Inter-α-trypsin Inhibitor

INHIBITION SPECTRUM OF NATIVE AND DERIVED FORMS*

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The conversion of inter-α-trypsin inhibitor (IaI) into active, acid-stable derivatives by proteolytic degradation has been tested with 10 different proteinases. Of these, only plasma kallikrein, cathepsin G, neutrophil elastase, and the Staphylococcus aureus V-8 proteinase were found to be effective, each releasing more than 50% of this activity. However, a strong correlation between inhibitor degradation and significant release of acid-stable activity could only be found with the V-8 enzyme. Inhibition kinetics for the interaction of native IaI, the inhibitory fragment released by digestion with S. aureus V-8 proteinase, or the related urinary trypsin inhibitor, with seven different proteinases indicated that all had essentially identical Kᵢ values with an individual enzyme and, where measurements were possible, nearly identical second order association rate constants. Significantly, none of the five human proteinases tested, including trypsin, chymotrypsin, plasmin, neutrophil elastase, and cathepsin G, would appear to have low enough Kᵢ values to be physiologically relevant. Thus, the role of native IaI or its degradation products in controlling a specific proteolytic activity is still unknown.

Human plasma contains a number of proteinase inhibitors whose total concentration represents approximately 10% of the protein in this fluid (1). Most of these inhibitors are specific for serine proteinases with several, including α₁-proteinase inhibitor and α₂-antichymotrypsin belonging to a superfamily referred to as serpins (2). However, at least two inhibitors, α₂-macroglobulin and inter-α-trypsin inhibitor (IaI), apparently inactivate serine proteinases by alternative schemes. While the mechanism of action of α₂-macroglobulin is well understood (1), data on IaI are still somewhat fragmentary and there is considerable controversy with regard to its structure, function, and mechanism of action.

Human IaI is a glycoprotein of mass near 200,000 Daltons first described by Steinbuch and Loel (3). It occurs at a concentration near 0.5 mg/ml plasma and accounts for less than 5% of the total trypsin inhibitory activity (4). Recent experimental data (5–7) indicate that the protein is synthesized as multipolypeptide chains, apparently joined through at least one carbohydrate linkage (8). It is known that the structure of one of these chains (light chain) shares homology with the Kunitz class of proteinase inhibitors (9) with the inhibitory site residing near the amino terminus (10), while the heavy chain contains both calcium-binding sites and sequences highly homologous to those at the reactive site of thiol-proteinase inhibitors (7). The inhibitor can inactivate a broad range of serine proteinases, including trypsin and chymotrypsin (11), neutrophil elastase (12), plasmin (13), and acrosin (14); however, its physiological function is unknown.

One of the unusual properties of IaI is the relative ease with which it may be degraded into lower Mᵢ polyepitides without loss of inhibitory activity (15). How this occurs as well as what relevance it might have to IaI function is not yet known, but it should be noted that small inhibitors related immunologically to IaI have been detected in urine, synovial fluid, cervical mucus, and purulent sputum (16). Indeed, the complete sequence of one of these derivatives, obtained from human urine, has been reported (9).

Because a detailed comparison of the inhibitory properties of IaI and its degradation products has not been made, and because little is known with regard to the mechanism by which conversion to the lower Mᵢ form occurs, we have begun a detailed kinetic analysis of this system in order to determine physiologically relevant target proteolytic enzyme(s). This report summarizes data which compare the inhibitory spectrum of three immunologically related forms of IaI, including the native inhibitor, a low molecular weight form obtained by proteinase degradation, and the naturally occurring urinary inhibitor.

Experimental Procedures

Materials

Human plasma was obtained from the American Red Cross, Atlanta, Georgia and human urine from a hospitalized patient with advanced malignancy. Antibodies to IaI were from Calbiochem. Human neutrophil elastase, cathepsin G, human cationic trypsin, and human chymotrypsin I were prepared by methods described previously by this laboratory (17–19). Porcine trypsin and bovine α-chymotrypsin were from Sigma, and low molecular weight urokinase was obtained from Calbiochem. Human plasmin was prepared by activation of human plasminogen (20). Human plasma kallikrein, the Staphylococcus aureus V-8 serine proteinase and metalbuminase, tissue plasminogen activator, and human thrombin were gifts of Drs. G. Salvesen (Duke University), A. Dubin (Jagiellonian University, Cracow, Poland), D. Loskutoff (Scripps Research Institute, La Jolla, CA), and J. Fenton (New York State Health Department, Albany, NY), respectively.
Inter-α-trypsin Inhibitor

Methods

Isolation of Inter-α-trypsin Inhibitor

Step A: Ammonium Sulfate Fractionation—One liter of citrated human plasma was brought to 0.3 saturation with solid ammonium sulfate and the mixture stirred for 30 min. The precipitate was pelleted by centrifugation at 25,000 x g for 10 min and the supernatant retained and brought to 0.45 saturation with ammonium sulfate. After centrifugation the pellet was re-dissolved in 100 ml of 0.05 M sodium phosphate buffer, pH 8.0, containing 0.005 M EDTA, 0.002 M phenylmethylsulfonyl fluoride, 0.05 M benzamidine, and 0.001 M sodium iodacetate to prevent proteolytic cleavage of Id.

This solution was dialyzed extensively versus 0.05 M sodium acetate buffer, pH 7.0, and any precipitate which formed was removed by centrifugation.

Step B: Anion Exchange Chromatography—The solution obtained above was added to a column of DE52 cellulose (6.6 x 14 cm) previously equilibrated with the pH 5.0 buffer and the column washed with 4 liters of 0.25 M sodium acetate buffer, 0.005 M EDTA, pH 5.0, followed by 2 liters of 0.5 M sodium acetate buffer, 0.005 M EDTA, pH 5.0. The column was then re-equilibrated with 0.05 M sodium acetate buffer, pH 5.0 (without EDTA), and a linear gradient (total volume = 1500 ml) from 0 to 0.25 M NaCl in the same buffer used to elute Id. Fractions (8.0 ml) containing inhibitory activity were pooled and concentrated on an Amicon PM-30 membrane.

Step C: Chromatography on Cibacron Blue-Sepharose—Protein-containing fractions from Step B were pooled, treated with phenylmethylsulfonyl fluoride (final concentration = 0.03 M), and loaded onto a column (1.8 x 9.0 cm) of Cibacron Blue-Sepharose (21) equilibrated with 0.05 M sodium phosphate buffer, pH 8.0. The column was washed with 0.05 M sodium phosphate buffer, pH 5.0, 0.4 M NaCl, pH 8.0, and the purified Id eluted with buffer containing 0.8 M NaCl.

Isolation of Urinary Trypsin Inhibitor

The urinary trypsin inhibitor was isolated as described previously (22) with the exception that a final step of purification by reverse phase chromatography on a C-18 column was added. Inhibitor was isolated by using a linear gradient from 0 to 100% acetonitrile in 0.05M trifluoroacetic acid.

Proteolytic Degradation of Plasma Inter-α-trypsin Inhibitor

In order to follow the release of low molecular weight, acid-stable, proteinase-derived forms of Id, native inhibitor was digested with several proteinases, using the following procedure with the S. aureus V-8 serine proteinase as an example. Briefly, 10 mg of purified Id (52 nmol of active inhibitor) was incubated with the V-8 proteinase (0.5 nmol) in 0.1 M Tris-HCl, pH 7.5, for up to 24 h at 37 °C. At given time intervals samples were removed, mixed with 10 μl of bovine serum albumin (100 mg/ml), followed by 70 μl of 15% perchloric acid. The resulting precipitate was removed by centrifugation and the supernatant mixed with 50 μl of 2 M potassium carbonate. Insoluble potassium perchlate was removed by centrifugation and the supernatant dialyzed versus 0.05 M Tris-HCl, pH 7.5, followed by testing for trypsin inhibitory activity. Similar experiments were carried out using trypsin, chymotrypsin, kallikrein, plasmin, thrombin, neutrophil elastase, cathepsin G, tissue plasminogen activator, and S. aureus metalloproteinase.

An acid-stable, active inhibitory fragment released by digestion of Id with the V-8 proteinase was purified to homogeneity by fast protein liquid chromatography (FPLC) treatment of the dialyzed supernatant, obtained as described above, on a mono-Q column in 0.05 M Tris-HCl, pH 7.5. Amino-terminal sequence analysis of this fragment was performed with an Applied Biosystems 4700A gas-phase Sequenator, using the program designed by the manufacturer.

Proteinase Inhibitor Assays

The inhibitory activities of Id, its V-8 proteinase-derived degradation products, and the urinary trypsin inhibitor were determined by measuring the residual enzyme activity of a given proteinase after incubation with inhibitor. Substrates utilized included OMe-Suc-Ala-Ala-Ala-pNA or Suc-Ala-Ala-Ala-pNA for neutrophil elastase, Suc-Ala-Ala-Pro-Phe-pNA for chymotrypsin and cathepsin G, Benz-pNA for trypsin, and O-Val-Leu-Lys-pNA for plasmin. All enzyme-inhibitor reactions were used as described previously (23, 24).

Assay of Elastinolytic Activity

The degradation of elastin by neutrophil elastase both in the presence and absence of Id or Id-derived products was monitored on elastin-agarose plates (25). Digestion was allowed to occur at 37 °C for given time intervals and zones of elastin digestion were measured either with or without protein staining.

Measurement of Association Rate Constants

The second order association rate constants for the interaction of Id and its derivatives with active site titrated proteinases was determined by the method of Biot (26). Equimolar mixtures of enzyme and inhibitor, based on activities, were incubated for given time periods, substrate added, and residual enzyme activity determined using the substrates described above. All experiments were carried out in 0.1 M Tris-HCl, pH 8.0, with the exception of cathepsin G where 0.8 M NaCl was added to the buffer. Final enzyme concentrations used in each determination were as follows: 5.7 nM for porcine and human trypsins, 20 nM for bovine α-chymotrypsin and human chymotrypsin I, 96 nM for plasmin, 60 nM for neutrophil elastase, and 200 nM for cathepsin G.

Determination of Equilibrium Constants

The measurement of Kᵢ for individual enzyme-inhibitor interactions described the protocol developed by Biot (26). Increasing amounts of inhibitor were added to a constant amount of proteinase and the mixture incubated at 25 °C for a given time period. Substrate was then added and residual enzyme activity measured. Kᵢ was determined by measuring residual enzyme activity after incubation with different concentrations of inhibitor, plotting 1/I vs. 1/[I, a where a is the fraction of total enzyme which is not bound to the inhibitor, and using the slope to calculate Kᵢ.

NaDodSO₄-Gel Electrophoresis and Histochemical Staining

Samples of purified Id (3.5 μM) were incubated with different proteinases at a molar ratio ranging from 20:1 to 500:1 in 0.02 M sodium phosphate buffer, pH 7.4, 0.15 M NaCl. At given time intervals aliquots were removed, denatured by boiling in 0.1% NaDodSO₄, and subjected to gel electrophoresis. Samples were also taken, treated with 0.1% NaDodSO₄ at room temperature for 30 min to retain inhibitory activity, and then subjected to gel electrophoresis. After separation the gel was soaked for 20 min in 0.1 M sodium phosphate buffer, pH 7.4, and stained for antiprotease activity (27). Briefly, the gel was incubated at 37 °C for 30 min with a solution of porcine trypsin (0.03 mg/ml) dissolved in phosphate buffer and then rinsed with buffer and immersed in a substrate solution (N-acetyl-dl-phenylalanine-β-naphthyl) ester. After 30 min the gel was removed and immersed in 2% acetic acid for a further 30 min. The zones of inhibition of proteolytic activity of the acid-stable inhibitor appeared as unstained bands against the reddish-brown background of the gel.

RESULTS

Isolation of Inter-α-trypsin Inhibitor—The purification of Id required four steps with an approximate yield of 21 mg of protein from 1 liter of plasma (5% yield). The inhibitor was stable for several months at -20 °C, but the addition of synthetic inhibitors in two of the steps was absolutely necessary in order to obtain a final preparation which did not autolyze over time. The protein migrated as a single polypeptide chain with Mr = 180,000 (Fig. 1), and this was confirmed by cross-gel immunoelectrophoresis where a single peak was obtained against rabbit antiserum to human plasma (not shown). Activity measurements indicated that the protein contained 97.5% active sites against trypsin, assuming a 1:1 interaction.

Release of Acid-stable Inhibitor by Proteolytic Digestion of Inter-α-trypsin Inhibitor—The conversion of Id into active, low Mr, forms by proteolytic digestion was attempted with 10 different proteinases (Table I). Only four, however, caused the release of significant activity (more than 50%). These were kallikrein, cathepsin G, neutrophil elastase, and the S. aureus V-8 serine proteinase. For each of these enzymes the
kinetics of release was followed (Fig. 2), and it was found that the most efficient enzyme was the S. aureus V-8 protease with a release rate of over 96% after 5-h incubation at a 100:1 inhibitor-enzyme molar ratio. Whether this is a normal function of proteinases from pathogenic bacteria has yet to be established. The fragment obtained from digestion of IaI with this enzyme was found to be of M, near 25,000 and to have a sequence identical with that beginning at residue 19 of the urinary inhibitor or the light chain of IaI. Thus, cleavage of the IaI to release this fragment must have occurred between Glu-18 and Val-19 (6, 9).

Figs. 3 and 4 show the degradation patterns obtained after incubation of IaI with these same four proteinases. Only for the V-8 protease is a good correlation found between the disappearance of native IaI and the liberation of the acid-stable inhibitor (Fig. 3). Indeed, in this case histochemical staining of the gel for trypsin inhibitory activity revealed that it was associated with two closely migrating protein bands between 22,000 and 24,000 Daltons. None of the other degradation products showed any major inhibitory activity by this method. Cathepsin G, despite its ability to completely degrade IaI after 4-h incubation (Fig. 4A), releases only 46% of the trypsin inhibitory activity of the native protein in this time period. This is consistent with a totally different pattern of active inhibitor bands shown by histochemical staining. In this case inhibitory activity was associated with four bands migrating near 22,000, 28,000, 53,000, and 120,000 Daltons. During the course of the incubation the first inhibitory derivatives of IaI were found to have higher mass and appeared to be further degraded to lower mass forms as the incubation time was prolonged. After precipitation with acid, inhibitory activity was only found in the M, 53,000, 28,000, and 22,000 fragments. Since lower M, derivatives appear to be resistant to degradation and inactivation, as has been shown for the conversion of IaI by the V-8 protease, the results with cathepsin G would indicate that some of the fragments of M, 53,000 and higher may undergo further proteolysis to give either active inhibitor or inactive degradation products.

Neutrophil elastase and plasma kallikrein are even slower in both degrading IaI (Fig. 4, B and C) and releasing acid-stable trypsin inhibitory activity (Table I). Significantly, prolonged incubation (12 h) with this latter pair of enzymes until the entire native inhibitor is degraded liberates only 30–35% of the inhibitory activity from the gel.

**TABLE I**

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>LE ratio</th>
<th>Activity recovered (%)</th>
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<tbody>
<tr>
<td>S. aureus metalloproteinase</td>
<td>20:1</td>
<td>0.0</td>
</tr>
<tr>
<td>Trypsin (human)</td>
<td>20:1</td>
<td>0.0</td>
</tr>
<tr>
<td>Urokinase</td>
<td>20:1</td>
<td>3.8</td>
</tr>
<tr>
<td>Tissue plasminogen activator</td>
<td>20:1</td>
<td>8.2</td>
</tr>
<tr>
<td>Plasmin</td>
<td>20:1</td>
<td>12.1</td>
</tr>
<tr>
<td>Thrombin</td>
<td>20:1</td>
<td>15.3</td>
</tr>
<tr>
<td>Neutrophil elastase</td>
<td>50:1</td>
<td>50.3</td>
</tr>
<tr>
<td>Plasma kallikrein</td>
<td>50:1</td>
<td>57.0</td>
</tr>
<tr>
<td>Neutrophil cathepsin G</td>
<td>100:1</td>
<td>81.2</td>
</tr>
<tr>
<td>S. aureus V-8 proteinase</td>
<td>100:1</td>
<td>100.0</td>
</tr>
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**FIG. 1.** NaDodSO₄-polyacrylamide gel electrophoresis of IaI at various stages of purification. Lane 1, standard proteins; lane 2, starting plasma; lane 3, (NH₄)₂SO₄ fraction; lane 4, after DEAE-cellulose; lane 5, after Cibacron Blue-Sepharose.

**FIG. 2.** Release of acid-stable trypsin inhibitory activity from IaI during protease digestion. Inhibitor and enzyme reaction mixtures were incubated at 37 °C, and aliquots were removed at specific times for analysis of acid-stable inhibitory activity, as described in the text. C, kallikrein (50:1); A, neutrophil elastase (50:1); Δ, cathepsin G (100:1); O, S. aureus V-8 proteinase (100:1); •, S. aureus V-8 proteinase (500:1). 100% inhibition represents the activity of control IaI before acidification.

**FIG. 3.** NaDodSO₄-polyacrylamide gel electrophoresis of IaI-S. aureus V-8 protease reaction mixtures. Enzyme and inhibitor reaction mixtures were incubated at 37 °C, and aliquots were removed at specific times for analysis by gel electrophoresis after treatment with 0.1% NaDodSO₄. Lane a, IaI control after 300 min incubation; lanes b–f, 1:100 E:I ratio for 45, 90, 180, 300, and 720 min; lanes g–j, 1:500 E:I ratio for 180, 360, 540, and 720 min; lane k, protein standards. Arrows represent areas of inhibitory activity as determined by histochemical staining.
Inter-α-trypsin Inhibitor-Three enzymes, human pancreatic trypsin, porcine trypsin, and bovine α-chymotrypsin, were utilized in an attempt to compare second order association rate constants with IαI, urinary trypsin inhibitor, and the S. aureus V-8 proteinase-derived IαI inhibitory fragment. The results (Table II) indicate that all three forms of the inhibitor were essentially the same in their rate of inactivation of either species of trypsin tested or with bovine chymotrypsin, although it must be realized that the values for IαI interaction are only approximations since this inhibitor is probably also being degraded during its interaction with these proteinases. Nevertheless, as shown in Table I, neither human trypsin nor plasmin were able to effect significant conversion of IαI into acid-stable forms. Yet, the $K_i$ values obtained for each of these with the native inhibitor, that derived by V-8 proteinase degradation, or the urinary trypsin inhibitor, were essentially the same. Thus, it is highly likely that the $K_i$ values are not significantly altered when IαI is converted into lower $M_r$ forms. The proteinases themselves interacted at somewhat different rates with the order being porcine trypsin > human trypsin > bovine α-chymotrypsin. Significantly, attempts to measure association rate constants for human chymotrypsin, neutrophil elastase, cathepsin G, and plasmin could not be made, presumably because of high off rates and/or low equilibrium constants. Indeed, the dissociation rate constant for neutrophil elastase and human urinary trypsin inhibitor, derived from IαI, has already been shown to be relatively fast ($3.2 \times 10^{-2}$ s$^{-1}$) (28). Equilibrium dissociation constants ($K_i$) for the reaction of IαI and its derivatives with the seven proteinases tested are also listed in Table II. Again, however, the $K_i$ values for IαI inhibition are only approximations because of inhibitor degradation. In general, these results indicate that none of the three forms of inhibitor are exceptionally useful in the control of the enzymes investigated since other inhibitors have already been shown to have dramatically higher $K_i$ values (1) with these proteinases. This conclusion is also supported by data (not shown) indicating that the elastinolytic activity of neutrophil elastase is not reduced by the presence of a 20-fold molar excess of any of the three IαI-related inhibitors.

**DISCUSSION**

One of the major problems involved in investigating the structure and function of IαI is stabilization of the purified protein. Early attempts at the isolation of this inhibitor in our laboratory had consistently resulted in the isolation of a highly purified preparation which was rapidly converted to lower $M_r$ products, yet without loss of inhibitory activity. Because we wanted to follow the degradation of native IαI with specific proteinases it was therefore necessary to alter our purification scheme and include small synthetic inhibitors at several steps during the isolation. As a result we were able to prepare a stable protein of $M_r$ near 180,000 with a yield equivalent to that described by others (29). However, it is still not known which endogenous proteinase(s) is responsible for the notorious instability of preparations of IαI made in the absence of inhibitors nor whether this apparent property is physiologically relevant.

Comparison of the kinetic data (Table II) indicates that the of the acid-stable trypsin inhibitory activity (Fig. 2). Once again, histochemical staining revealed the type of active degradation products obtained after incubation of IαI with either enzyme. In the case of kallikrein, the major inhibitory product had $M_r$ 150,000 and was precipitated by formic acid. In the supernatant a weak inhibitory component of $M_r$ 48,000 was detectable. For neutrophil elastase the pattern found indicated four inhibitory fractions, two of $M_r$ 160,000 and 72,000 which were acid-precipitable, and two of $M_r$ 40,000–43,000 which represented acid-stable trypsin inhibitors.

Interaction of Serine Proteinases with Native and Derived Forms of Inter-α-trypsin Inhibitor—Three enzymes, human pancreatic cationic trypsin, porcine pancreatic trypsin, and bovine α-chymotrypsin, were utilized in an attempt to compare second order association rate constants with IαI, urinary trypsin inhibitor, and the S. aureus V-8 proteinase-derived IαI inhibitory fragment. The results (Table II) indicate that all three forms of the inhibitor were essentially the same in their rate of inactivation of either species of trypsin tested or with bovine chymotrypsin, although it must be realized that the values for IαI interaction are only approximations since this inhibitor is probably also being degraded during its interaction with these proteinases. Nevertheless, as shown in Table I, neither human trypsin nor plasmin were able to effect significant conversion of IαI into acid-stable forms. Yet, the $K_i$ values obtained for each of these with the native inhibitor, that derived by V-8 proteinase degradation, or the urinary trypsin inhibitor, were essentially the same. Thus, it is highly likely that the $K_i$ values are not significantly altered when IαI is converted into lower $M_r$ forms. The proteinases themselves interacted at somewhat different rates with the order being porcine trypsin > human trypsin > bovine α-chymotrypsin. Significantly, attempts to measure association rate constants for human chymotrypsin, neutrophil elastase, cathepsin G, and plasmin could not be made, presumably because of high off rates and/or low equilibrium constants. Indeed, the dissociation rate constant for neutrophil elastase and human urinary trypsin inhibitor, derived from IαI, has already been shown to be relatively fast ($3.2 \times 10^{-2}$ s$^{-1}$) (28). Equilibrium dissociation constants ($K_i$) for the reaction of IαI and its derivatives with the seven proteinases tested are also listed in Table II. Again, however, the $K_i$ values for IαI inhibition are only approximations because of inhibitor degradation. In general, these results indicate that none of the three forms of inhibitor are exceptionally useful in the control of the enzymes investigated since other inhibitors have already been shown to have dramatically higher $K_i$ values (1) with these proteinases. This conclusion is also supported by data (not shown) indicating that the elastinolytic activity of neutrophil elastase is not reduced by the presence of a 20-fold molar excess of any of the three IαI-related inhibitors.

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Comparison of the kinetic data (Table II) indicates that the
native, protease-converted, and urinary forms of the inhibitor behave identically in their interactions with a specific protease. Thus, proteolytic conversion of IaI to lower M, forms, as noted above, has little effect on either rates of inhibition or inhibitory capacity. Significantly, K values and rate constants in all cases indicated weak interactions with the proteinases examined. It has been suggested (26) that for an inhibitor to be efficient it should have a high affinity for its target enzyme and an P/K ratio higher than 100, where P is the concentration of the inhibitor in plasma. For IaI this ratio is 47.0 for cathepsin G, 23.1 for human plasmin, 20.0 for neutrophil elastase, 3.8 for human chymotrypsin, and 0.5 for cathepsin G. This indicates that enzyme-IaI complexes are so loosely formed that some of the enzymes are likely to be in free rather than inhibited states. It also explains why none of the inhibitors could reduce the elastinolytic activity of neutrophil elastase, even at a high molar excess over proteinase.

Human IaI is one of the few plasma proteinase inhibitors whose physiological role is unknown (30). Indeed, no deficiency syndrome for this protein has as yet been reported. However, it has been shown that IaI levels are depleted in cancer patients (31) and after renal failure (32), while urinary concentrations are significantly increased, often up to 100 times normal. Since malignancy is often accompanied by a considerable increase in proteolytic activity primarily associated with plasminogen activators, two enzymes of this type, tissue plasminogen activator and urokinase, were tested in this study for their ability to convert IaI into lower M, forms. However, neither of these nor plasmin or thrombin had any significant effect on the transformation of IaI into an acid-stable form. Therefore, it is doubtful that any of these enzymes could be responsible for the dramatic increase in acid-stable inhibitory activity found in the urine of cancer patients (22).

It has also been recently suggested that IaI might act as a secondary regulator of plasmin (30), because its K values are comparable to that of α2-macroglobulin. However, it is unlikely that this is true because one would expect that with the high concentration of α2-macroglobulin in plasma this inhibitor would reduce free plasmin activity in patients on tissue plasminogen activator therapy. This apparently does not occur because there is continued massive degradation of fibrinogen, indicative of free plasmin in the circulation in such individuals. Because K values are nearly equivalent for IaI-plasmin interactions this inhibitor would likely be just as inefficient in regulating the activity of this enzyme.

Inflammation is always accompanied by the accumulation and degranulation of neutrophils and, inevitably, the extracellular release of the proteolytic enzymes elastase and cathepsin G (33). These enzymes, therefore, could be involved in the conversion of IaI to lower M, forms under pathological conditions. Previously (34), it was shown that elastase could, indeed, process IaI; however, such experiments utilized an excess of proteinase over inhibitor. Our results were obtained with inhibitor in excess, and under these conditions cathepsin G was much more efficient in liberating an acid-stable IaI derivative than any of the other human proteinases investigated. It has recently been reported (35) that carbohydrate attached to residue 10 of the light chain of IaI (36) forms a covalent linkage with the heavy chain(s) (8) to give this protein its unique structure. Thus, cathepsin G and other proteinases must attack peptide bonds downstream from this residue in order to release low molecular weight inhibitors. In the case of the V-8 proteinase cleavage occurs specifically between Glu-18 and Val-19. However, for cathepsin G the cleavage point has not been elucidated, although a likely candidate is between Leu-15 and Val-16, in line with the specificity of this enzyme (37). Significantly, a Leu-Val cleavage is required for the release of angiotensin I from human angiotensinogen by the enzyme renin (38). Preliminary studies (not shown) indicate that this enzyme can also release a low molecular weight inhibitor from native IaI, although the cleavage site has not yet been established. Thus, if the conversion of IaI to the urinary inhibitor takes place in the kidney, renin would appear to be an excellent candidate as the protease involved in this reaction.

It should be pointed out that IaI degradation in plasma by all of the proteinases tested is extremely slow due to the presence of high concentrations of other proteinase inhibitors. The question, therefore, still remains as to where and how IaI conversion takes place in tissues as well as to how the low and high M, forms function as proteinase inhibitors. Part of the answer may lie in the fact that IaI is synthesized as individual heavy and light chains (39) and that small inhibitors derived from the latter may be secreted directly. However, the depletion of IaI in the plasma of cancer patients paralleled by the significant increase in their urinary trypsin inhibitor levels would argue against this supposition and support IaI proteolytic conversion (22). Alternatively, individual chain synthesis and conversion of IaI may be occurring simultaneously. It remains to be established which mechanisms are operative in this system.

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\footnote{J. Potempa, unpublished observations.}
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