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Proteolytic Activity of α_2-Macroglobulin–Enzyme Complexes toward Human Factor VIII/von Willebrand Factor†

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ABSTRACT: The low level of enzymatic activity of certain α_2-macroglobulin–proteinase complexes could be important to the function of factor VIII/von Willebrand glycoprotein since it is especially sensitive to proteolytic cleavage. To test this possibility, complexes of α_2-macroglobulin with plasmin, trypsin, and thrombin were formed in at least a 2:1 molar ratio of α_2-macroglobulin:proteinase and tested for effects on the factor VIII procoagulant activity of the factor VIII/von Willebrand glycoprotein. Neither the α_2-macroglobulin–trypsin complex nor the α_2-macroglobulin–plasmin complex affected factor VIII procoagulant activity. The behavior of the α_2-macroglobulin–thrombin complex was different. When α_2-macroglobulin and thrombin were incubated in a mole ratio of 3:1 or less, factor VIII procoagulant activity was enhanced to about the same extent as with free thrombin. Even at a 24:1 mole ratio, the mixture could produce 45% of the increase in factor VIII activity obtained with free thrombin. The isolated α_2-macroglobulin–thrombin complex could also activate the factor VIII procoagulant function to about 45% of the level obtained with an identical amount of uncomplexed thrombin. Analysis of the α_2-macroglobulin–^{125}I-labeled thrombin complexes by rechromatography or by polyacrylamide gel electrophoresis in sodium dodecyl sulfate indicated that this activation was not due to free thrombin. We conclude that the α_2-macroglobulin–thrombin complex retains sufficient proteolytic activity to activate the procoagulant function of factor VIII/von Willebrand glycoprotein despite the latter being a very large substrate, having an estimated molecular weight of 1–20 million.

Proteinases appear in the circulation during blood clotting, fibrinolysis, or activation of the complement system. These proteinases can be inactivated by one or more of the several proteinase inhibitors in blood (Muller-Eberhard, 1975; Laurell & Jeppson, 1975; Heimburger, 1975). The present study focuses on α_2-macroglobulin, which is normally present in plasma in a concentration of 2–4 mg/mL (Laurell & Jeppson, 1975). This inhibitor is a large protein composed of four identical subunits and has a molecular weight of 725 000 (Swenson & Howard, 1979). The observation that α_2-macroglobulin binds proteinases and is cleaved by them led to the formulation of the "bait-trap hypothesis" in which the proteinase cleaves α_2-macroglobulin and then becomes entrapped as conformational changes occur (Barrett & Starkey, 1973). Certain proteinases remain bound even after the dissociation of α_2-macroglobulin into half-molecules in 4 M urea; under these conditions 2 mol of chymotrypsin remain bound per mol of α_2-macroglobulin (Pochon et al., 1978). More recent studies have shown that the strength of binding of α_2-macroglobulin to different proteinases varies widely; for example, analyses of α_2-macroglobulin–trypsin and α_2-macroglobulin–papain complexes show that 61.2% of the trypsin, but only 8.3% of the papain, becomes bound with a stability indicative of a covalent bond (Salvesen & Barrett, 1980).

Previous studies suggest that α_2-macroglobulin–proteinase complexes may retain low levels of proteolytic activity against

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synthetic and certain naturally occurring protein substrates (Harpel, 1976; Harpel & Mosesson, 1973; Rinderknecht & Geokas, 1973; Bieth et al., 1981). Of particular significance to blood coagulation and fibrinolysis, α_2-macroglobulin–plasmin or α_2-macroglobulin–thrombin complexes can still slowly hydrolyze fibrinogen (Harpel, 1976; Harpel & Mosesson, 1973; Rinderknecht & Geokas, 1973). In view of such activity, others have proposed that in the in vivo function of α_2-macroglobulin may be to preserve some of the biological activity of the bound proteinase in the presence of other, more effective circulating inhibitors (Rinderknecht & Geokas, 1973; Harpel & Rosenberg, 1976).

The studies reported here focus on another potential substrate for α_2-macroglobulin–proteinase complexes, namely, the circulating human plasma protein, factor VIII/von Willebrand factor. This glycoprotein complex has a molecular weight of $(1–21) \times 10^6$ (Fass et al., 1978) and corrects the plasma defect in classic hemophilia (factor VIII activity) as well as the prolonged bleeding time in von Willebrand's disease (von Willebrand activity) (Langdell et al., 1953; Hoyer, 1976; Weiss et al., 1973). The factor VIII procoagulant function is extremely sensitive to activation and inactivation by trace proteinases such as thrombin, trypsin, and plasmin (Kirby et al., 1974; McKee et al., 1975). We found that α_2-macroglobulin complexes of trypsin or plasmin have no effect on factor VIII procoagulant activity; however, α_2-macroglobulin–thrombin complexes continue to possess a significant ability to activate the factor VIII procoagulant function.

Experimental Procedures

Materials. All chemical were reagent grade or better and were used without further purification. Tosylphenylalanine chloromethyl ketone (TPCK)–trypsin, soybean trypsin inhibitor, and α-casein were obtained from Worthington Biochemical Corp. (Freehold, NJ). Rivanol (2-ethoxy-6,9-diaminoacridine lactate) was purchased from K & K Laboratories (Plainview, NY), bentonite was from Sigma Chemical Co. (St. Louis, MO), Trasylol (kallikrein inhibitor, bovine pancreatic trypsin inhibitor) was from Mobay Chemical Corp. (New York, NY), human fibrinogen was from Kabi (Stockholm, Sweden), and urokinase and alumina Cγ gel were from Calbiochem (La Jolla, CA). Poly(ethylene glycol) 4000 (Carbowax 4000) was obtained from Union Carbide Corp. (South Charleston, WV) and carrier-free $Na^{125}I$ (20 mCi/mL) from New England Nuclear (Boston, MA). The chromogenic substrates tosyl-Phe-Val-Arg-*p*-nitroanilide (S2160) and tosyl-Gly-Pro-Arg-*p*-nitroanilide acetate (Chromozym TH) were products of A. B. Bofors (Molndale, Sweden) and Boehringer Mannheim Biochemicals (Indianapolis IN); these were used at final concentrations of 0.07 and 0.1 mg/mL, respectively. M-Partigen α_2-macroglobulin plates were obtained from Behring Diagnostics, Hoechst Corp. (Somerville, NJ). Blue Sepharose CL-6B, Sepharose 4B, Sephadex G-100, and Sephadex G-25 were purchased from Pharmacia Fine Chemicals (Piscataway, NJ) and Bio-Gel A-5m was from Bio-Rad Laboratories (Richmond, CA). Bovine serum albumin was purchased from Sigma Chemical Co. (St Louis, MO) and rendered proteinase free by adsorption with alumina Cγ gel followed by treatment with 2.5 mM diisopropyl fluorophosphate as previously described (Switzer & McKee, 1980). Human plasminogen, prepared by affinity chromatography using lysine covalently coupled to 4% agarose, had a specific activity of 22 CTA units/mg (Walther et al., 1974). Purified human thrombin with a specific activity of 1000 units/mg was supplied by Dr. D. L. Aronson, Bureau of Biologics, Bethesda, MD. Thrombin was labeled with $Na^{125}I$ by using insolubilized lactoperoxidase as we have described before (Switzer & McKee, 1980), except that unreacted $Na^{125}I$ was removed by gel filtration on Sephadex G-25. Purified human antithrombin III, isolated by heparin–agarose chromatography, was the gift of Dr. Michael J. Griffith, University of North Carolina at Chapel Hill.

Electrophoretic Analyses. Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was performed in the presence of Tris–borate buffers by a modification of the method of Sykes & Bailey (1971). Samples were prepared by boiling for 2–3 min in 40 mM Tris–borate–2% $NaDodSO_4$, with or without 5% 2-mercaptoethanol. The electrophoreses were carried out in 100 mM Tris–60 mM boric acid, pH 8.6, containing 0.1% $NaDodSO_4$–4% acrylamide, with 0.08% methylenebis(acrylamide). The running buffer was 100 mM Tris–60 mM boric acid–0.1% $NaDodSO_4$, pH 8.6. The gels were stained for 15 min at 60 °C in 0.125% Coomassie brilliant blue in 50% methanol–9.2% acetic acid and destained in 14% acetic acid–10% methanol.

Purification of α_2-Macroglobulin. The α_2-macroglobulin used in these studies was purified by two different methods. The first was a combination of two published procedures (Harpel, 1976; Steinbuch et al., 1965). Blood was collected and the plasma was separated by the method of Harpel (1976). The soybean trypsin inhibitor was added to a final concentration of 0.08 mg/mL, and the plasma was adsorbed with $BaCl_2$ and $BaSO_4$. As described in this procedure, the fibrinogen was selectively precipitated by 4% poly(ethylene glycol). The supernatant was then removed and adjusted to 12% poly(ethylene glycol) to precipitate the α_2-macroglobulin. Further purification involved dissolution and reprecipitation with rivanol and adsorption with bentonite (Steinbuch et al., 1965). The α_2-macroglobulin concentrate was then gel filtered on a 2.5 × 100 cm column of Bio-Gel A-5m; the fractions eluting in the void volume contained all of the α_2-macroglobulin as determined by radial immunodiffusion on M-Partigen plates. For some of these studies, α_2-macroglobulin was purified by affinity chromatography on Blue Sepharose CL-6B (Virca et al., 1978). The α_2-macroglobulin prepared by either method was >97% pure by analysis on $NaDodSO_4$–polyacrylamide gel electrophoresis in the presence and absence of reducing reagents (Straight & McKee, 1982). The activity of the α_2-macroglobulin preparation was assessed by one of the inhibitor assays described below or by the method of Ganrot (1966). By this technique, we found that our α_2-macroglobulin preparation bound the expected 2 mol of trypsin/mol (Straight & McKee, 1982).

Purification and Assay of Factor VIII/von Willebrand Glycoprotein. Human factor VIII/von Willebrand glycoprotein was purified as previously described (Switzer & McKee, 1979). Factor VIII procoagulant activity was determined by a partial thromboplastin time method (Langdell et al., 1953) except that kaolin (5 mg/mL) was added to the hemophilic substrate plasma (<1% of normal factor VIII activity) and stirred for 6 min at 37 °C to maximize the activation of the contact pathway of blood coagulation. The concentration of the factor VIII/von Willebrand complex was estimated by the absorbance at 280 nm by using a scattering correction based on the absorbance at 320 nm and an $E^{1\%}_{280nm}$ of 12.3 (Switzer & McKee, 1980).

α_2-Macroglobulin–Proteinase Complexes. Unless otherwise indicated, complexes were formed by incubating thrombin or trypsin with the indicated amount of α_2-macroglobulin for 30 min at 37 °C. When specified, the α_2-macroglobulin–thrombin complex was isolated by gel filtration on Sephadex G-100. For

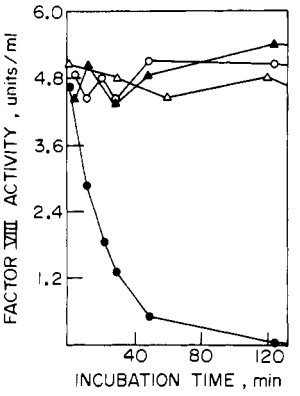

FIGURE 1: Effect of α_2-macroglobulin on the inactivation of factor VIII procoagulant activity by plasmin. At a concentration of 1.14 μg (0.025 CTA unit)/mL, plasmin rapidly inactivated the procoagulant activity of 0.21 mg/mL factor VIII/von Willebrand glycoprotein (●). This effect was completely abolished when the plasmin was complexed with 50 μg of α_2-macroglobulin/mL to give a 5:1 mole ratio of α_2-macroglobulin:plasmin (○). In control experiments, buffer (▲) or α_2-macroglobulin alone (△) had no effect on factor VIII procoagulant activity.

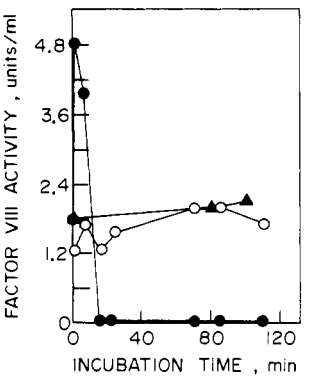

FIGURE 2: Effect of α_2-macroglobulin on the activation and inactivation of factor VIII procoagulant activity by trypsin. At a concentration of 0.2 μg/mL, trypsin alone (●) first activated and then inactivated the factor VIII procoagulant activity of 0.45 mg/mL factor VIII/von Willebrand glycoprotein. A 2:1 mole ratio of α_2-macroglobulin:trypsin had no effect on factor VIII procoagulant activity (○). Control experiments with buffer are indicated (▲).

the α_2-macroglobulin–plasmin complexes, plasmin was first prepared by incubating 480–560 CTA units of urokinase with each mg of plasminogen for 20 min at 37 °C. Then selected amounts of α_2-macroglobulin were added to the plasmin solution, and the incubation was continued for another 30 min at 37 °C. Experiments investigating proteinase inhibition by α_2-macroglobulin used trypsin at concentrations of 0.2–0.66 μg/mL, thrombin at 0.04–0.18 μg/mL, plasmin at 1.14–29.2 μg/mL, and α_2-macroglobulin at concentrations of 40–1800 μg/mL; experiments measuring the binding of thrombin to α_2-macroglobulin were done at thrombin concentrations of 2.1–71.8 μg/mL and α_2-macroglobulin concentrations of 202–1190 μg/mL. Several methods were used to evaluate the proteolytic activities of the complexes as described in the next section.

Measurement of Proteolytic Activity of α_2-Macroglobulin–Enzyme Complexes. The α_2-macroglobulin–trypsin and α_2-macroglobulin–plasmin complexes were tested for enzymatic activity by measuring the cleavage of radioactive peptides from ^{125}I-labeled α-casein (Highsmith & Rosenberg, 1977). The α_2-macroglobulin–thrombin complex was examined for peptidase activity toward tosyl-Gly-Pro-Arg-*p*-nitroanilide and for fibrinogen clotting activity. Finally, each α_2-macroglobulin–protease complex was examined for its effect on factor VIII procoagulant activity.

Results

Inhibitory Activity of α_2-Macroglobulin Preparations. When α_2-macroglobulin:plasmin or α_2-macroglobulin:trypsin mixtures were preincubated in mole ratios of 1:2, 1:4, or 1:8 for 5 min at room temperature and then added to ^{125}I-labeled casein at room temperature for an additional 5 min, the proteolysis that occurred was proportional to the excess concentration of proteinase. With α_2-macroglobulin:proteinase mole ratios of 2:1 or 1:1, however, complete inhibition was observed. Just as reported by others, we found that α_2-macroglobulin–plasmin complexes retained a percent or two of activity toward the carboxyl-terminal region of the Aα chain of fibrinogen (Harpel & Mosesson, 1973). Since thrombin has no activity toward casein, fibrinogen was used as the substrate to test the ability of α_2-macroglobulin to inhibit thrombin. When compared with free thrombin, the isolated α_2-macroglobulin–thrombin complex possessed about 4% fibrinogen clotting activity. The activity of the α_2-macroglobulin–thrombin complex toward a synthetic tripeptide substrate will be discussed later.

Effect of α_2-Macroglobulin Complexes with Plasmin or Trypsin on Factor VIII Procoagulant Activity. Figures 1 and 2 show the effects of plasmin and trypsin and their corresponding α_2-macroglobulin complexes on factor VIII procoagulant activity. As apparent from Figure 1 and as previously demonstrated (Andersen et al., 1980), free plasmin produced an immediate, rapid decrease in factor VIII procoagulant activity, with essentially no activity detected after 2 h of incubation. When α_2-macroglobulin and plasmin were incubated in a 2:1 mole ratio, about 20% of the factor VIII activity was destroyed within 30 min. However, complexes formed by preincubating α_2-macroglobulin with plasmin in 3:1, 4:1, and 5:1 mole ratios produced no significant loss of factor VIII procoagulant activity during a 2-h incubation period. As expected, α_2-macroglobulin alone had no effect on factor VIII procoagulant activity.

Figure 2 shows that free trypsin rapidly activated and inactivated factor VIII procoagulant activity and that a 2:1 mole ratio of α_2-macroglobulin:trypsin totally abolished this activity. As expected, complexes formed by using α_2-macroglobulin:trypsin ratios of 3:1, 4:1, and 7:1 also had no effect on factor VIII procoagulant activity. Thus, according to the data of Figures 1 and 2, α_2-macroglobulin inhibits trypsin even more effectively than plasmin.

Effect of α_2-Macroglobulin–Thrombin Complexes on Factor VIII Procoagulant Activity. α_2-Macroglobulin–thrombin complexes had a considerably different effect on factor VIII procoagulant activity. Even when α_2-macroglobulin and thrombin were incubated in a mole ratio of 6:1 for 30 min at 37 °C, there was only about 20% inhibition of thrombin toward the factor VIII/von Willebrand substrate (Figure 3). Whether or not α_2-macroglobulin was present, factor VIII procoagulant activity was rapidly enhanced and then gradually inactivated. As will be shown below the activity at a 6:1 mole ratio of α_2-macroglobulin to thrombin is due to a mixture of free thrombin and the α_2-macroglobulin–thrombin complex. Figure 4 expands our observations by showing the ratio of factor VIII activation by 0.18 μg/mL thrombin in the presence of α_2-macroglobulin to activation in the absence of α_2-macroglobulin plotted against the ratio of α_2-macroglobulin to thrombin. When the mole ratio of α_2-macroglobulin to thrombin was less than 3:1, the mixture had essentially the same activity as free thrombin. Then as the amount of α_2-macroglobulin was increased, the activity of the mixture de-

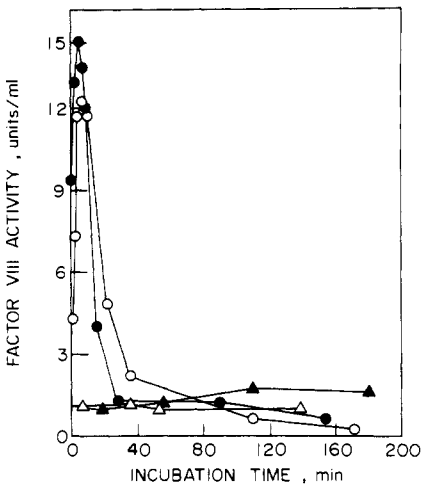

FIGURE 3: Effects of α_2-macroglobulin on the activation and inactivation of factor VIII procoagulant activity by thrombin. Factor VIII/von Willebrand factor, 0.17 mg/mL, was reacted either with 0.04 μg (0.04 unit)/mL thrombin alone (●) or with the same amount of thrombin complexed with 5 μg/mL α_2-macroglobulin to give a 6:1 mole ratio of α_2-macroglobulin:thrombin (○). In both cases, the rate and extent of activation and inactivation were similar. No activation was observed when α_2-macroglobulin alone (▲) or buffer alone (▲) was added to the factor VIII/von Willebrand glycoprotein.

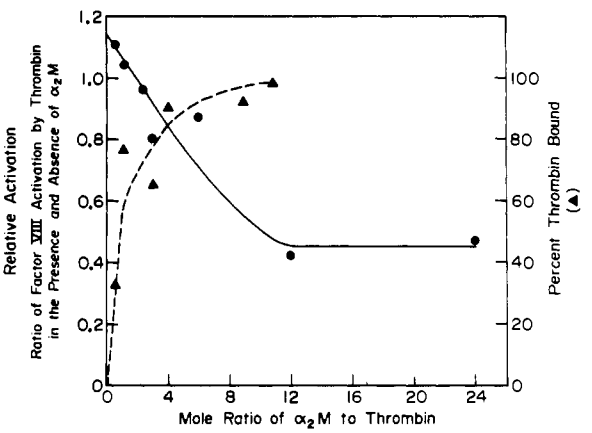

FIGURE 4: Activation of factor VIII procoagulant activity by thrombin in the presence and absence of α_2-macroglobulin and binding of thrombin to α_2-macroglobulin. Thrombin was incubated with α_2-macroglobulin or buffer for 30 min at 37 °C. The extent of activation of factor VIII procoagulant activity in the presence of different amounts of α_2-macroglobulin divided by the activation observed in the absence of α_2-macroglobulin is expressed as a function of the mole ratio of α_2-macroglobulin to thrombin (●). There was little inhibition of factor VIII activation when the α_2-macroglobulin:thrombin ratio was <3:1. Even at large molar excesses of α_2-macroglobulin, the enhancement of factor VIII procoagulant activity was still about 45% of that produced by thrombin alone. For the activation experiments, the final concentration of factor VIII/von Willebrand glycoprotein was 0.39 mg/mL, that of thrombin was 0.18 μg/mL, and the α_2-macroglobulin concentration ranged from 2.2 to 88.1 μg/mL. Figure 4 also shows the percent thrombin bound to α_2-macroglobulin as a function of the mole ratio of α_2-macroglobulin to thrombin (▲). The percent thrombin bound increased with the concentration of α_2-macroglobulin; essentially all of the thrombin was bound when the mole ratio of α_2-macroglobulin to thrombin was 10.7:1. For the binding experiments, the α_2-macroglobulin concentration ranged from 202 to 1190 μg/mL and the thrombin concentration from 2.1 to 71.8 μg/mL.

creased. But even in the presence of a 24-fold molar excess of α_2-macroglobulin, factor VIII procoagulant activity could still be increased to about 45% of the level produced by thrombin alone.

Figure 4 also shows the binding of thrombin to α_2-macroglobulin. ^{125}I-Labeled thrombin was incubated with α_2-macroglobulin for 30 min at 37 °C and then chromatographed on Sephadex G-100. The α_2-macroglobulin–thrombin complex eluted in the void volume and free thrombin eluted about halfway between the void volume and the salt volume. Figure 4 shows that the percent of bound thrombin increased with the concentration of α_2-macroglobulin and was approximately 100% when a 10.7-fold molar excess of α_2-macroglobulin was reached. Hence, the binding data are strong evidence that the activation of factor VIII in the presence of a 12–24-fold molar excess of α_2-macroglobulin is due to the inherent activity of the α_2-macroglobulin–thrombin complex rather than to uncomplexed free thrombin.

To provide further evidence that the activity attributed to the complex was not that of residual free thrombin, we reacted thrombin first with α_2-macroglobulin and then with antithrombin III, which has been reported to bind free thrombin but not thrombin that is complexed with α_2-macroglobulin (Fischer et al., 1981). In this experiment, 0.16 μg/mL thrombin was reacted with 9.5 μg of α_2-macroglobulin/mL for 30 min at 37 °C to give a 3:1 molar excess of inhibitor. The mixture was then incubated for 60 min at 37 °C with 2.4 μg of antithrombin III/mL to give an 8-fold molar excess relative to total thrombin. The reaction mixture that did not contain antithrombin III was also incubated for an additional hour. As a control, 0.16 μg of thrombin/mL was incubated for 60 min at 37 °C with 2.4 μg of antithrombin III/mL but without any α_2-macroglobulin, in order to give an 8-fold molar excess of antithrombin III. Reference to the binding curve in Figure 4 predicts that in the presence of a 3-fold molar excess of α_2-macroglobulin, 75–80% of the thrombin should be in the form of the α_2-macroglobulin–thrombin complex. Thus, the added antithrombin III, which would actually be approximately a 32–40-fold molar excess relative to free thrombin, should be sufficient to irreversibly complex any free thrombin (Fischer et al., 1981). In the control experiment in which thrombin was incubated with an 8-fold molar excess of antithrombin, activation was only about 3-fold. This mixture should have contained more free thrombin and hence more activity toward factor VIII than when α_2-macroglobulin was also present; however, this was not the case. As shown in Figure 5, the mixture of α_2-macroglobulin and thrombin without antithrombin III activated factor VIII about 8-fold. When antithrombin III was also present, factor VIII could still be activated about 7-fold and inactivation was only slightly delayed. Hence, even if we handicap our data and subtract the thrombin activity not inhibited by an 8:1 mole ratio of antithrombin III:thrombin, rather than the calculated 32–40:1 mole ratio, our results predict that at least a 4-fold activation can be attributed to the α_2-macroglobulin–thrombin complex.

Isolation and Characterization of the α_2-Macroglobulin–Thrombin Complex. Since the activity of α_2-macroglobulin–thrombin complexes toward factor VIII procoagulant activity appeared so much greater than those containing plasmin or trypsin, we isolated the complex for further characterization. Excess thrombin plus tracer amounts of ^{125}I-labeled thrombin were incubated with α_2-macroglobulin for 30 min at 37 °C, and the mixture was then chromatographed on Sephadex G-100. A peak of radioactivity corresponding to the α_2-macroglobulin–^{125}I-labeled thrombin complex was eluted in the void volume and a second peak corresponding to free ^{125}I-labeled thrombin was eluted approximately halfway between the void volume and the salt volume. Paired samples of α_2-macroglobulin–^{125}I-labeled thrombin and ^{125}I-labeled thrombin alone were adjusted to contain identical amounts of radiolabel and then tested for peptidolytic and proteolytic

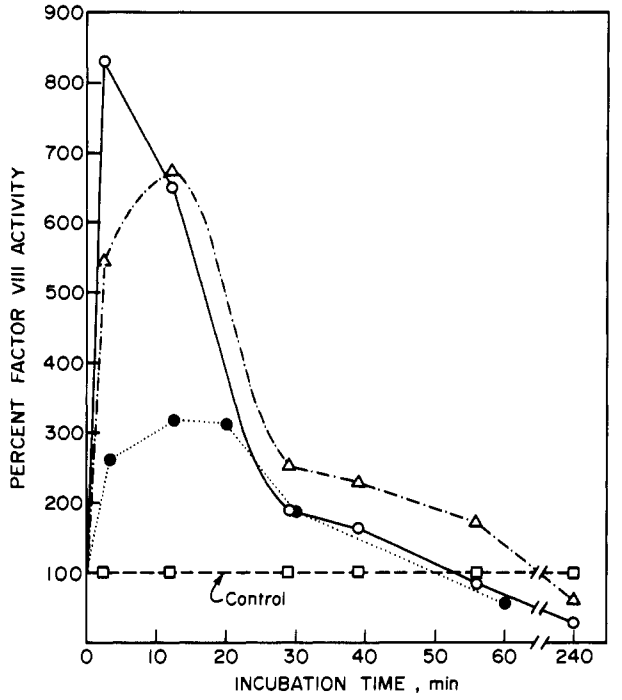

FIGURE 5: Activation of factor VIII by a mixture of thrombin and α_2-macroglobulin in the presence and absence of antithrombin III. Thrombin at a concentration of 0.16 μg/mL was reacted for 30 min at 37 °C with α_2-macroglobulin in a 3:1 mole ratio (○). Then half of the mixture was reacted with an 8-fold molar excess of antithrombin III, relative to total thrombin (△). Thrombin was also incubated with an 8-fold molar excess of antithrombin III without any α_2-macroglobulin (●). Then each of these solutions was tested for its ability to activate 187 μg/mL factor VIII/von Willebrand glycoprotein. The control line represents factor VIII without added thrombin (□). The final concentration of α_2-macroglobulin was 9.5 μg/mL and that of antithrombin III was 2.4 μg/mL.

activity. Although both the α_2-macroglobulin–thrombin complex and free thrombin cleaved the synthetic tripeptide substrate, tosyl-Gly-Pro-Arg-*p*-nitroanilide acetate, the initial rate of hydrolysis by the complex was decreased to 56% of the rate of free thrombin. The fibrinogen clotting times obtained with the isolated complex were much longer than with free thrombin. For example, 0.7 unit/mL thrombin clotted 3.8 mg of fibrinogen/mL in 0.8 min, compared to 36 min when the α_2-macroglobulin–thrombin complex was used. Over a concentration range of 0.2–0.7 unit of free thrombin/mL, the α_2-macroglobulin–thrombin complex had only 4% of the fibrinogen clotting activity of free thrombin. These results agree with previous studies demonstrating that α_2-macroglobulin–thrombin complexes can still clot fibrinogen, but at a markedly slower rate than free thrombin (Rinderknecht & Geokas, 1973).

As shown in Figure 6, both free thrombin and the isolated α_2-macroglobulin–thrombin complex activated and then gradually inactivated factor VIII procoagulant activity; however, both activation and inactivation of factor VIII occurred more slowly with the α_2-macroglobulin–thrombin complex. The extent to which the isolated α_2-macroglobulin–thrombin complex could still activate factor VIII remained significant. Instead of being completely or nearly completely inhibited, the complex still enhanced factor VIII procoagulant activity to about 45% of the level obtained with the same amount of free thrombin. Given the fact that factor VIII activation is not directly proportional to thrombin concentration (Switzer & McKee, 1980), we determined the percent activity of the isolated α_2-macroglobulin–thrombin complex by comparing the amount of activation that we observed with the complex

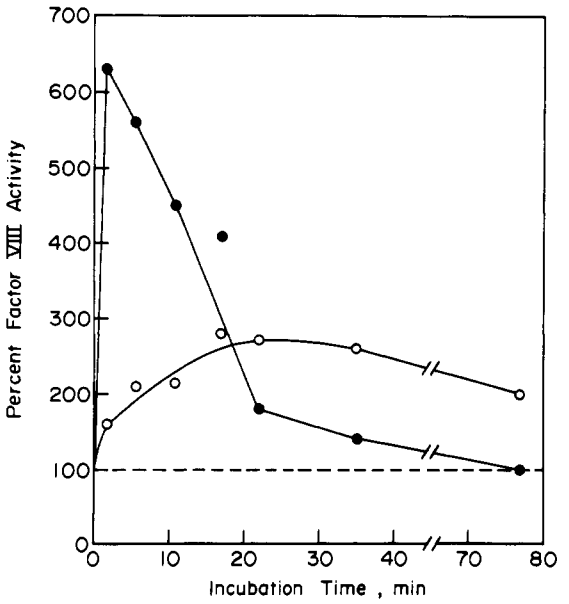

FIGURE 6: Activation of factor VIII activity by free thrombin (●) and the isolated α_2-macroglobulin–thrombin complex (○). Free thrombin and complexed thrombin were each isolated by chromatography on Sephadex G-100, adjusted by radioactivity to contain 0.05 thrombin unit/mL, and then incubated with 0.41 mg/mL factor VIII/von Willebrand glycoprotein.

Table I: Factor VIII Procoagulant Activation by Free Thrombin and the Isolated α_2-Macroglobulin–Thrombin Complex

thrombin concn (unit/mL)	% factor VIII activation[a] by free thrombin	by α_2-macroglobulin–thrombin complex	% activity of α_2-macroglobulin–thrombin complex[b]
0.16	690	400	9.4
0.08	570	240	4.0
0.032	490	170	5.2
			$\bar{X}$ = 6.2

[a] Relative to control. [b] Based on the activation of factor VIII procoagulant activity relative to that of free thrombin.

to the level of factor VIII activation obtained with serial dilutions of free thrombin. These results are presented in Table I, which shows that the α_2-macroglobulin–thrombin complex had only about 6.2% of the activity of free thrombin, yet this was sufficient to activate factor VIII 45% as well as the same amount of free thrombin. As will be described below, the maximum amount of thrombin that we were able to dissociate from the α_2-macroglobulin without reduction was <1% by $NaDodSO_4$–polyacrylamide gel electrophoresis. Even if this amount of thrombin could be dissociated in neutral buffers, it is much less than that required to account for this activity.

Instantaneous Effects of α_2-Macroglobulin and Antithrombin III on the Reaction of Thrombin with Factor VIII. The previous experiments tested the effect of α_2-macroglobulin on thrombin after incubation for 30 min. An experiment that might better simulate the in vivo effects of inhibitors is shown in Figure 7. Factor VIII was mixed with plasma levels of α_2-macroglobulin and antithrombin III and the reaction begun by adding 0.14 unit/mL thrombin. Even in the presence of 1.8 mg of α_2-macroglobulin/mL (a 650-fold molar excess) and 0.26 mg of antithrombin III/mL (a 1050-fold molar excess), the rate and extent of factor VIII activation were about the same as in the absence of inhibitors. Addition of heparin to the antithrombin III reduced the effect of thrombin to that of buffer alone. When thrombin was preincubated at 37 °C

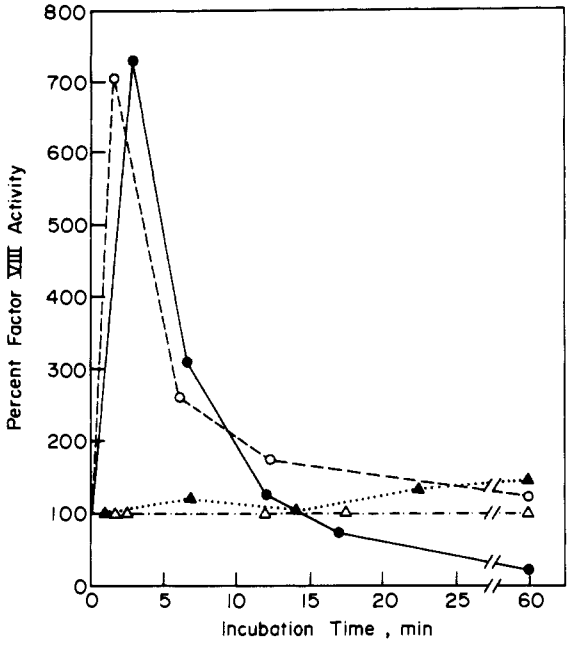

FIGURE 7: Activation of factor VIII by thrombin in the presence of plasma levels of α_2-macroglobulin and antithrombin III. Thrombin (0.14 μg) was added to 84 μg of factor VIII/von Willebrand glycoprotein in 1 mL of buffer with (○) and without (●) 1.8 mg of α_2-macroglobulin and 0.26 mg of antithrombin III. Each mixture was immediately assayed for factor VIII activating ability. As indicated, the mixture of thrombin and α_2-macroglobulin plus antithrombin III activated factor VIII as well as thrombin alone. In the presence of 0.02 unit of heparin/mL (▲), essentially all thrombin activity was abolished instantaneously. No activation was observed with a control solution of factor VIII/von Willebrand glycoprotein without thrombin or inhibitors (△).

with the two inhibitors, complete inhibition of the enzyme did not occur until approximately 20 min. This experiment suggests that even though thrombin is released in vivo into a medium rich in inhibitors, some slight level of thrombin activity may remain.

Rechromatography of the α_2-Macroglobulin–Thrombin Complex. Recent studies suggest that complexes of α_2-macroglobulin with certain proteinases are quite labile (Salvesen & Barrett, 1980). To gain a better understanding of the strength of binding between α_2-macroglobulin and thrombin and to determine whether the activity of the α_2-macroglobulin–thrombin complex might be due to residual thrombin or that which dissociates from the complex, rechromatography was performed in dilute neutral buffer. When the peak void volume fraction from Sephadex G-100 chromatography of α_2-macroglobulin and ^{125}I-labeled thrombin in a mole ratio of 1:11, was rechromatographed on the same column, the results shown in Figure 8 were obtained. Most of the radioactivity was recovered in the void volume with less than 2% eluting in the salt volume, well after the position for free thrombin and therefore likely due to a low molecular weight radioactive peptides or free Na^{125}I. Control experiments showed that ^{125}I-labeled thrombin alone eluted approximately halfway between the void volume and the salt volume; no radioactivity was ever detected in the void volume. Besides showing that α_2-macroglobulin–thrombin complexes withstand gel filtration in neutral buffers, these experiments provide additional evidence that the α_2-macroglobulin–thrombin complex rather than free thrombin is responsible for the increase in factor VIII procoagulant activity that we observed in the preceding experiments.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis of the α_2-Macroglobulin–Thrombin Complex. To test further the strength of α_2-macroglobulin–thrombin complexes and to analyze for the possibility of contaminating free thrombin, we used NaDodSO$_4$ gel electrophoresis to analyze the α_2-macroglobulin–^{125}I-labeled thrombin complexes that had been used to activate factor VIII. Figure 9 shows the radioactivity profile from the electrophoretic analysis of the α_2-macroglobulin–thrombin complex. Essentially all of the radioactivity coeluted with the band corresponding to the α_2-macroglobulin protein, suggesting that in our experiments the α_2-macroglobulin–thrombin complex, and not free thrombin, is responsible for the activation of factor VIII.

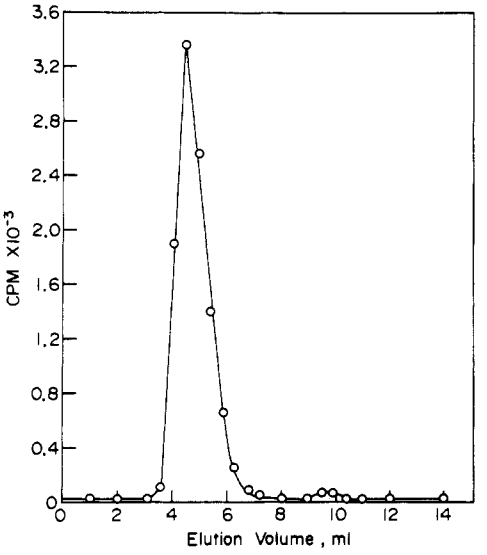

FIGURE 8: Rechromatography of the α_2-macroglobulin–thrombin complex on Sephadex G-100 in 0.05 M Tris–0.15 M NaCl. The complex was isolated initially by chromatography on the same column. Then the peak void volume fractions were made to 0.9 mg/mL in proteinase-free bovine serum albumin and rechromatographed on the same column to give the results shown here. In this experiment, >98% of the total radioactivity eluted in the void volume, and the remainder eluted well after the position of free thrombin, indicating thrombin did not dissociate from the complex in neutral buffers.

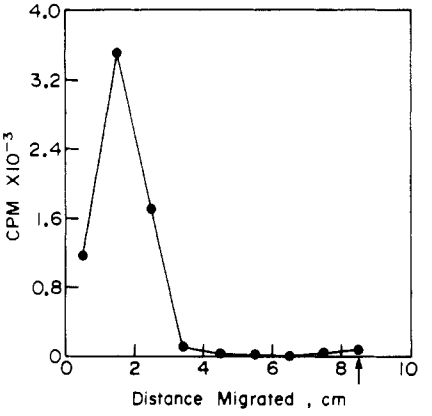

FIGURE 9: Radioactivity profile of the NaDodSO$_4$–polyacrylamide gel electrophoresis of the α_2-macroglobulin–^{125}I-labeled thrombin complex. Essentially all of the thrombin radioactivity was found at the top of the gel. The position of free thrombin is indicated by the upright arrow on the abscissa; small peptides would also migrate in this position.

Discussion

Our results expand the observations that α_2-macroglobulin–proteinase complexes retain low levels of proteolytic activity toward certain macromolecular substrates. Although it is uncertain how proteinases are inhibited by α_2-macroglobulin, data from several laboratories indicate that the active

site of the proteinase can still hydrolyze low molecular weight peptides and esters (Harpel, 1976; Harpel & Mosesson, 1973; Rinderknecht & Geokas, 1973; Bieth et al., 1981). As previously reported by others (Harpel & Mosesson, 1973; Rinderknecht & Geokas, 1973) even large protein substrates such as fibrinogen (M_r 330000) can be cleaved by certain α_2-macroglobulin–proteinase complexes. We began our studies by verifying that α_2-macroglobulin complexes of plasmin or thrombin do slowly cleave fibrinogen. In our experiments, the latter activities were equivalent to only about 1% and 4% of that for free plasmin or thrombin, respectively.

A mole ratios of 3:1 or higher, α_2-macroglobulin completely eliminated the effects of plasmin or trypsin on factor VIII procoagulant activity, despite the latter being extraordinarily sensitive to inactivation by either proteinase (Kirby et al., 1974; McKee et al., 1975). In contrast, when incubated with α_2-macroglobulin in a mole ratio of 3:1, thrombin retained essentially full activity toward factor VIII/von Willebrand protein. Even at α_2-macroglobulin:thrombin mole ratios of 24:1, for which the binding curve suggests all of the thrombin to be complexed, factor VIII procoagulant activity was still enhanced about half as well as with free thrombin. That all of the thrombin was complexed to α_2-macroglobulin at this mole ratio is supported by our finding that the chromatographically isolated α_2-macroglobulin–thrombin complex gave the same percentage of activation (45% of that produced by free thrombin).

In all of the above studies, α_2-macroglobulin was preincubated with thrombin for 30 min at 37 °C. However, a better simulation of in vivo events might be to assess the effects of thrombin immediately after its addition to factor VIII/von Willebrand glycoprotein when both major plasma inhibitors of thrombin, α_2-macroglobulin, and antithrombin III (Vogel et al., 1979), are present in their plasma concentrations of 1.8 and 0.26 mg/mL, respectively. When we performed this experiment, we found that despite 650–1050-fold molar excesses of the two inhibitors, thrombin was not inhibited very rapidly since factor VIII activity could still be stimulated about 7-fold over the next several minutes. With continuing exposure to both inhibitors, the ability of thrombin to cause any enhancement of the procoagulant activity of factor VIII was totally lost by 20 min. In the presence of heparin, however, no thrombin activity could be detected, presumably due to the accelerated rate of complex formation with antithrombin III (Vogel et al., 1979; Rosenberg & Damus, 1973).

A major concern is whether the limited proteolysis attributed to α_2-macroglobulin–proteinase complexes is actually due to slow dissociation of free proteinase. Several considerations make this unlikely. First, the activities of the α_2-macroglobulin–proteinase complexes were assayed in the presence of excess α_2-macroglobulin. With α_2-macroglobulin–trypsin and α_2-macroglobulin–plasmin complexes in 2:1 and 3:1 mole ratios, respectively, factor VIII procoagulant activity was unaffected. Our binding data show that at a 11:1 mole ratio of α_2-macroglobulin to thrombin, essentially all of the thrombin is tightly bound to α_2-macroglobulin. Second, even in the presence of antithrombin III, which inhibits free thrombin but not the α_2-macroglobulin–thrombin complex (Fischer et al., 1981), an α_2-macroglobulin–thrombin mixture activates factor VIII about half as well as thrombin without α_2-macroglobulin. Third, studies demonstrating the activity of α_2-macroglobulin–thrombin mixtures were repeated by using the chromatographically isolated complex, and these verified that the complex itself possesses activity toward the factor VIII/von Willebrand protein. Fourth, when the isolated α_2-macroglobulin–^{125}I-labeled thrombin complex was rechromatographed in 0.05 M Tris–0.15 M NaCl, there was no dissociation. Finally, $NaDodSO_4$–polyacrylamide gel electrophoresis of the same sample of the isolated α_2-macroglobulin–thrombin complex used for the factor VIII activation studies in Figure 6 showed that essentially all of the thrombin remained complexed despite the detergent effects of $NaDodSO_4$. This experiment strongly suggests that thrombin is not likely to be dissociating from the complex under the conditions of our experiments and that the α_2-macroglobulin–thrombin complex itself can activate factor VIII. Although the evidence outlined above is consistent with thrombin and α_2-macroglobulin being covalently bonded, this is not a necessary condition for inhibition. For example, the experiments of Salvesen and Barrett (1980) indicate that α_2-macroglobulin–trypsin complexes are only 61% covalent and α_2-macroglobulin–plasmin complexes only 54% covalent. Yet the fact that the α_2-macroglobulin complexes of plasmin and trypsin have no activity toward factor VIII (Figures 1 and 2) shows that these proteinases are completely inhibited, at least toward the factor VIII substrate, even when noncovalently bonded to α_2-macroglobulin. Of note, the complex between α_2-macroglobulin and thrombin may be stronger than that between α_2-macroglobulin and trypsin or plasmin, since >99% appears tightly bound to the α_2-macroglobulin by $NaDodSO_4$ electrophoresis (Figure 9); even when boiled with $NaDodSO_4$ under reducing conditions, >92% of the thrombin remained bound.

Our results have several potential implications for the regulation of proteinase activity in plasma. First, some α_2-macroglobulin–proteinase complexes might retain very specific proteolytic activity toward certain substrates, such as the factor VIII/von Willebrand glycoprotein. Second, proteins that are strictly substrates for the free proteinase might not be degraded by the α_2-macroglobulin–proteinase complex. Third, the proteinase complexed to α_2-macroglobulin would be protected from other circulating inhibitors. Such mechanisms may be especially important in the control and regulation of thrombin activity. For example, recent data show that fibrinopeptides A and B are present in the plasma of normal individuals (Eckhardt et al., 1981). Since the fibrinopeptides are rapidly cleared from plasma (Eckhardt et al., 1981; Nossel et al., 1974), it follows that they must be generated continuously, presumably by low levels of thrombin in the circulation. The experiments of Vogel et al. (1979) indicate that about 79% of this thrombin becomes complexed to antithrombin III and inactivated, but the remaining 21% should become complexed to α_2-macroglobulin. More recently, studies performed by Fischer et al. (1981) show that the α_2-macroglobulin–thrombin complex does not react with antithrombin III. Since our data indicate that the α_2-macroglobulin–thrombin complex remains active toward factor VIII, we propose that such complexes may actually provide the amount of thrombin activity that many believe requisite for the in vivo generation and regulation of factor VIII procoagulant activity (Rapaport et al., 1963; Hultin & Nemerson, 1978; Switzer et al., 1979; van Dieijen et al., 1981). Additional studies will be required to determine whether α_2-macroglobulin–proteinase complexes are indeed important in physiologic and pathophysiologic phenomena.

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