Inhibition of Prothrombinase Complex by Plasma Proteinase Inhibitors†

Vincent Ellis, Michael F. Scully,* and Vijay V. Kakkar

ABSTRACT: The rate of inactivation of human coagulation factor Xa by the plasma proteinase inhibitors antithrombin III and α_1-antitrypsin has been studied in the presence of the accessory components which constitute the prothrombinase complex. The rate of inactivation of factor Xa by antithrombin III was found to be decreased in the presence of phospholipid vesicles with high affinity for factor Xa. The second-order rate constant for the reaction fell from 6.21×10^4 to 3.40×10^4 M^{-1} min^{-1} in the presence of 20 μM phospholipid. Purified factor Va had no effect on the rate of inactivation of factor Xa in the absence of phospholipid. In the presence of phospholipid, factor Va increased the protective effect displayed by phospholipid, further reducing the rate constant to 2.20×10^4 M^{-1} min^{-1}. The rate of inactivation of factor Xa by α_1-antitrypsin was unaffected under these conditions. Platelet-bound prothrombinase complex was formed by incubation of factor Xa with washed human platelets activated by a mixture of collagen and thrombin. The prothrombinase activity was inhibited by antithrombin III with a second-order rate constant of 0.85×10^4 M^{-1} min^{-1}. This rate was obtained in both the presence and absence of exogenous factor Va. Platelet factor 3 vesicles, isolated from platelet aggregation supernatants, also formed prothrombinase complex in the presence of factor Va, and this was inhibited by antithrombin III at the same rate as the platelet-bound complex. There was no protection of the platelet-bound prothrombinase complex from inhibition by α_1-antitrypsin.

Factor Xa is the coagulation enzyme responsible for the generation of thrombin by the limited proteolysis of its zymogen prothrombin (Davie & Ratnoff, 1964; MacFarlane, 1964). The rate of prothrombin activation by factor Xa is very low but is greatly accelerated when factor Xa is bound to a negatively charged phospholipid surface in the presence of factor Va and calcium ions (Jobin & Esnouf, 1967; Esmon et al., 1974; Miletich et al., 1978; Nesheim et al., 1979) to form the prothrombinase complex. Phospholipid and factor Va, when present individually, each accelerate the rate of thrombin generation. Phospholipid has been shown to do this by lowering the K_m for prothrombin (Rosing et al., 1980), while factor Va increases the V_{max} of prothrombin activation (Nesheim et al., 1979b; Rosing et al., 1980). The rate of thrombin generation by the prothrombinase complex is up to 300 000-fold faster than with free factor Xa (Nesheim et al., 1979b; Rosing et al., 1980; Miletich et al., 1978). Due to this great difference in rates, the activation of prothrombin by free factor Xa is thought to play a very minor role in vivo.

The prothrombinase complex in vivo is thought to be assembled on the surface of platelets (Miletich et al., 1978; Dahlback & Stenflo, 1978; Tracy et al., 1979). The prothrombinase complex components, factor Xa and factor Va, have been shown to bind to the surface of unstimulated platelets, in 1:1 stoichiometry, with approximately 900 sites per platelet (Tracy et al., 1981). Platelets also provide a source of factor V, in addition to the plasma factor V, which is released from α-granules upon platelet activation (Osterud et al., 1977). Thrombin, collagen, and calcium ionophore A23187 have been shown to release platelet factor V (Osterud et al., 1977; Dahlback & Stenflo, 1978; Miletich et al., 1977). It has been suggested that platelet factor V is released in a form which does not require further proteolytic modification for activity (Osterud et al., 1977; Miletich et al., 1977). However, Kane et al. (1980) showed that thrombin activation of the platelet factor V was necessary for factor Xa binding. This has been confirmed by Bevers et al. (1982a), who also showed that an increase in prothrombinase activity[1] occurred when platelets were stimulated by a mixture of collagen and thrombin. This increased prothrombinase activity was correlated with an exposure of phosphatidylserine on the platelet surface (Bevers et al., 1982b). Sandberg et al. (1982) have reported the isolation of vesicles, released from platelets after activation, which can act as sites for the formation of the prothrombinase complex.

We have previously reported the inactivation of uncomplexed human factor Xa by the plasma proteinase inhibitors antithrombin III, α_1-antitrypsin, and α_2-macroglobulin (Ellis et al., 1982, 1983) and have shown α_1-antitrypsin to be the major inhibitor of factor Xa under these conditions. There have been various reports that the inhibition of bovine factor Xa is reduced in the presence of phospholipid and factor Va (Marciniak, 1973), phospholipid alone (Yin, 1974), or both (Walker & Esmon, 1979). However, Marciniak (1972) has shown that there are differences in the inhibition of human and bovine factor Xa in plasma, and no reports have been made on the effect of α_1-antitrypsin. The work presented here studies the effect of the components of the prothrombinase complex on the inhibition of human factor Xa by antithrombin III and α_1-antitrypsin and also studies the effect of these inhibitors on platelet-bound prothrombinase complex.

Materials and Methods

Materials. Phosphatidylserine (bovine brain) (PS),[2] phosphatidylcholine (dioleoyl), soybean trypsin inhibitor, *p*-nitrophenyl *p*-guanidinobenzoate, Russell's viper venom, and bovine serum albumin were purchased from Sigma. *N*-

† From the Thrombosis Research Unit, King's College School of Medicine and Dentistry, London SE5 8RX, U.K. *Received February 1, 1984.*

[1] Prothrombinase activity is defined in this paper as the procoagulant activity expressed in the presence of factors Xa and Va.

[2] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; RVV-X, factor X activator purified from Russell's viper venom; *p*-NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PF3, platelet factor 3; PEG 6000, poly(ethylene glycol) (average M_r 6000–7500); S-2222, *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine-*p*-nitroanilide dihydrochloride; S-2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride.

0006-2960/84/0423-5882$01.50/0

Benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine-*p*-nitroanilide dihydrochloride (S-2222) and H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride (S-2238) were purchased from Kabi Diagnostica, Stockholm, Sweden. Sepharose CL-4B and Sephadex G-50 were products of Pharmacia. Collagen was obtained from Horm Chemical Co., Munich, West Germany. Prostacyclin was obtained from Upjohn Co., Kalamazoo, MI. All reagents used were of the highest grade commercially obtainable.

Proteins. Human factor X, human antithrombin III, and human α_1-antitrypsin were prepared as previously described (Ellis et al., 1982, 1983). These proteins were quantitated by using $E^{1\%}_{280nm}$ values of 11.6 (DiScipio et al., 1977), 6.1 (Jesty et al., 1974), and 4.84 (Bloom & Hunter, 1978), respectively. Factor Xa was prepared by RVV-X activation of factor X (Ellis et al., 1982) and quantified by active-site titration with *p*-NPGB (Smith, 1973).

Human prothrombin was purified as described for factor X, with prothrombin being separated by chromatography on sulfated Sephadex (Miletich et al., 1980). Prothrombin was assayed by the hydrolysis of S-2238 after activation with *Echis carinatus* venom and its concentration determined by using an $E^{1\%}_{280nm}$ value of 13.8 (Kisiel & Hanahan, 1973).

Bovine factor V was purified from freshly collected bovine blood by the method of Esmon (1979), as modified by Lindhout et al. (1982), and its concentration determined by using an $E^{1\%}_{280nm}$ value of 9.6 (Nesheim et al., 1979a). Bovine factor Va was obtained by incubating 0.3 mg mL^{-1} factor V with 0.2 μg mL^{-1} human α-thrombin in 0.05 M Tris-HCl, 0.1 M NaCl, and 3 mM $CaCl_2$, pH 7.4 at 37 °C, for 20 min. Factor Va was assayed by the method of Lindhout et al. (1982) and used within 8 h of preparation.

Human α-thrombin was a gift of Dr. J. W. Fenton, New York State Department of Health, Albany, NY.

SDS–polyacrylamide gel electrophoresis (Weber & Osborn, 1969) was used to determine the homogeneity of all the protein preparations.

Phospholipid Vesicle Preparation. Phospholipid vesicles were prepared by sonication of mixtures of dioleoylphosphatidylcholine and bovine brain phosphatidylserine in 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.4 at 0 °C, by the method of De Kruijff et al. (1975).

Measurement of Protein–Phospholipid Binding. The binding of factor X and factor Xa to phospholipid vesicles was measured as an increase in relative molecular weight as determined by relative 90° light scattering intensities, essentially as described by Nelsestuen & Lim (1977). Measurements were made in an Aminco-Bowman SP-4 spectrofluorometer, with excitation and emission monochromators set at 320 nm. The instrument was zeroed with buffer in the cuvette. A fixed concentration of the varying mole percent of phosphatidylserine vesicles was added and the light scattering measured. The phospholipid was then titrated with the protein, and light scattering measurements were made after the attainment of equilibrium at each protein concentration. Prior to use, all solutions were centrifuged for 15 min at 10000*g* to eliminate contamination by dust particles. The concentration of free and bound protein was estimated from comparison of the observed molecular weight to the theoretical molecular weight if all the protein were bound. Dissociation constants were calculated from double-reciprocal plots of free vs. bound protein, which also gave the concentration of protein binding sites.

Platelet Preparation. Blood from healthy volunteers, who had been without medication for 2 weeks, was collected into acid–citrate–dextrose, and washed platelets were prepared by the albumin density gradient separation method of Walsh (1972). Platelets were also prepared by collection and washing in prostacyclin-containing buffers, as described by Radomski & Moncada (1983). Platelet counts were performed with a Coulter counter. Washed platelet preparations were maintained at room temperature and used within 8 h of preparation.

Platelets were activated by addition of 0.2 NIH unit mL^{-1} α-thrombin, 10 μg mL^{-1} collagen, and 3 mM $CaCl_2$ at 37 °C for 10 min, either with or without stirring, at a platelet concentration of 3×10^8 mL^{-1}.

PF3 vesicles were prepared from the supernatant of thrombin–collagen aggregated platelets by centrifugation at 3000*g* for 20 min (Sandberg et al., 1982).

Determination of Inhibition Rate. The prothrombinase inhibition assays were performed by incubating factor Xa with antithrombin III (0.5–5 μM) or α_1-antitrypsin (0.8–3.2 μM) in the presence of either phospholipid vesicles, factor Va, or platelets, or a combination of these, in 0.5 M Tris-HCl, 0.1 M NaCl, 5 mM $CaCl_2$, and 0.1% PEG 6000, pH 7.4 at 37 °C. The proteinase inhibitor was added after the equilibration of the prothrombinase components (generally less than 5 min as judged by assay). At timed intervals after the addition of the proteinase inhibitor, 10-μL aliquots were removed into 5 μL of prothrombin (9 μM). After 1-min incubation at 37 °C, the thrombin generated was determined by the addition of 200 μL of 0.4 mM S-2238 and 20 mg mL^{-1} soybean trypsin inhibitor in 0.05 M Tris-HCl, 0.1 M NaCl, 10 mM $CaCl_2$, and 0.1% PEG 6000, pH 7.4, and the initial change in absorbance was measured by using an Abbott bichromatic analyzer fitted with a 380/450-nm filter. Rates of thrombin generation were calculated by comparison with calibrations made by using active-site-titrated human α-thrombin.

The first-order plots obtained were linear and extrapolated to within 10% of the activity present prior to addition of proteinase inhibitor; this had no significant effect on the apparent first-order rate of inhibition calculated from the data. Second-order rate constants were calculated by the method of Kitz & Wilson (1962).

Results

Binding of Factor Xa to Phospholipid Vesicles. The affinity of factor Xa for phospholipids of varying compositions was measured in the presence of calcium ions by 90° light scattering. Figure 1 shows the dissociation constant for the factor Xa–phospholipid interaction plotted against increasing mole percent of PS, from 5 to 50 mol %. It can be seen that the affinity of factor Xa for the vesicles increases with increasing percentage of PS. The maximum affinity is given when the vesicles are composed of 40% PS and 60% PC. With this composition, the dissociation constant is 0.035 μM, and the concentration of factor Xa binding sites is 0.80 μM per 100 μM phospholipid. When the PS concentration was increased above 40%, the measurements became variable, presumably due to calcium ion induced vesicle aggregation. Using this methodology, we observed no binding of factor Xa to vesicles of less than 10% PS. The binding of factor X to phospholipid vesicles was studied for comparison. A similar dependence on the mole percent of PS was observed, but factor X had a lower affinity for the vesicles throughout the range of phospholipid compositions. The minimum dissociation constant observed was 0.16 μM, which is approximately 5 times higher than that for factor Xa–phospholipid binding.

Vesicles composed of 40% PS and 60% PC were used throughout the following studies, due to their high affinity for factor Xa.

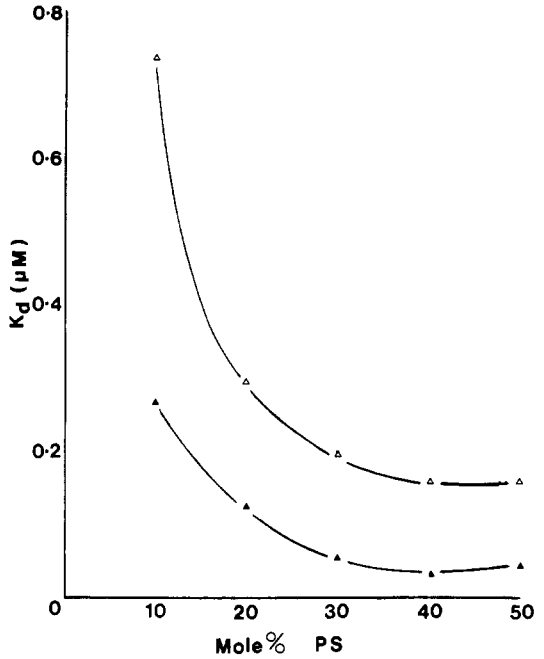

FIGURE 1: Dissociation constant for factor X (△) and factor Xa (▲) binding to phospholipid plotted agginst mole percent of phosphatidylserine. Fixed concentrations of vesicles were titrated with protein in 0.05 M Tris, 0.1 M NaCl, and 5 mM $CaCl_2$, pH 7.4 at 25 °C. The scattered light intensity of the protein–phospholipid complex was measured relative to the scattering intensity of the vesicles alone. Comparison of the relative molecular weights gives the protein bound to the vesicles. K_d was calculated from [free protein]$^{-1}$ vs. [bound protein]$^{-1}$.

Effect of Phospholipid on Factor Xa Inhibition. We have previously determined the rates of inhibition of free factor Xa by antithrombin III and α_1-antitrypsin, measuring the residual factor Xa activity with factor Xa specific chromogenic peptide substrates (Ellis et al., 1982, 1983). The assay used in these present experiments measures residual factor Xa activity by prothrombin activation, and in the presence of phospholipid essentially measures only phospholipid-bound factor Xa. Using this assay with 0.30 μM factor Xa, it was possible to measure the rates of inhibition of free factor Xa in the presence of 5 mM Ca^{2+}. The rate of inhibition of free factor Xa by antithrombin III was found to be 6.21×10^4 M^{-1} min^{-1}, and the rate of inhibition by α_1-antitrypsin was 1.57×10^4 M^{-1} min^{-1}. These compare favorably with the rates of 6.33×10^4 and 1.50×10^4 M^{-1} min^{-1} determined previously with the S-2222 assay (Ellis et al., 1982).

When vesicles containing 40% PS were incubated with 0.30 μM factor Xa, the rate of inhibition by antithrombin III was found to be decreased (Figure 2). This effect was maximal at a phospholipid concentration of 20 μM, which caused the second-order rate constant to fall to 3.40×10^4 M^{-1} min^{-1}. At higher phospholipid concentrations, a slight increase in the rate constant was observed, with a value of 4.05×10^4 M^{-1} min^{-1} at 80 μM phospholipid.

To ensure that the inhibition rate of 3.40×10^4 M^{-1} measured at 20 μM phospholipid is actually the rate of inhibition of bound factor Xa, the rate was also measured with the S-2222 assay. This gave a second-order rate constant of 4.90×10^4 M^{-1} min^{-1} at 20 μM phospholipid, which represents the combined rates of inhibition of free and bound factor Xa. The amount of factor Xa bound to the phospholipid was calculated from the dissociation constant and concentration of binding sites for factor Xa–phospholipid binding and under these conditions was found to be 0.14 μM or 46% of the total factor Xa. This gives a second-order rate constant for the inhibition

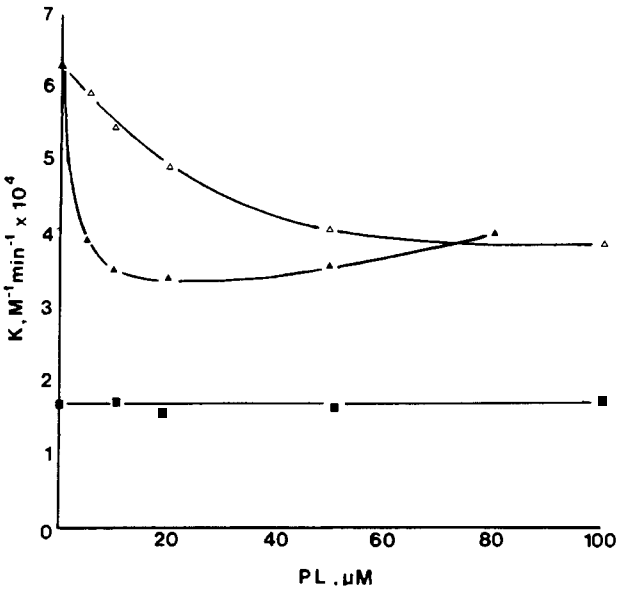

FIGURE 2: Rate of inhibition of factor Xa by antithrombin III and α_1-antitrypsin in the presence of varying concentrations of 40/60 PS/PC vesicles. The second-order rate constant was calculated from residual factor Xa activity measured by prothrombin activation (closed symbols) or S-2222 hydrolysis (open symbols): (△, ▲) antithrombin III; (■) α_1-antitrypsin.

of the bound factor Xa by antithrombin III of 3.28×10^4 M^{-1} min^{-1}, in agreement with the rate determined by using the prothrombin activation assay. At high phospholipid concentrations when virtually all the factor Xa is bound, the same rate of inhibition is obtained with either assay (Figure 2).

When α_1-antitrypsin was used as the inhibitor, phospholipid concentrations up to 100 μM had no effect on the rate of inhibition of factor Xa when determined by the prothrombin activation assay (Figure 2).

Effect of Factor Va on Factor Xa Inhibition. Factor Va had no effect on the rate of inhibition of factor Xa by either antithrombin III or α_1-antitrypsin, up to a concentration of 0.8 μM. Using the factor Xa–Va binding data of Lindhout et al. (1982), it can be calculated that essentially all of the factor Xa will be bound at this concentration of factor Va.

Effect of Factor Va and Phospholipid on Factor Xa Inhibition. When factor Xa is incubated with factor Va in the presence of phospholipid, the complete prothrombinase complex is formed. In the presence of 3.6 nM factor Xa and 9.6 nM factor Va, increasing the phospholipid concentration from 0 to 30 μM resulted in a decrease in the rate constant for the factor Xa–antithrombin III reaction from 6.21×10^4 to 2.20×10^4 M^{-1} min^{-1} (Figure 3). Increasing the phospholipid concentration above 30 μM resulted in a slight increase in the rate constant to 3.25×10^4 M^{-1} min^{-1} at 100 μM, a similar effect to that observed in the absence of factor Xa (although much lower factor Xa concentrations are employed in these experiments). When the factor Va concentration was increased up to 96 nM at a fixed phospholipid concentration (30 μM), no effect was observed on the inhibition reaction. Factor Va therefore appears to enhance the protective effect of phospholipid on the inhibition of factor Xa by antithrombin III.

The reaction between α_1-antitrypsin and factor Xa was found to be unaffected by the incorporation of factor Xa into the prothrombinase complex and proceeds with the same rate constant of 1.6×10^4 M^{-1} min^{-1} at all concentrations of phospholipid and factor Va used.

Prothrombinase Activity of Platelets. The prothrombinase activity of platelets was studied by incubating albumin density

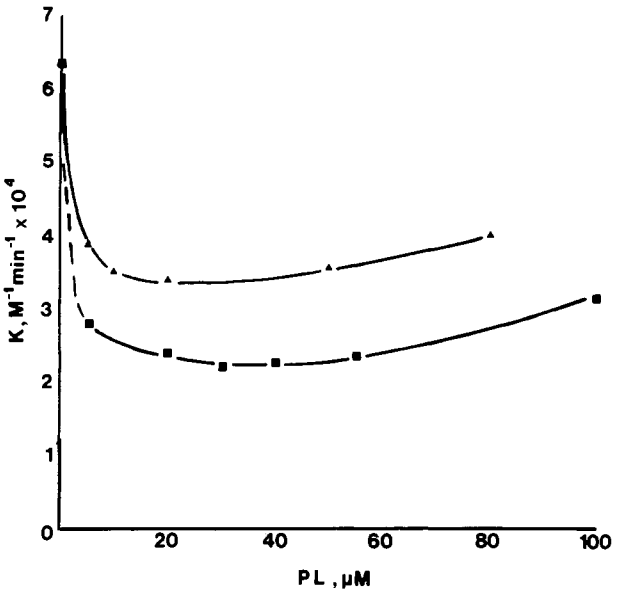

FIGURE 3: Rate of inhibition of prothrombinase complex by antithrombin III. The second-order rate constant for the inhibition of factor Xa is plotted against phospholipid concentration in the presence (■) and absence (▲) of factor Va.

Table I: Effect of Platelet Activation on the Rate of Thrombin Generation[a]

agonist	thrombin generation rate [nM min⁻¹ (10⁸ platelets)⁻¹] −Va	+Va
none	5	96
thrombin	112	534
collagen	57	1347
thrombin and collagen	590	3042

[a] Prostacyclin-washed platelets were activated for 10 min with 0.2 NIH unit/mL human α-thrombin or 10 μg/mL collagen, or a mixture of both, in the presence of 3 mM $CaCl_2$. Measurements of thrombin generation after incubation with factor Xa were made in the presence and absence of exogenous factor Va.

gradient separation washed platelets at concentrations between 3×10^6 and 3×10^7 mL^{-1} with 3.6 nM factor Xa in the presence or absence of 9.6 nM factor Va. In the absence of factor Va, these unstimulated platelets generated thrombin at a rate of 10 nM min^{-1} $(10^8$ platelets$)^{-1}$. When factor Va was present, the rate of thrombin generation was found to be 363 nM min^{-1} $(10^8$ platelets$)^{-1}$. Platelets which had been activated by a mixture of thrombin and collagen had much greater prothrombinase activity, generating thrombin at a rate of 250 nM min^{-1} $(10^8$ platelets$)^{-1}$ in the absence of factor Va and 2050 nM min^{-1} $(10^8$ platelets$)^{-1}$ in the presence of factor Va, confirming the findings of Bevers et al. (1982a). Increasing the concentrations of factor Xa and factor Va had no effect on the rate of thrombin generation, showing that all the platelet sites had been saturated. These experiments were repeated by using prostacyclin-washed platelets, and the thrombin generation rate of the unstimulated platelets was found to be lower than 5 nM min^{-1} $(10^8$ platelets$)^{-1}$ in the absence of factor Va. The rates obtained after thrombin–collagen stimulation were slightly higher. It appears that platelets washed by this method are less affected by the washing procedures, and therefore, these platelets were used in subsequent experiments. The rates of thrombin generation of these platelets after stimulation by thrombin and collagen, individually, were also determined and found to be lower than when the agonists were present together (Table I). It was found that the same rates of thrombin generation were given whether the platelets were activated with or without stirring. All subsequent experiments were performed with prostacyclin-washed platelets, activated with thrombin and collagen without stirring.

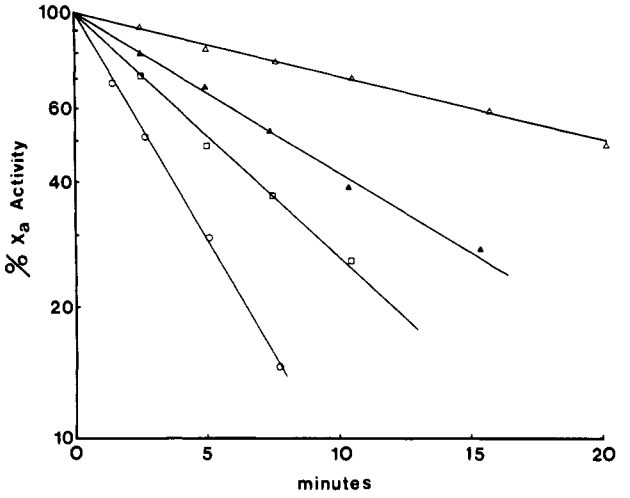

FIGURE 4: Comparison of the inhibition of platelet-bound prothrombinase complex (△), phospholipid-bound prothrombinase complex (▲), phospholipid-bound factor Xa (□), and free factor Xa (○) by antithrombin III. The apparent first-order rate of inhibition is plotted at an antithrombin III concentration of 4.0 μM, which is close to the physiological concentration found in plasma.

Effect of Inhibitors on Platelet-Bound Prothrombinase Complex. Platelets activated with collagen plus thrombin were incubated at a concentration of 5×10^6 mL^{-1} with 3.6 nM factor Xa for 2 min prior to the addition of the proteinase inhibitors. The second-order rate constant obtained for the inhibition of platelet-bound factor Xa by antithrombin III was 0.85×10^4 M^{-1} min^{-1}. This value is 2.6-fold lower than that obtained for the prothrombinase complex assembled on synthetic vesicles with bovine factor Va and 7.4-fold lower than the value for free factor Xa (Figure 4). When bovine factor Va was added to the incubations, the same rate of inhibition of prothrombinase activity was observed, showing that bovine plasma factor Va is indistinguishable from endogenous human platelet factor Va in these experiments. The second-order rate constant obtained for the inhibition of platelet-bound factor Xa by α_1-antitrypsin was 1.52×10^4 M^{-1} min^{-1}, which is virtually identical with the rate obtained for free factor Xa.

PF3 vesicles, prepared by gel filtration of the supernatant of collagen plus thrombin-aggregated platelets, were found to account for 12–33% of the prothrombinase activity of platelet suspensions in the presence of exogenous factor Va. In the presence of PF3 vesicles and factor Va, factor Xa was inhibited at a rate of 0.85×10^4 M^{-1} min^{-1}, which is identical with the rate obtained by using platelet suspensions.

Discussion

The results presented here show that human factor Xa is protected from inactivation by antithrombin III when bound to phospholipid vesicles. The binding of factor Xa to phospholipid vesicles is accompanied by a 44% decrease in the rate of inhibition by antithrombin II, from 6.21×10^4 to 3.40×10^4 M^{-1} min^{-1}. This is in contrast to the results of Marciniak (1972), who observed that bovine factor Xa was protected by phospholipid only in the presence of factor Va and concluded that the protection was dependent on the Xa–Va association. Walker & Esmon (1979) observed a 9-fold decrease in the rate of antithrombin III inhibition of bovine factor Xa using an amidolytic assay and demonstrated this to be due to a reduced

affinity between the inhibitor and the enzyme. However, Marciniak & Tsukamura (1972) have observed that the inhibition of bovine factor Xa and human factor Xa in plasma is markedly different. We have obtained a rate constant of 4.73×10^5 M^{-1} min^{-1} for the inhibition of bovine factor Xa by human antithrombin III (unpublished experiments), which is 7.6-fold higher than with the human enzyme, confirming these earlier observations.

At phospholipid concentrations above 20 μM, at which the second-order rate constant for inhibition by antithrombin III is 3.40×10^4 M^{-1} min^{-1}, a slight increase in the rate of inhibition is observed. At these higher concentrations of phospholipid, factor Xa is less densely packed on the vesicle surface. From the data obtained in the factor Xa–phospholipid binding experiments, it can be calculated that at 20 μM phospholipid 85% of the available sites are occupied, whereas at 100 μM phospholipid only 35% are occupied. This suggests that the protective effect displayed by phospholipid on the factor Xa–antithrombin III reaction is due to steric hindrance of antithrombin III binding, which is increased when the enzyme is densely packed on the vesicle surface.

Factor Va, in the absence of phospholipid, was found to have no effect on the inhibition of factor Xa. As factor Va is primarily responsible for the increased enzymatic activity of factor Xa in the prothrombinase complex, by increasing the V_{max} for prothrombin activation (Nesheim et al., 1979b), it appears that this increased activity has no effect on the rate of the reaction with antithrombin III. In the presence of phospholipid, the protective effect is enhanced by factor Va, with the rate constant falling to 2.20×10^4 M^{-1} min^{-1}. This is the rate of inhibition of the complete prothrombinase complex and represents a 65% decrease from the rate of inhibition of free factor Xa. The increased protection of factor Xa by the prothrombinase complex is not due to the increased binding of factor Xa to phospholipid in the presence of factor Va, as the assay employed does not measure the inhibition of free factor Xa and is therefore unaffected by the amount of factor Xa bound. The enhanced protective effect from antithrombin III inhibition is unlikely to be due to the altered proteolytic activity of the prothrombinase complex, as this would be expected to be displayed by factor Va alone (factor Va increases the V_{max} for prothrombin activation). It is therefore probable that the effect is due to factor Va altering the binding of factor Xa on the phospholipid surface in such a way that the steric hindrance to antithrombin III binding is increased, with the large bulk of the factor Va molecule perhaps contributing to this effect.

The rate of reaction between α_1-antitrypsin and factor Xa is unaffected by the incorporation of factor Xa into the prothrombinase complex. This observation was unexpected as α_1-antitrypsin and antithrombin III are proteinase inhibitors of similar molecular size and have extensive sequence homology (Hunt & Dayhoff, 1982). The inhibitors share a common inhibitory mechanism (Travis & Salvesen, 1983), which can be written

$$\text{Xa} + \text{I} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Xa–I} \xrightarrow{k_3} \text{Xa–I}^*$$

where Xa–I represents the reversible factor Xa–inhibitor complex and Xa–I* represents the covalently or tetrahedrally stabilized complex. In this scheme, the stabilized complex is essentially irreversible. For the thrombin–antithrombin III reaction, the equilibrium constant for the formation of the reversible complex (K_I) has been shown to be 1.4×10^{-3} M (Olson & Shore, 1982). This relatively weak association is followed by the formation of the stable complex with a rate constant (k_3) of 600 min^{-1}. The reaction between factor Xa and antithrombin III is thought to follow a similar kinetic mechanism, a theory which is supported by the similarity of the action of heparin on the antithrombin III inhibition of both factor Xa and thrombin. Heparin has been shown to exert its action on the thrombin–antithrombin III reaction by lowering the K_I to 4.0×10^{-6} M (Olson & Shore, 1982), and a comparable reduction must be expected with factor Xa. Antithrombin III has as its reactive site arginine-393, which corresponds to the primary specificity of factor Xa and thrombin. The reactive site residue in α_1-antitrypsin, however, is not an arginine residue, but methionine-368, highlighting the major role of α_1-antitrypsin as an inhibitor of elastase, an enzyme which has this specificity. The rate of stable complex formation between factor Xa and α_1-antitrypsin might therefore be expected to be lower than that with antithrombin III. Measurement of these kinetic parameters is hampered by the high concentrations of inhibitor which have to be employed, but extrapolations of data obtained at lower α_1-antitrypsin concentrations (up to 10 μM) give a k_3 of 0.25 min^{-1} and a K_I of 2×10^{-5} M for the reaction with factor Xa (unpublished experiments). Pixley & Danishefsky (1983), however, have observed no saturation of the thrombin–α_1-antitrypsin reaction at inhibitor concentrations approaching 100 μM. This is perhaps surprising in view of the 4000-fold increase in the thrombin–α_1-antitrypsin reaction rate observed by Owen et al. (1983) for an α_1-antitrypsin molecule with a methionine-358 → arginine mutation.

It is possible that this stronger association between factor Xa and α_1-antitrypsin is responsible for the difference in effect between α_1-antitrypsin and antithrombin III inhibition of phospholipid-bound factor Xa. We have proposed that the reduced rate of reaction between phospholipid-bound factor Xa and antithrombin III is due to steric hindrance of the relatively weak binding between the inhibitor and the enzyme, therefore giving an increased K_I. The affinity of α_1-antitrypsin for factor Xa is higher than that of antithrombin III, and it appears that due to this increased affinity there is no inhibition of α_1-antitrypsin binding on assembly of the prothrombinase complex.

We have previously calculated that α_1-antitrypsin is the major plasma inhibitor of factor Xa using the experimentally determined second-order rate constants and the plasma concentrations of the inhibitors (Ellis et al., 1982), and this was confirmed by using dilute plasma in place of the purified inhibitors (Scully et al., 1983). These experiments were carried out at α_1-antitrypsin concentrations of less than 4 μM, which is only 10% of the plasma concentration. If the K_I for the factor Xa–α_1-antitrypsin reaction is 2×10^{-5} M, then this reaction will have reached saturation at plasma concentrations of α_1-antitrypsin and the rate will be less than that predicted by the second-order rate constant. Therefore, in plasma α_1-antitrypsin will only account for approximately 28% of the total inhibitory rate rather than the 60% previously calculated. This possibly explains why thrombotic tendency is not observed in α_1-antitrypsin-deficient individuals. This observation highlights the possible dangers of extrapolating second-order rate constants to pseudo-first-order rates in plasma without first demonstrating that saturation kinetics do not prevail at plasma concentrations of the inhibitor, especially for α_1-antitrypsin which is present at a high molar concentration.

The prothrombinase activity of platelets has been shown by Bevers et al. (1982a) to be dependent on the agonist used for platelet activation. The results obtained here confirm these findings, with a mixture of collagen and thrombin giving a

maximal thrombin generation rate of 3042 nM min^{-1} $(10^8$ platelets$)^{-1}$. This activity was found to be inhibited by antithrombin III with a second-order rate constant of 0.85×10^4 M^{-1} min^{-1}. This rate is 62% lower than that obtained with the phospholipid vesicle bound prothrombinase complex, and 86% lower than that obtained with free factor Xa.

It seems from these results that after activation the platelet provides an environment which makes factor Xa less susceptible to inhibition by antithrombin III, suggesting that under these conditions a further constraint is imposed upon the factor Xa molecule. As the increase in prothrombinase activity upon platelet activation is due to exposure of PS (Bevers et al., 1982a), it is possible that a particular phospholipid composition defines this further constraint, as recently suggested for prothrombin activation (Van Rijn et al., 1983). Alternatively, the factor Xa–Va binding site on the platelet surface may involve not only negatively charged phospholipids but also other protein components as postulated by Miletich et al. (1979).

PF3 was purified from the supernatant of thrombin–collagen-aggregated platelets and found to account for between 12% and 33% of the prothrombinase activity of the platelet suspension. Sandberg et al. (1982) have shown that these PF3 vesicles are genuine platelet-release products, not artifacts due to platelet lysis, and consist of closely similar membranous particles with a discreet size distribution. The vesicles have a well-defined phospholipid composition and contain 40% protein. The rate of inhibition of PF3-bound prothrombinase complex of antithrombin III was found to be identical with the rate of inhibition obtained in platelet suspensions, and therefore, the inhibition of the prothrombinase complex on the platelet surface and on released PF3 vesicles is identical.

Data presented by Miletich et al. (1978) have suggested that platelet-bound factor Xa is completely protected from inactivation by the heparin–antithrombin III complex, and Teitel & Rosenberg (1983) have presented similar results for factor Xa in whole blood. The results obtained here would appear to be in disagreement with these findings, and preliminary results from experiments performed with heparin–antithrombin III complex and platelet-bound prothrombinase would appear to support this (M. F. Scully, V. Ellis, and V. V. Kakkar, unpublished experiments).

Acknowledgments

We are grateful to Professor H. C. Hemker and Dr. M. J. Lindhout, Rijksuniversiteit Limburg, Maastricht, The Netherlands, for providing the facilities for the purification of bovine factor V.

Registry No. Dioleoyl-PC, 10015-85-7; blood coagulation factor X, 9001-29-0; blood coagulation factor Xa, 9002-05-5; antithrombin, 9000-94-6; α_1-antitrypsin, 9041-92-3; blood coagulation factor Va, 65522-14-7; thrombin, 9002-04-4; blood platelet factor 3, 37270-93-2; prothrombinase, 72162-96-0.

References

Bevers, E. M., Comfurius, P., Van Rijn, J. L. M. L., Hemker, H. C., & Zwaal, R. F. A. (1982a) *Eur. J. Biochem. 122*, 429–436.

Bevers, E. M., Comfurius, P., & Zwaal, R. F. A. (1982b) *Eur. J. Biochem. 122*, 81–85.

Bloom, J. W., & Hunter, M. J. (1978) *J. Biol. Chem. 253*, 547–559.

Chase, T. C. & Shaw, E. (1969) *Biochemistry 8*, 2212–2224.

Dahlback, B., & Stenflo, J. (1978) *Biochemistry 17*, 4938–4945.

De Kruijff, B., Cullis, P. R., & Radda, G. K. (1975) *Biochim. Biophys. Acta 406*, 6–20.

Di Scipio, R. G., Hermodson, M. A., Yates, S. G., & Davie, E. W. (1977) *Biochemistry 16*, 698–706.

Ellis, V., Scully, M. F., MacGregor, I., & Kakkar, V. V. (1982) *Biochim. Biophys. Acta 701*, 24–31.

Ellis, V., Scully, M. F., & Kakkar, V. V. (1983) *Biochim. Biophys. Acta 747*, 123–129.

Esmon, C. T. (1979) *J. Biol. Chem. 254*, 964–973.

Hunt, L. T., & Dayhoff, M. O. (1982) *Biochem. Biophys. Res. Commun. 95*, 864–871.

Jesty, J., Spencer, A. K., & Nemerson, Y. (1974) *J. Biol. Chem. 249*, 5614–5622.

Jobin, F., & Esnouf, M. P. (1967) *Biochem. J. 102*, 666–674.

Kisiel, W., & Hanahan, D. J. (1973) *Biochim. Biophys. Acta 304*, 103–113.

Kitz, R., & Wilson, B. (1962) *J. Biol. Chem. 237*, 3245–3249.

Lindhout, T., Govers-Riemslag, J. W. P., Van de Waart, P., Hemker, H. C., & Rosing, F. (1982) *Biochemistry 21*, 5494–5503.

MacFarlane, R. G. (1964) *Nature (London) 202*, 498–499.

Marciniak, E. (1973) *Br. J. Haematol. 24*, 391–400.

Marciniak, E., & Tsukamura, S. (1972) *Br. J. Haematol. 22*, 341–351.

Miletich, J. P., Jackson, C. M., & Majerus, P. W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4033–4036.

Miletich, J. P., Jackson, C. M., & Majerus, P. W. (1978) *J. Biol. Chem. 253*, 6908–6916.

Miletich, J. P., Kane, W. H., Hoffmann, S. L., Stanford, N., & Majerus, P. W. (1979) *Blood 45*, 1015–1022.

Miletich, J. P., Broze, G. J., Jr., & Majerus, P. W. (1980) *Anal. Biochem. 105*, 304–310.

Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164–4171.

Nesheim, M. E., Myrmel, K. H., Hibbard, L., & Mann, K. G. (1979a) *J. Biol. Chem. 254*, 508–517.

Nesheim, M. E., Taswell, J. B., & Mann, K. G. (1979b) *J. Biol. Chem. 254*, 10952–10962.

Olson, S. T., & Shore, J. D. (1982) *J. Biol. Chem. 257*, 14891–14895.

Osterud, B., Rapaport, S. I., & Lavine, K. (1977) *Blood 49*, 819–834.

Pixley, R., & Danishefsky, I. (1983) *Biochemistry 22*, 4810–4813.

Radomski, M., & Moncada, S. (1983) *Thromb. Res. 30*, 383–389.

Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Zwaal, R. F. A., & Hemker, H. C. (1980) *J. Biol. Chem. 255*, 274–283.

Sandberg, H., Andersson, L.-O., & Hoglund, S. (1982) *Biochem. J. 203*, 303–311.

Scully, M. F., Ellis, V., & Kakkar, V. V. (1983) *Thromb. Res. 29*, 387–388.

Smith, R. L. (1973) *J. Biol. Chem. 248*, 2418–2423.

Teitel, J. M., & Rosenberg, R. D. (1983) *J. Clin. Invest. 71*, 1383–1391.

Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655–709.

Van Rijn, J. L. M. L., Comfurius, P., Van Diejen, G., Rosing, J., Bevers, E. M., & Zwaal, R. F. A. (1983) *Thromb. Haemostasis 50*, 251.

Walker, F. J., & Esmon, C. T. (1979) *Biochem. Biophys. Res. Commun. 90*, 641–647.

Walsh, P. N. (1972) *Br. J. Haematol. 22*, 205–212.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Yin, E. T. (1974) *Thromb. Diath. Haemorrh. 33*, 43–50.