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Structural and Functional Characterization of the Inhibition of Urokinase by α_2-Macroglobulin†

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ABSTRACT: We have investigated the interaction of α_2-macroglobulin (α_2M) with the serine proteinase urokinase, an activator of plasminogen. Urokinase formed sodium dodecyl sulfate stable complexes with purified α_2M and with α_2M in plasma. These complexes could be visualized after polyacrylamide gel electrophoresis by protein blots using ^{125}I-labeled anti-urokinase antibody or by fibrin autography, a measure of fibrinolytic activity. According to gel electrophoretic analyses under reducing conditions, urokinase cleaved α_2M subunits and formed apparently covalent complexes with α_2M. Urokinase cleaved only about 60% of the α_2M subunits maximally at a mole ratio of 2:1 (urokinase:α_2M). Binding of urokinase to α_2M protected the urokinase active site from inhibition by antithrombin III–heparin and inhibited, to a significant extent, plasminogen activation by urokinase. Reaction of urokinase with α_2M caused an increase in intrinsic protein fluorescence and, thus, induced the conformational change in α_2M that is characteristic of its interactions with active proteinases. Our results indicate that both in plasma and in a purified system the α_2M–urokinase reaction is functionally significant.

α_2-Macroglobulin (α_2M)[1] has been shown to bind and inhibit a wide variety of proteinases, while leaving the active site of the proteinase free to attack small substrates (Mehl et al., 1964; Barrett & Starkey, 1973; Rinderknecht et al., 1975). The proteinase cleaves some or all of the four α_2M subunits (M_r ~185 000) during binding and is subsequently protected from other macromolecular inhibitors (Haverback et al., 1962; Ganrot, 1966). Binding of the proteinase to α_2M is considered to be essentially irreversible under nondenaturing conditions

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[1] Abbreviations: α_2M, α_2-macroglobulin; SDS, sodium dodecyl sulfate; ATIII, antithrombin III; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane.

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(Saunders et al., 1971; Barrett & Starkey, 1973), with some proteinase molecules forming covalent bonds with α_2M (Harpel, 1977; Salvesen & Barrett, 1980). The reaction of α_2M with a proteinase is accompanied by a conformational change, resulting in changes in several of α_2M's physical parameters (Marelis et al., 1969; Jacquot-Armand & Krebs, 1973; Barrett et al., 1979; Richman & Verpoorte, 1981; Straight & McKee, 1982; Bjork & Fish, 1982; Gonias et al., 1982). These characteristics are generally considered to be measures of functional binding of a proteinase to α_2M.

Urokinase, a plasminogen activator, is one proteinase that has been shown to bind to α_2M, but perhaps not in a functional manner. Previous studies showed that urokinase incubated in human plasma was bound by α_2M, ATIII, and α_1-antitrypsin (Murano et al., 1980; Walker & Ogston, 1982; Waller et al., 1983). Some investigators using purified proteins also concluded that urokinase does bind to α_2M (Ogston et al., 1973; Waller et al., 1983) while others concluded it does not (Vahtera & Hamberg, 1978; Harpel, 1973). Most recently, Waller et al. (1983) demonstrated that plasma or purified α_2M incubated at 37 °C with ^{125}I-labeled urokinase resulted in radioactive bands on SDS–polyacrylamide gels attributable to complexes between α_2M and urokinase. This study suggested that urokinase does bind to α_2M in a way similar to other proteinases.

Our study was undertaken to fully characterize the α_2M–urokinase interaction and in particular to determine whether urokinase cleaves α_2M subunits, thus triggering the conformational change in the α_2M molecule that traps the proteinase, and whether urokinase bound to α_2M is inhibited toward its physiological substrate plasminogen. We have employed SDS–polyacrylamide gel electrophoresis, intrinsic protein fluorescence, macromolecular inhibitor assays, fibrin autography, protein blots, and plasminogen activation assays to show that while the reaction of urokinase with α_2M at 37 °C is slow, it occurs via the same structure–function changes that typify other α_2M–proteinase reactions.

Experimental Procedures

Materials. Urokinase was purchased from Winthrop Laboratories (Sterling Drug Inc., ~120 000 CTA units/mg). Heparin sodium (beef lung) was a sterile solution of 10 000 units/mL from Upjohn. Human ATIII was provided by Dr. Michael Griffith, University of North Carolina, Chapel Hill. The synthetic substrates S-2251 (plasmin) and S-2444 (urokinase) were purchased from Kabi. Affi-Gel Blue was from Bio-Rad and Sepharose 4B from Pharmacia. All chemicals were of reagent grade, and all reaction mixtures were buffered by 0.05 M Tris–0.10 M NaCl, pH 8.0, and carried out at 37 °C unless otherwise stated.

Purification of Proteins. Plasminogen was purified from fresh frozen human plasma by using lysine–Sepharose 4B as previously described (Deutsch & Mertz, 1970). α_2M was purified on Affi-Gel Blue, followed by gel filtration on Sepharose 4B (Arnaud & Gianazza, 1982). Protein concentrations were determined with the following extinction coefficients and molecular weights: plasminogen, $E^{1\%}_{280}$ = 17.0 and M_r 92 000 (Violand & Castellino, 1976); α_2M, $E^{1\%}_{280}$ = 8.9 (Hall & Roberts, 1978) and M_r 725 000 (Jones et al., 1972).

Active Site Titration of Urokinase. The concentration of the active sites of the urokinase used in these studies was determined as described by Chase & Shaw (1970).

Preparation of Anti-Urokinase IgG. A healthy New Zealand white rabbit was immunized with subcutaneous injections of urokinase (1 mg in 1.0 mL of Freund's complete adjuvant) and boosted with injections of 0.5 mg of urokinase every 2 weeks. After 6 weeks the rabbit was bled and IgG was isolated from the serum by chromatography on DEAE-Affi-Gel Blue (Bio-Rad).

Analysis of the Reaction of Urokinase with α_2M by SDS–Polyacrylamide Gel Electrophoresis and Subsequent Radioimmunoelectrophoretic (Western) Blotting and Fibrin Zymography. α_2M (1.5 μM) was reacted with urokinase (2.7 μM) at 37 °C. Samples were denatured and reduced (2% SDS–5% 2-mercaptoethanol, 37 °C) at selected time intervals and analyzed on 4% polyacrylamide gels as previously described (Sykes & Bailey, 1971). The protein content of each band on the gel was determined by densitometric scanning after staining with Coomassie Blue R250.

To determine the stoichiometry of the urokinase–α_2M reaction, α_2M (0.4 μM) was reacted with various concentrations of urokinase (0–1.2 μM) at 37 °C for 3.5 h. The samples were then analyzed by SDS–polyacrylamide gel electrophoresis after reduction and denaturation as described above. Gels were stained, destained, and then scanned to determine the protein content of each band. Protein bands of apparent M_r greater than 85 000 were derived from α_2M and, other than the intact α_2M subunit (M_r 185 000), were considered to be products of the α_2M–urokinase interaction.

Under similar conditions, α_2M (0.4 μM) was reacted with urokinase (1.5 μM) that had been radiolabeled with ^{125}I by the method of David & Reisfeld (1974). The sample was subjected to SDS–polyacrylamide gel electrophoretic analysis as above. After scanning, the gel was sliced and analyzed for radioactivity in the protein bands visualized during staining.

Two samples of individual plasmas were diluted with 0.05 M Tris–0.1 M NaCl, pH 8.0, and incubated with urokinase (1.0 μM, final plasma dilution 1:8) at 37 °C for 60 min. Alternatively, purified α_2M (0.5 μM) was reacted with various concentrations of urokinase (0.1–1.5 μM, 60 min, 37 °C). The samples were then mixed with an equal volume of 2% SDS and subjected to electrophoretic analysis on a gradient slab gel (5–8% polyacrylamide) as previously described (Laemmli, 1970). The slab gels were then further analyzed by the fibrin plate zymographic technique of Granelli-Piperno & Reich (1978) or the Western blotting technique of Towbin et al. (1979). Immune overlay of the Western blots was with ^{125}I-labeled IgG specific for urokinase and prepared as described above.

Effect of ATIII on the Activity of Urokinase and Urokinase Reacted with α_2M. Urokinase (1.4 μM) was reacted with α_2M (1.6 μM) or, in the case of the control sample, without α_2M at 37 °C for 3 h. ATIII (9.5 μM) was then added to both samples. After incubation at 37 °C for selected times, samples were removed and reacted with the synthetic substrate S-2444 (0.3 mM) to determine residual urokinase activity. Absorbance at 410 nm was measured after stopping the urokinase–S-2444 reaction with glacial acetic acid. After 70 min heparin (50 units/mL) was added to the urokinase and urokinase–α_2M samples to accelerate the reaction of ATIII with the enzyme. Portions of each reaction mixture were assayed with S-2444 with time.

Activation of Plasminogen by Urokinase and Urokinase–α_2M Complexes. Urokinase (1.4 μM) was incubated at 37 °C for 3 h with or without α_2M (1.6 μM). Urokinase or α_2M–urokinase (17 nM) was then added to plasminogen (6.2 μM in 0.05 M Tris–0.1 M lysine, pH 8.0) and again allowed to incubate at 37 °C. Samples were removed at selected intervals, and plasmin activity was measured at 410 nm by hydrolysis of the synthetic tripeptide S-2251. Alone, plasminogen or urokinase showed no activity with the synthetic

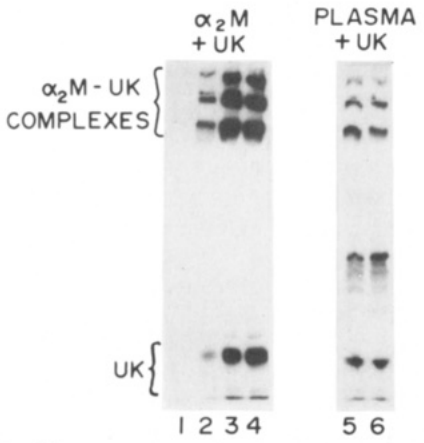

FIGURE 1: Radioimmunoelectrophoretic analyses of the reaction of urokinase with α_2M and plasma. α_2M (0.5 μM) or fresh human plasma (diluted 1:8 in 0.05 M Tris–0.1 M NaCl, pH 8.0) was incubated with various concentrations of urokinase and then subjected to SDS–polyacrylamide gel electrophoresis as described under Experimental Procedures. The gel was then electrophoretically transferred to nitrocellulose paper and overlayed with ^{125}I-labeled anti-urokinase IgG. The protein complexes were then visualized by autoradiography. Lanes 1–4: α_2M (0.5 μM) plus 0.1, 0.5, 1.0, or 1.5 μM urokinase, respectively. Lanes 5 and 6: plasma (two different individuals) plus 1.0 μM urokinase; the unidentified bands just above free urokinase must represent complexes of urokinase with other circulating inhibitors.

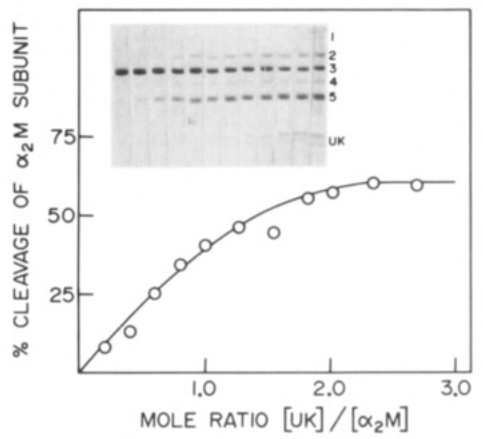

FIGURE 2: Extent of cleavage of α_2M by various concentrations of urokinase. α_2M (0.4 μM) was reacted with urokinase (0–1.2 μM, 37 °C, 0.05 M Tris–0.1 M NaCl, pH 8.0), and after 3.5 h the samples were treated with SDS and 2-mercaptoethanol (1% and 5%, respectively, at 37 °C for 45 min) and then subjected to electrophoretic analysis as described under Experimental Procedures. The gels were stained and destained, and the protein content of the various bands was determined by densitometric scans.

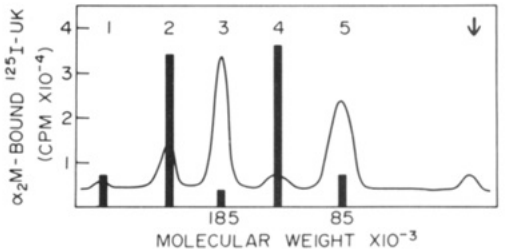

FIGURE 3: Electrophoretic analysis of the binding of ^{125}I-labeled urokinase to α_2M. α_2M (0.4 μM) was reacted with ^{125}I-labeled urokinase (1.5 μM) and analyzed by SDS–polyacrylamide gel electrophoresis under reducing conditions as described in the legend to Figure 2. After staining, destaining, and densitometric scanning, protein bands were excised, and the radioactive content of each was determined by γ counting. Arrow (↓) indicates position of unbound urokinase.

substrate. To ensure that activation of plasminogen was due to α_2M–urokinase complexes and not free urokinase, α_2M–urokinase (0.7 μM) as prepared above was incubated with ATIII (1.7 μM) and heparin (45 units/mL) for 1.5 h at 25 °C. This mixture (28 nM α_2M–urokinase) was then incubated with plasminogen (13 μM) at 37 °C. Developing plasmin activity was measured with S-2251 as stated above.

Intrinsic Protein Fluorescence Studies of the Reaction of α_2M and Urokinase. α_2M (0.5 μM) was incubated with urokinase (1.0 μM) at 37 °C. Fluorescence emission at 340 nm was measured after excitation at 285 nm at various times.

RESULTS

Specific Cleavage of α_2M Subunits by Urokinase. In analyses by polyacrylamide gel electrophoresis, followed by protein Western blotting with ^{125}I-labeled anti-urokinase IgG, we found high molecular weight immunoreactive bands resulting from the reaction of urokinase with purified α_2M (lanes 1–4, Figure 1). Since the antibody used was specific for urokinase and the bands migrated much more slowly than free urokinase, they must represent α_2M–urokinase complexes. When urokinase was reacted with human plasma, similar immunoreactive bands were observed, thus indicating urokinase and α_2M form complexes in the plasma milieu (lanes 5 and 6, Figure 1). The lower molecular weight bands (above urokinase) in lanes 5 and 6 are apparently the result of the reaction of urokinase with other plasma inhibitors such as α_1-antitrypsin and antithrombin III (Clemmensen & Christensen, 1976; Clemmensen, 1978). Similarly, α_2M–urokinase complexes subjected to electrophoresis in the presence of SDS and then analyzed by fibrin plate autography showed high molecular weight bands of fibrinolytic activity apparently corresponding to the various forms of α_2M–urokinase complexes (not shown). To further explore this interaction, samples of α_2M reacted for various times (37 °C) with urokinase were analyzed by SDS gel electrophoresis after reduction and denaturation. Cleavage of the α_2M subunits reached a maximum after about 3 h. α_2M was then reacted with various concentrations of urokinase for 3.5 h and analyzed as in the previous experiment. Results in Figure 2 show that not only is maximum (~60%) cleavage of α_2M subunits (measured as the increase in bands 1, 2, 4, and 5) achieved at a mole ratio of 2:1 (urokinase:α_2M) but the cleavage pattern is essentially the same as that observed for other proteinases (Harpel, 1973). On these gels, the protein bands that represent urokinase–α_2M covalent complexes were identified by electrophoretic analysis of ^{125}I-labeled urokinase reacted with α_2M. Results shown in Figure 3 indicate the majority of α_2M-bound urokinase is present in bands (1, 2, and 4) that correspond to neither the unreacted α_2M subunit (band 3) nor the cleaved subunit of α_2M (band 5).

Functional Binding of Urokinase to α_2M. Functional binding of proteinases to α_2M has been defined by several methods. First, once the proteinase is bound to α_2M, it is usually protected to some extent from other macromolecular inhibitors such as ATIII. The results shown in Figure 4 are in accord with those previously reported (Clemmensen, 1978), which show that urokinase is inhibited very slowly if at all by ATIII alone. The same is true of urokinase bound to α_2M. However, when heparin is added to the reaction mixtures, free urokinase is inactivated rapidly and completely by ATIII. The urokinase previously reacted with α_2M is, on the other hand, protected from ATIII even in the presence of heparin.

Second, when a proteinase is bound to α_2M, the activity of the proteinase toward macromolecular substrates is largely if not totally inhibited (Barrett & Starkey, 1973). We, therefore,

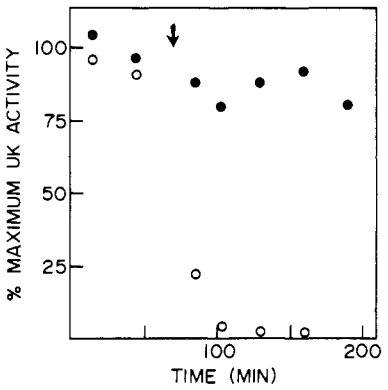

FIGURE 4: Reaction of antithrombin III with urokinase and urokinase bound to α_2M. Urokinase (1.4 μM, O) or the urokinase–α_2M complex (●) was incubated with ATIII (9.5 μM). Samples were removed and assayed vs. S-2444 (0.3 mM). After 70 min, heparin (50 units/mL, ↓) was added to each mixture and the residual urokinase activity measured with S-2444 at various times. All incubation mixtures were buffered with 0.05 M Tris–0.1 M NaCl, pH 8.0, and maintained at 37 °C.

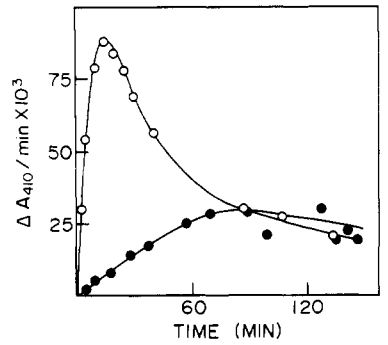

FIGURE 5: Activation of plasminogen by urokinase (O) and the α_2M–urokinase complex (●). Urokinase or the urokinase–α_2M complex (17 nM) was incubated with plasminogen (6.2 μM). Developing plasmin activity was measured by using S-2251. Activation mixtures were in 0.05 M Tris–0.1 M lysine, pH 8.0 and 37 °C.

measured the activity of α_2M-bound urokinase toward plasminogen. As shown in Figure 5, free urokinase activated plasminogen rapidly, the activity reaching a maximum after about 15 min under the conditions of our assay. In contrast, α_2M-bound urokinase activated plasminogen very slowly relative to free urokinase. It is unlikely that this activity is due to residual free urokinase rather than the α_2M–urokinase complex itself. We base this conclusion on the observations (Figure 4) that ATIII with heparin totally inactivates urokinase and that the α_2M–urokinase complex in the presence of ATIII and heparin still activates plasminogen (results not shown).

The rapid decrease in plasmin activity after 15 min in the presence of urokinase and the apparent maximum of plasmin activity reached with α_2M–urokinase are due to plasmin autolysis and its consequent inactivation (Takada & Takada, 1981). Autolysis is a significant competing reaction early in the urokinase activation of plasminogen, since the rate of autolysis depends on the plasmin concentration. On the other hand, the plasmin concentration, and thus autolysis, must be very low in the early stages of α_2M–urokinase activation of plasminogen. Autolysis makes it difficult to quantitate the relative rates of activation but should decrease the apparent initial rate of activation by urokinase more than the rate of α_2M–urokinase activation of plasminogen.

As a third measure of urokinase binding to α_2M, we determined the effect of urokinase on the intrinsic fluorescence of α_2M. We and others have previously shown that reaction

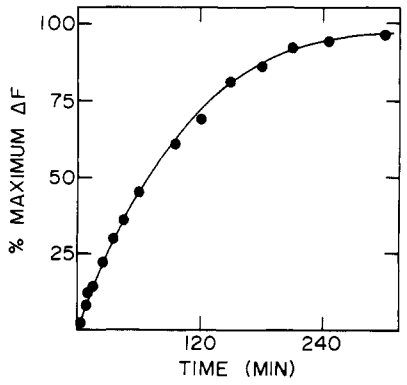

FIGURE 6: Effect of urokinase treatment on α_2M's intrinsic fluorescence. α_2M was reacted with a 2-fold molar excess of urokinase (37 °C, 0.05 M Tris–0.1 M NaCl, pH 8.0). The fluorescence emission was monitored at 340 nm (excitation, 285 nm) until a maximum value was obtained. The data are presented as a percentage of this maximum value.

of an active proteinase with α_2M causes an increase in fluorescence (Richman & Verpoorte, 1981; Bjork & Fish, 1982; Straight & McKee, 1982). On the other hand, inactive proteinases do not cause this effect, and thus it has been concluded that the fluorescence increase is a measure of the conformational change that proteolytic cleavage triggers in the α_2M molecule. Our results (Figure 6) show that urokinase causes a similar increase in protein fluorescence upon reaction with α_2M.

Discussion

There are several structural properties of α_2M that are unique among proteinase inhibitors. These features have yet to be fit neatly into a mechanism for the reaction of α_2M with a proteinase or for events such as clearance from blood that occur after binding. Thus, characterization of α_2M–proteinase interactions, it is hoped, will add to our understanding of the role of the various structural elements critical to its function.

The binding interactions of several proteinases with α_2M have been described, and subsequently criteria for what constitutes functional binding to α_2M have been established as detailed above. Some disagreement exists among previous reports as to whether α_2M does (Ogston et al., 1973; Murano et al., 1980; Walker & Ogston, 1982; Waller et al., 1983) or does not (Harpel, 1973; Vahtera & Hamberg, 1978) inhibit urokinase.

Perhaps the most persuasive evidence to date that α_2M and urokinase form stable complexes is the recent study by Waller et al. (1983) that showed α_2M–^{125}I-labeled urokinase complexes were not dissociable in SDS. While our observations using the Western blot technique (Figure 1) confirm their findings, neither our study nor that of Waller et al. (1983) directly addresses the question of whether urokinase proteolytically cleaves α_2M in the manner of other proteinases. The previous reports of Harpel (1973) and Vahtera & Hamberg (1978) suggest that urokinase does not cleave α_2M subunits.

The results we report here show that urokinase cleaved the α_2M subunit in a manner identical with that of other proteinases (Figure 2). In addition, cleavage was saturable just as with trypsin, plasmin, and thrombin (Swensen & Howard, 1979a; Straight & McKee, 1982). The earlier study indicating that urokinase does not cleave α_2M, even after very long times (Vahtera & Hamberg, 1978), was performed at 25 °C, as opposed to 37 °C in the present study. In the study by Harpel (1973) a relatively short incubation at 37 °C was used. We believe this may explain the different results. Also of interest is our observation that urokinase cleavage of α_2M appeared

to reach a maximum at a 2:1 mole ratio (proteinase:α_2M). Although, as we have described before, this is not necessarily indicative of a 2:1 binding ratio, it is characteristic of other proteinases we have studied (Straight & McKee, 1982). The extent of α_2M cleavage by urokinase is also interesting, because it only involved ~60% of the α_2M subunits. As described earlier, plasmin and thrombin also cleave only 50–75% of α_2M subunits (Sottrup-Jensen et al., 1981; Straight & McKee, 1982). This seems to be characteristic of larger proteinases that react relatively slowly in vitro with α_2M and stands in contrast to trypsin, for example, which cleaves 100% of α_2M subunits (Swensen & Howard, 1979a).

To further explore the nature of the α_2M–urokinase complex, ^{125}I-labeled urokinase was reacted with α_2M and analyzed on SDS–polyacrylamide gels under reducing conditions. Bands were observed that represent covalent complexes of urokinase and α_2M. Just as described for other proteinases (Harpel, 1977; Harpel et al., 1979; Wang et al., 1984), urokinase appeared to form complexes with both proteolytically cleaved and/or uncleaved (Figure 3, bands 1, 2, and 4) α_2M subunits. Two additional points related to cleavage and covalent binding should be made concerning these gel experiments. First, the extent of urokinase cleavage of α_2M subunits (~60%) and the shape of the curve in Figure 2 suggest that the enzyme may bind to α_2M at a mole ratio between 1:1 and 2:1. This conclusion is based on similar results obtained by using plasmin and thrombin. These enzymes are more fully characterized with respect to α_2M binding and appear to bind to α_2M at a mole ratio of between 1 and 2 (Straight & McKee, 1982, 1984; Howell et al., 1983; Strickland & Bhattacharya, 1984). Second, our gel study of ^{125}I-labeled urokinase binding to α_2M indicates that, at a mole ratio of 2:1 (urokinase:α_2M), about 40% of the recovered radioactivity is associated with α_2M. This suggests that the extent of covalent binding is quite significant and may approach 1 molecule of urokinase per molecule of α_2M. These results are similar to those obtained with the thrombin–α_2M complex (Straight & McKee, 1984).

ATIII in the presence of heparin inactivates urokinase (Clemmensen, 1978), but as our results show, when urokinase is bound to α_2M, it is protected from ATIII (Figure 4). Protection of a proteinase from other macromolecular inhibitors while the proteinase retains small-substrate activity is characteristic of binding to α_2M. Perhaps the most important point to be made about this result is that α_2M-bound urokinase was not progressively inhibited by ATIII. This indicates that binding is very tight and that the urokinase molecule is inaccessible as is characteristic of most proteinase–α_2M complexes. Vahtera & Hamberg (1978) in their study of the α_2M–urokinase reaction at 25 °C suggested that binding was reversible. On the basis of our results with ATIII, this does not appear to be the case when the α_2M–urokinase complex is formed at 37 °C.

Next we measured the effectiveness of α_2M-bound urokinase as a plasminogen activator. The rate of activation by α_2M–urokinase was estimated to be less than 2% of that for free urokinase (Figure 5). Whether the residual activity in the α_2M–urokinase sample was due to activity of the complex or to a small amount of undetectable free urokinase might be debatable, but even in the presence of ATIII–heparin, α_2M–urokinase could activate plasminogen, indicating that the complex does retain activity. This would not be unusual since α_2M complexes with other proteinases have been shown to retain small amounts of activity toward macromolecular substrates (e.g., fibrinogen, antihemophilic factor) and inhibitors (Harpel & Mosesson, 1973; Switzer et al., 1983; Bieth et al., 1981; Wang et al., 1981).

During the reaction of proteinases with α_2M some or all of the putative thiol ester bonds of α_2M are broken (Sottrup-Jensen et al., 1980; Swensen & Howard, 1979b). The scission of these bonds, either by a proteinase or by methylamine, results in an increase in intrinsic protein fluorescence due to a major conformational change in α_2M (Bjork & Fish, 1982; Straight & McKee, 1982). Thus, the ability of a proteinase to cause a fluorescence change during its reaction with α_2M is a measure of functional binding to the inhibitor. As shown in Figure 6, urokinase causes an increase in α_2M fluorescence. As expected, the time course of the fluorescence change is similar to the rate of urokinase cleavage of α_2M subunits.

We conclude on the basis of our results that urokinase reacts with α_2M, albeit relatively slowly. Once bound, urokinase proteolytically cleaves α_2M subunits, and a conformational change in the structure of α_2M then follows. The urokinase–α_2M interaction is essentially irreversible and to a large extent covalent. When urokinase is added to plasma, a significant amount of this exogenous urokinase also binds to α_2M. Our observations here expand and extend those of Waller et al. (1983) by suggesting that the α_2M–urokinase interaction may indeed be a significant control mechanism in the activation of plasminogen in vivo.

Registry No. Urokinase, 9039-53-6; antithrombin, 9000-94-6; heparin, 9005-49-6.

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5-Oxoprolinal: Transition-State Aldehyde Inhibitor of Pyroglutamyl-Peptide Hydrolase†

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ABSTRACT: Pyroglutamyl-peptide hydrolase (EC 3.4.11.8) removes the N-terminal pyroglutamyl residue from pyroglutamyl-containing peptides such as thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LH-RH), neurotensin, and bombesin. The aldehyde analogue of pyroglutamate, 5-oxoprolinal, was synthesized as an active site directed transition-state inhibitor of the enzyme. 5-Oxoprolinal was found to be a potent (K_i = 26 nM) and specific competitive inhibitor of pyroglutamyl-peptide hydrolase. Other aldehydes tested inhibited the enzyme only weakly or not at all. 5-Oxoprolinal blocked the degradation of LH-RH by purified pyroglutamyl-peptide hydrolase. The inhibitor, when injected into mice, inhibited the enzyme after 10 and 30 min. 5-Oxoprolinal should be of value in studies probing the biological significance of pyroglutamyl-peptide hydrolase.

Pyroglutamyl-peptide hydrolase (EC 3.4.11.8), an enzyme classified as a thiol protease, cleaves the N-terminal pyroglutamyl residue from pyroglutamyl-containing peptides such as thyrotropin-releasing hormone (TRH).[1] This enzyme, initially found by Doolittle & Armentrout (1968) in a strain of *Pseudomonas fluorescens* and later purified from other bacteria (Szewczuk & Mulczyk, 1969), was found to be distributed in animal tissues (Szewczuk & Kwiatkowska, 1970). It has recently been purified from guinea pig brain (Browne & O'Cuinn, 1983). It might differ from a M_r 260 000 TRH-degrading serum enzyme which also cleaves the pyroglutamyl residue from TRH (Bauer & Kleinkauf, 1980; Taylor & Dixon, 1978). Pyroglutamyl-peptide hydrolase can catalyze the removal of the N-terminal pyroglutamyl residue from proteins such as fibrinogen and human serum mucoid and also from fibrinopeptides (Szewczuk & Mulczyk, 1969; Ar-

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[1] Abbreviations: TRH, thyrotropin-releasing hormone; LH-RH, luteinizing hormone-releasing hormone; DNPH, 2,4-dinitrophenylhydrazine; Me_2SO, dimethyl sulfoxide; DTT, dithiothreitol; TCA, trichloroacetic acid; PTFA, pyridinium trifluoroacetate; HMe_3Si, trimethylsilane; TLC, thin-layer chromatography; <Glu, pyroglutamate; 2NA, 2-naphthylamide; pNA, *p*-nitroanilide; SM, sulfamethoxazole; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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