of 40 nM chymotrypsin by using 40 μM MeOSuc-Arg-Pro-Tyr-pNA (B). Correction was made for substrate hydrolysis by proteinases already present. (a) Control IαI; (b) IαI digested with 2 μM trypsin for 1.5 h; (c) IαI digested with 1 μM chymotrypsin for 1 h; (d) IαI digested with 2 μM plasmin for 2.5 h; (e) IαI digested with 0.4 μM leukocyte elastase for 1 h; (f) IαI digested with 1 μM pancreatic elastase for 1 h.

Identification of Inhibitor Fragments. Because the inhibitory capacity of IαI remained constant following proteolysis by a variety of enzymes, a study was made to identify the fragments of IαI responsible for inhibition. IαI was incubated with trypsin, chymotrypsin, plasmin, leukocyte elastase, or pancreatic elastase, under conditions for complete proteolysis, and the reaction products were separated by $NaDodSO_4$ electrophoresis. The inhibitory activity of sections of each lane was tested with trypsin and chymotrypsin, as described under

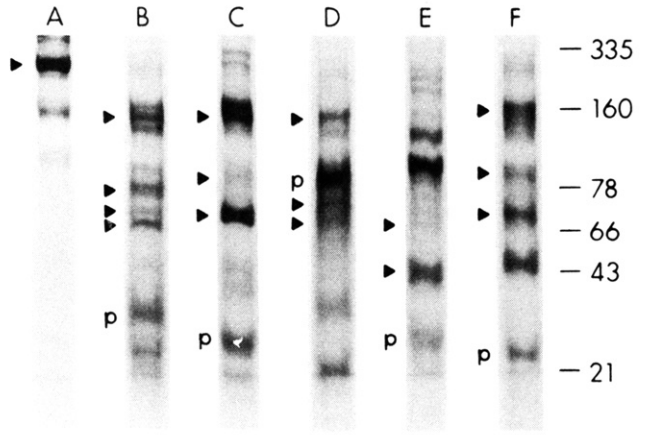


FIGURE 4: Inhibition of trypsin and chymotrypsin by proteolyzed fragments of IαI. 2μM (11 μg) IαI was digested with proteinase and then subjected to $NaDodSO_4$ electrophoresis. The inhibition activity of slices cut from each lane was tested as described under Materials and Methods. Conditions of digestion were identical with those described in Figure 3. (A) IαI; (B) IαI digested with trypsin; (C) IαI digested with chymotrypsin; (D) IαI digested with plasmin; (E) IαI digested with leukocyte elastase; (F) IαI digested with pancreatic elastase. The positions of proteinases are indicated by p. Arrows indicate protein bands associated with inhibitory activity against trypsin and chymotrypsin. Molecular weights were determined from the mobility of the following standards: thyroglobulin (335 000), immunoglobulin G (160 000), transferrin (78 000), bovine serum albumin (66 000), ovalbumin (43 000), and soybean trypsin inhibitor (21 000).

Materials and Methods. In all cases, the profile of trypsin inhibition was identical with that of chymotrypsin inhibition. A duplicate stained gel is shown (Figure 4) with arrows to indicate protein bands localized to areas containing inhibitory activity. Control samples containing only proteinase did not interfere with trypsin or chymotrypsin activity. Inhibitory activity was assigned to protein bands corresponding to molecular weights of 50 000–100 000, in addition to 160 000, which is the apparent molecular weight of the major contaminant of the starting material. In digests produced by trypsin, plasmin, leukocyte elastase, and pancreatic elastase, inhibitory activity was also localized in areas corresponding to molecular weights less than 40 000, where discrete protein bands were difficult to visualize.

Proteolysis of IαI in the Presence of a Second Inhibitor. To demonstrate that proteinase not inhibited by IαI was responsible for degradation of the inhibitor, a second proteinase inhibitor was introduced in order to decrease the concentration of free enzyme. When 10 μM soybean trypsin inhibitor was added at intervals to samples containing 2 μM trypsin and 2 μM IαI, proteolysis of IαI by trypsin was prevented (Figure 5). Moreover, soybean trypsin inhibitor, which has an inhibition constant comparable to that of IαI (Kassell, 1970b), promoted dissociation of the IαI–trypsin complex. Because this reaction did not result in additional proteolysis, it was concluded that the proteolysis reaction was not directly linked to dissociation of the enzyme–inhibitor complex.

Proteolysis of IαI and Its Inhibitory Complexes. To determine whether proteolysis involved only free inhibitor or both free inhibitor and inhibitor in complex with proteinase, degradation of IαI and IαI–trypsin complexes by pancreatic elastase was examined. Because complexes containing trypsin are the most stable formed by IαI, trypsin was not expected to dissociate and thereby proteolyze IαI. Pancreatic elastase, which is not inhibited, served as a model enzyme for the study of proteolysis. A time course of proteolysis by pancreatic elastase, shown as the $NaDodSO_4$ electrophoretogram (Figure 6), revealed comparable rates of proteolysis of IαI and IαI

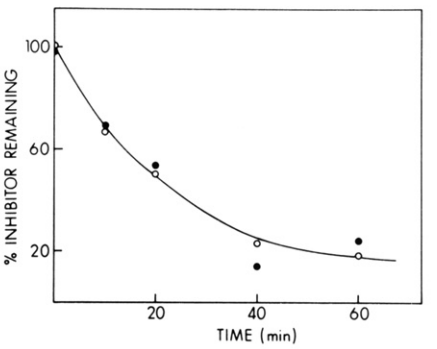


FIGURE 5: Prevention of proteolysis by addition of a second proteinase inhibitor. 2.2 μM (10 μg) IαI was digested with 2.2 μM trypsin at 37 °C. At intervals samples were denatured for $NaDodSO_4$ electrophoresis or were supplemented with 10 μM soybean trypsin inhibitor and incubated at 37 °C to give a total incubation time of 1 h before denaturation. The extent of proteolysis of IαI was determined by densitometry and expressed as a percentage of the initial IαI. (Open circles) IαI digested with trypsin; (closed circles) IαI digested with trypsin and then incubated with soybean trypsin inhibitor for the remainder of the hour.

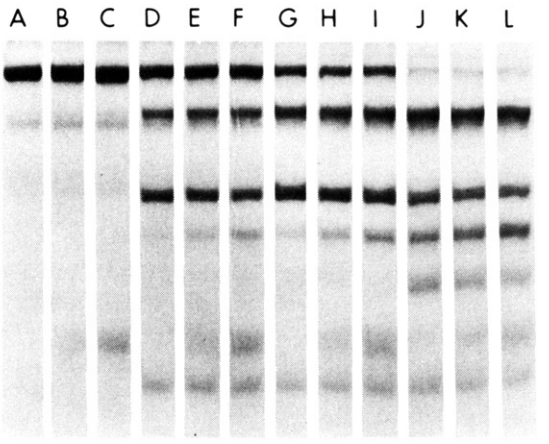


FIGURE 6: Proteolysis of IαI and IαI–trypsin complex by pancreatic elastase. 2.2 μM (15 μg) IαI was reacted with molar ratios of 0.3 (0.7 μM) or 0.6 (1.4 μM) trypsin, then incubated with 1.1 μM pancreatic elastase, and subjected to $NaDoSO_4$ electrophoresis. (A) IαI; (B) IαI reacted with a molar ratio of 0.3 trypsin; (C) IαI reacted with a molar ratio of 0.6 trypsin; (D–F) same as (A–C) incubated with elastase 2 min; (G–I) same as (A–C) incubated with elastase 5 min; (J–L) same as (A–C) incubated with elastase 15 min.

complexes with 0.3 or 0.6 mol of trypsin/mol of inhibitor. In addition, the presence of trypsin in an inhibitory complex did not affect the pattern of proteolysis, indicating that trypsin did not sterically hinder proteolysis by pancreatic elastase and suggesting that proteolysis occurred independently of inhibition.

Kinetics of Inhibition. Numeric parameters describing the inhibition and proteolysis reactions of IαI with trypsin, chymotrypsin, plasmin, and leukocyte elastase were measured as described under Materials and Methods and are presented in Table II. Inhibition constants (K_i), a measure of the stability of proteinase inhibition, varied with a difference of more than a thousandfold between trypsin and plasmin. The rate constants for the association (k_i) of chymotrypsin, plasmin, and leukocyte elastase were virtually identical; the rate of dissociation of these complexes (k_{-i}) therefore determined the strength of inhibition. Trypsin exhibited the most stable inhibition as well as the greatest association rate. The inhibition constants (K_i') and rate constants (k_i and k_{-i}') for the proteolyzed form of the inhibitor were similar to the values for intact IαI, further indicating that proteolysis did not affect inhibition.

Table III: Half-Times for Inhibition of Plasmin and Leukocyte Elastase by Plasma Proteinase Inhibitors

inhibitor	plasma concn (μM)	plasmin inhibition		leukocyte elastase inhibition	
		k_i (M^{-1} s^{-1})	$t_{1/2}$ (s)	k_i (M^{-1} s^{-1})	$t_{1/2}$ (s)
inter-α inhibitor	3.1[a]	6.2×10^5	0.52	8.0×10^5	0.40
α_1-proteinase inhibitor	42[b]	1.9×10^2[e]	130	6.5×10^7[e]	0.00037
α_2-macroglobulin	2.8[c]	5×10^5[f]	0.71	4.1×10^7[h]	0.0087
α_2-proteinase inhibitor	1.0[d]	3.8×10^7[g]	0.026		

[a] Heimburger et al. (1970). [b] Kueppers & Black (1974). [c] Starkey & Barrett (1977). [d] Moroi & Aoki (1976). [e] Beatty et al. (1980). [f] Christensen & Sottrup-Jensen (1984). [g] Wiman & Collen (1978). [h] Virca & Travis (1984).

To test whether proteolysis occurred during measurement of inhibition equilibrium or rate constants, ^{125}I-IαI was incubated with proteinase under the same conditions used for kinetic measurements. No proteolysis occurred with trypsin, plasmin, or elastase, as demonstrated by gel electrophoresis and autoradiography (data not shown). In the case of chymotrypsin, IαI was almost completely proteolyzed during measurement of K_i, while proteolysis during measurement of k_i was less than 20% at the highest ratio of enzyme to inhibitor.

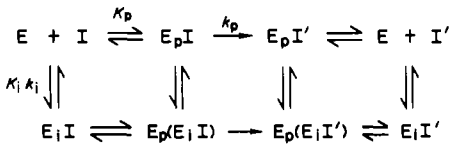
Kinetics of Proteolysis. The Michaelis constant for proteolysis (K_p) and first-order proteolysis constants (k_p) for all four enzymes varied within about 1 order of magnitude. The most efficient proteolysis, based on K_p or k_p, was accomplished by chymotrypsin, followed by leukocyte elastase, trypsin, and plasmin. The estimated second-order rate constants for the association of inhibitor and proteinase that leads to proteolysis (k_p/K_p) provided an additional indicator of the likelihood of degradation by different proteinases: chymotrypsin was most likely to proteolyze IαI; plasmin, the least likely.

The ratio of the Michaelis constant for proteolysis (K_p, an apparent equilibrium constant) and the equilibrium constant for inhibition (K_i) described the relative stability of the inhibitory complex and the complex that leads to proteolysis. The highest ratio, indicating that the inhibitory complex predominated, belonged to trypsin; following trypsin were chymotrypsin, plasmin, and leukocyte elastase. It was assumed that once proteolysis was complete, the proteolytic complex dissociated such that it would not contribute to inhibition.

The ratio of forward rate constants for inhibition and proteolysis [$k_i/(k_p/K_p)$] provided a parameter to describe the relative likelihood of formation of a complex of IαI and a given proteinase leading to either inhibition or proteolysis. The values given in Table II represented an upper limit for this ratio, since the quotient k_p/K_p represented a lower limit for the rate constant for association of proteinase and inhibitor. Trypsin exhibited the greatest ratio, followed by plasmin and then leukocyte elastase. With a ratio of less than unity, chymotrypsin exhibited a greater tendency toward proteolysis than inhibition. These predictions of relative rates of association leading to inhibition or to proteolysis for each enzyme clearly differed from predictions based on comparisons of equilibrium constants. The stability of inhibition is thus not a reliable indicator of the likelihood of proteolysis.

Physiological Half-Time of Inhibition. The half-time of inhibition of plasmin and leukocyte elastase by IαI was calculated and compared to values calculated from literature reports of the rates of inhibition of the same enzymes by several other plasma proteinase inhibitors. As shown in Table III, the half-time of inhibition of plasmin by IαI was greater than that of α_2-proteinase inhibitor but comparable to that of α_2-macroglobulin. The half-time of inhibition of leukocyte elastase by IαI was greater than that of both α_1-proteinase inhibitor and α_2-macroglobulin. Although the association rate constants for proteinase inhibitors other than IαI were measured at 23 or 25 °C, they are useful as approximations of physiological rates.

Scheme I



Discussion

IαI is unique among the plasma proteinase inhibitors in that it can be proteolyzed by the same enzymes that are inhibited, without loss of inhibitory activity. The results of this study suggest a model (Scheme I) for the interaction of IαI with proteinase where E, I, and I′ represent proteinase, IαI, and active fragments of IαI, respectively. The subscript i refers to the inhibitory complex; p refers to the complex that leads to proteolyis. On a molecular level, E_i represents enzyme reacting with the inhibitory site of IαI; E_p represents enzyme reacting with the proteolysis site. Inhibition is reversible and is characterized by an equilibrium constant (K_i) and an association rate constant (k_i). Proteolysis is characterized by a Michaelis constant (K_p) and a hydrolysis constant (k_p). Because inhibition is reversible, free enzyme is present and can proteolyze the inhibitor (or the inhibitor–proteinase complex, E_iI) to produce smaller proteinase inhibitors. Proteolysis does not diminish the inhibitory capacity of IαI since the product of proteolyis (I′) is also a proteinase inhibitor with properties similar to IαI. Scheme I describes two possible ways in which proteinase and IαI can reversibly combine; both reactions can occur simultaneously such that an E_iI or E_pI complex becomes an $E_p(E_iI)$ complex. It is not possible to measure the kinetic parameters of the reactions that yield complexes containing proteinase at both the inhibitory and proteolytic sites of IαI. Scheme I does not preclude the possibility that proteolysis of the inhibitor may occur in stages or at more than one site. It is assumed that proteolysis of the inhibitor is irreversible and that dissociation of the E_pI' or $E_p(E_iI')$ complex is rapid.

Kinetic parameters for both inhibition and proteolysis reactions can be used to predict the relative importance of each reaction for a given proteinase. This reaction mechanism differs significantly from the standard mechanism shared by other plasma proteinase inhibitors (Laskowski & Kato, 1980), in which inhibition is essentially irreversible and proteolysis, when it occurs, destroys inhibitory activity. The proposed model for the mechanism of IαI applies to a variety of proteinases under physiological conditions and is consistent with previously observed properties of IαI.

The inhibitory properties of IαI are similar to those reported for other proteinase inhibitors related to IαI. IαI and a urinary proteinase inhibitor (Balduyck et al., 1985) exhibit broad proteinase specificity, as does the Kunitz basic pancreatic trypsin inhibitor (Kassell, 1970a), with which IαI shares sequence homology (Hochstrasser & Wachter, 1979). The demonstration of an IαI–proteinase complex that is reversible and unstable in $NaDodSO_4$ is consistent with observations of urinary proteinase inhibitor complexes with the same pro-

teinases (Jonsson & Ohlsson, 1984; Balduyck et al., 1985).

Numerous cases of proteolysis of plasma proteinase inhibitors have been described; in general, these are of three types. Nonspecific proteolysis of the inhibitor may destroy inhibitory activity, as is the case with macrophage elastase and α_1-proteinase inhibitor (Banda et al., 1985) or with leukocyte elastase and α_2-plasmin inhibitor (Brower & Harpel, 1982). Alternatively, in an inhibitor–proteinase complex, proteolysis at the reactive site of the inhibitor may occasionally result in the slow release of active enzyme and inactive inhibitor (Oda et al., 1977; Wong et al., 1982). α_1-Macroglobulin exhibits a third type of proteolysis: inhibition of proteinase requires proteolysis of a "bait region" within the inhibitor subunit which induces a conformational change that physically entraps the proteinase (Barrett et al., 1979). Proteolysis of IαI represents a fourth and novel case in which proteolysis that occurs concurrently with inhibition is not essential for inhibition and does not diminish inhibition of the same or a different proteinase.

Studies of the proteolysis of IαI in the presence of a second proteinase inhibitor demonstrated that dissociation of the enzyme from the inhibitory complex was not accompanied by proteolysis but that proteolysis was due to the presence of uninhibited enzyme in equilibrium with IαI. Thus, proteolysis of IαI does not represent an intramolecular reaction of the IαI–proteinase inhibitory complex. Moreover, because both IαI and the IαI–trypsin complex served as substrates for pancreatic elastase, indicating that bound proteinase does not hinder proteolysis by a second enzyme, it can by concluded that the inhibition and proteolysis reactions occur at independent sites. This is consistent with the amino-terminal IαI sequence presented by Reisinger et al. (1985), which contains Kunitz-type inhibitory domains flanked by sequences highly sesceptible to proteolysis.

The proteolytic generation of active fragments of IαI, as proposed in the model of the mechanism of IαI, was confirmed by identification of such inhibitors in $NaDodSO_4$ electrophoresis. Inhibitory activity was assigned to a number of fragments of varied molecular weight. This contrasts with the findings of Hochstrasser et al. (1976), in whose hands only two small inhibitor fragments were recovered; this discrepancy might result from the use of prolonged tryptic digestion and acid precipitation steps in the purification of such inhibitor species. In the present study, the acid stability of IαI digestion products was not determined. The molecular weights of inhibitor fragments generated by four proteinases of different specificity, ranging from 50 000 to 100 000, resemble those of urinary proteinase inhibitors, whose reported molecular weights vary from 44 000 (Hochstrasser et al., 1973) to 72 000 (Barthelemy-Clavey et al., 1979). Moreover, urinary proteinase inhibitors can be proteolyzed to smaller inhibitors of molecular weight 19 000 (Tanaka et al., 1982) to 30 000 (Muramatu et al., 1980), again consistent with results of the present study. Proteolysis of urinary proteinase inhibitors is accomplished by the same proteinases that are inhibited (Jonsson & Ohlsson, 1984; Balduyck et al., 1985). Further studies to determine accurate yields and sizes of products of IαI are in progress.

In support of the model presented for the mechanism of action of IαI, the kinetics of inhibition and proteolysis reactions involving IαI and four proteinases were determined. This study represents the first report of the kinetics of proteolysis and includes two physiologically important proteinases, plasmin and leukocyte elastase. Experiments were designed to mimic physiological conditions of pH and temperature. Kinetic parameters allow comparison of the stability of proteinase inhibition by IαI, as well as comparison of the association rates of proteinases with IαI and other plasma proteinase inhibitors. In addition, the likelihood of inhibition or proteolysis for a given proteinase can be predicted.

The stability of inhibition, assessed by inhibition constants (K_i or K_i'), is greatest for trypsin, followed by chymotrypsin, leukocyte elastase, and plasmin, in order of decreasing stability. The values of inhibition constants are in good agreement with those reported for IαI and IαI derivatives for the inhibition of trypsin, chymotrypsin, and leukocyte elastase (Aubry & Bieth, 1976; Albrecht et al., 1983b; Gast & Bieth, 1985). Our results further indicate that IαI and its proteolyzed forms are comparable with respect to both the rate and the stability of inhibition.

The ratio of K_p and K_i describes the distribution of proteinase between the inhibitory complex (E_iI) and the complex that leads to proteolysis (E_pI). The K_i values determined for plasmin and leukocyte elastase may be erroneously low due to the effect of the E_pI complex on free enzyme concentration, since the ratio of K_p to K_i is only 23 for plasmin and 15 for leukocyte elastase. In any case, the calculation of uninhibited proteinase concentration from K_i was sufficiently accurate for the graphical determination of K_p.

A comparison of the inhibition equilibrium constant (K_i) and the apparent equilibrium constant for proteolysis (K_p) for a given proteinase indicates the relative affinity of the proteinase for the inhibition or proteolysis site of IαI but does not predict the likelihood of occurrence of either reaction. The likelihood of inhibition or proteolysis can be assessed by comparing second-order rate constants that govern the initial association rates at the inhibition and proteolysis sites. Values for $k_i/(k_p/K_p)$ do not necessarily reflect the values of K_p/K_i. In the case of chymotrypsin, where the inhibitory complex is 550 times stronger than the proteolysis complex, the rate constants indicate that the complex leading to proteolysis (E_pI) forms slightly more rapidly than the inhibitory complex (E_iI). Because of this, the inhibition equilibrium is reached, and K_i can be measured only after IαI is completely converted to I'. The enzymes most likely to proteolyze IαI, on the basis of the ratio $k_i/(k_p/K_p)$ are, in order, chymotrypsin, leukocyte elastase, plasmin, and trypsin.

Proteolysis of IαI in vivo might be accomplished by either plasmin or leukocyte elastase. However, the contribution of either of these reactions to the production of IαI derivatives such as urinary proteinase inhibitors cannot be assessed without precise knowledge of the physiological concentrations of the proteinases, which might be relatively high at the surface of thrombi (Rakoczi et al., 1978) or at sites of inflammation (Unkeles et al., 1974; Gordon, 1976). Predictions of the efficiency of proteolysis by particular enzymes, as shown by Dietl et al. (1979), must account for the effect of inhibition on the enzyme concentration available for proteolysis of IαI. Thus, the initial concentrations of proteinase and inhibitor, as well as the rates of proteolysis, affect the production of IαI-derived inhibitors.

The contribution of IαI to proteinase inhibition in vivo can be assessed by comparison of the half-times of inhibition of various plasma proteinase inhibitors with plasmin and leukocyte elastase. IαI is relatively ineffective as an inhibitor of leukocyte elastase; however, it is comparable to α_2-macroglobulin in inhibition of plasmin. This feature may be physiologically important when α_2-proteinase inhibitor, the primary inhibitor of plasmin, is depleted during fibrinolytic therapy (Nilehn & Ganrot, 1967; Mullertz & Clemmensen, 1976). As previously suggested (Pratt & Pizzo, 1986), IαI

may function as a proteinase shuttle in vivo since complexes between IαI and plasmin form rapidly but are reversible. The ability of IαI to transfer proteinases to other plasma proteinase inhibitors has been corroborated by reports of similar activity for a urinary proteinase inhibitor (Jonsson & Ohlsson, 1984).

The physiological importance of proteolysis of IαI is not known. IαI derivatives, measured as urinary proteinase inhibitors, increase during pregnancy (Proksch & Routh, 1971; Barthelemy-Clavey et al., 1979) and cancer (Chawla et al., 1978); however, these increases are not clearly correlated with decreases in circulating IαI concentration (Sesboue et al., 1983; Chawla et al., 1984). Because proteinase inhibitors derived from IαI by proteolysis do not differ in proteinase specificity or stability of inhibition, their function may be related to altered kinetics of inhibition, to altered accessibility to proteinases in vivo, or to some other property unrelated to proteinase inhibition.

Registry No. IαI, 39346-44-6; Bz-Arg-pNA, 6208-93-1; MeO-Suc-Arg-Pro-Tyr-pNA, 82564-18-9; Val-Leu-Lys-pNA, 64816-15-5; MeOSuc-Ala-Pro-Val-pNA, 99248-89-2; trypsin, 9002-07-7; chymotrypsin, 9004-07-3; plasmin, 9001-90-5; elastase, 9004-06-2.

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Interaction of Human Calpains I and II with High Molecular Weight and Low Molecular Weight Kininogens and Their Heavy Chain: Mechanism of Interaction and the Role of Divalent Cations†

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ABSTRACT: Calpain I prepared from human erythrocytes was half-maximally and maximally activated at 23 and 35 μM calcium ion, and two preparations of calpain II from human liver and kidney were half-maximally activated at 340 and 220 μM calcium ion and maximally activated at 900 μM calcium ion, respectively. High molecular weight (HMW) and low molecular weight (LMW) kininogens isolated from human plasma and the heavy chain prepared from these proteins inhibited calpain I as well as calpain II. The molar ratios of calpains to HMW kininogen to give complete inhibition of calpains were 1.4 for calpain I and 2.0 for calpain II, and those of calpains to heavy chain were 0.40–0.66 for calpain I and 0.85 for calpain II. LMW kininogen did not completely inhibit the calpains even with an excess amount of kininogen. The apparent binding ratio of calpain to HMW kininogen estimated from the disc gel electrophoretic analysis, however, was found to be 2:1, whereas those of calpain to LMW kininogen and of calpain to heavy chain were found to be 1:1. Calpains and kininogens failed to form complexes in the absence of calcium ion. In the presence of calcium ion, however, they formed the complexes, which were dissociable by the addition of ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid. The minimum concentrations of calcium ion required to induce complex formation between calpain I and kininogens and calpain II and kininogens were 70 and 100 μM, respectively. Some other divalent cations such as Mn^{2+}, Sr^{2+}, and Ba^{2+} were also able to induce the complex formation between calpains and kininogens. Calpain I was activated by these divalent cations except by Mn^{2+}, while calpain II was not activated by any of these divalent cations. These results indicated that the divalent cations required to activate calpains and the divalent cations required to induce the complex formation are not necessarily the same. The ionic radii of the effective divalent cations varied, ranging from 0.8 to 1.3 Å including calcium ion.

Calpain (calcium-dependent cysteine proteinase, EC 3.4.22.17) is known as a cytosolic proteinase widely distributed in mammalian and avian cells and has been isolated from various tissues (Murachi et al., 1981). Calpain has two distinct subclasses of the enzyme: calpain I, which requires low concentrations of Ca^{2+} (micromolar order) for activation, and calpain II, which requires high concentrations of Ca^{2+} (millimolar order) (De Martino, 1981; Yoshimura et al., 1983; Kitahara et al., 1984). These enzymes are composed of two different subunits, a large subunit with M_r 80 000 and a small subunit with M_r 30 000. Recently, amino acid sequences of calpain I of porcine, rabbit, and human and calpain II of rabbit were predicted by analyzing the base sequences of cDNA (Sakihama et al., 1985; Emori et al., 1986a,b; Aoki et al., 1986). Comparison of the amino acid sequences of rabbit calpains revealed that the small subunit of calpain I is identical with that of calpain II, whereas the large subunits of calpains I and II are different from each other, although they share some extent of homology (approximately 50%).

Calpastatin, a calpain-specific inhibitor, is also present in cytosol (Murachi et al., 1981). It was shown that the calpastatin formed a complex with calpain I in the presence of high concentrations of Ca^{2+} (millimolar order) and further that the calpain–calpastatin complex was easily dissociated by adding chelating reagents (Melloni et al., 1982).

On the other hand, Ohkubo et al. (1984) have isolated and characterized cDNA coding for human α_2TPI[1] and revealed that α_2TPI is identical with low molecular weight kininogen

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[1] Abbreviations: HMW kininogen, high molecular weight kininogen; LMW kininogen, low molecular weight kininogen; TPI, thiol proteinase inhibitor; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; β-ME, β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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