Regulation of Factor X_a In Vitro in Human and Mouse Plasma and In Vivo in Mouse

ROLE OF THE ENDOTHELIUM AND PLASMA PROTEINASE INHIBITORS

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ABSTRACT The regulation of human Factor X_a was studied in vitro in human and mouse plasma, and in vivo in mouse. In human plasma, ^{125}I-Factor X_a bound to α_1-proteinase inhibitor, antithrombin III, and α_2-macroglobulin in a ratio of 4.9:1.9:1 as determined by gel electrophoresis and by adsorption to IgG-(antiproteinase inhibitor)-Sepharose beads. The distribution of Factor X_a in mouse plasma was similar. The clearance of Factor X_a in mice was rapid (50% clearance in 3 min) and biphasic. α_1-Proteinase inhibitor-trypsin, even at a 2,000-fold molar excess, failed to inhibit the clearance of Factor X_a, while α_2-macroglobulin-trypsin inhibited only the later phase of clearance. The plasma clearance of diisopropylphosphoryl-Factor X_a was more rapid than native Factor X_a (50% clearance in 2.5 min), and the clearance was blocked by diisopropylphosphoryl-thrombin. Electrophoresis experiments confirmed that by 2 min after injection into the murine circulation, 90% of the bound Factor X_a was on α_2-macroglobulin, in marked contrast to the in vitro results. Organ distribution studies at 3 and 15 min with ^{125}I-Factor X_a demonstrated that the majority of radioactivity was in the liver, with significant radioactivity also present in lung and kidney. Autopsies performed 30 s after injection of ^{125}I-Factor X_a also demonstrated significant binding to the aorta and vena cava. These studies indicate that Factor X_a binds to specific thrombin-binding sites on endothelial cells, and that this binding alters its proteinase inhibitor specificity. Factor X_a binds to α_2-macroglobulin in vivo, whereas the predominant in vitro inhibitor of Factor X_a is α_1-proteinase inhibitor.

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INTRODUCTION

Factor X is a vitamin K-dependent coagulation protein activated by both the intrinsic and extrinsic pathways (1). The regulation of Factor X_a depends on the presence of the plasma proteinase inhibitors. α_2-Macroglobulin (α_2M),[1] antithrombin III (ATIII), and α_1-proteinase inhibitor (α_1-antitrypsin, α_1PI) all are capable of inactivating Factor X_a in vitro. α_2M is unique in its ability to inhibit proteinases from all four classes (2), including the homologous serine proteinases thrombin and Factor X_a (3–7). ATIII only inhibits serine proteases such as thrombin and Factor X_a (8–12). Its inhibitory potential is greatly enhanced by heparin as determined by in vitro studies (8, 13, 14). α_1PI is homologous to ATIII in its sequence and reaction mechanism (15) and is responsible for >90% of the trypsin inhibitory capacity of human plasma (16). Like ATIII, it is capable of inhibiting thrombin and Factor X_a in vitro (6, 17, 18).

While the in vitro inhibition of a number of the coagulation enzymes has been studied, much less is known about the in vivo inactivation of these proteinases with the exception of thrombin. ATIII appears to be the most important plasma proteinase inhibitor of thrombin (19). In vivo studies demonstrate that thrombin binds to high affinity sites on endothelial cells. The binding of thrombin to the endothelium then leads to an acceleration of ATIII-thrombin interactions and the resulting complex is cleared from the circulation by hepatocytes (20, 21). One class of endothelial cell thrombin-binding sites is the endothelial cell surface

[1] *Abbreviations used in this paper:* ATIII, antithrombin III; α_2M, α_2-macroglobulin; α_1PI, α_1-proteinase inhibitor, α_1-antitrypsin; DIP, diisopropylphosphoryl; RVV, Russell's viper venom.

protein, thrombomodulin. Thrombin bound to thrombomodulin activates protein C, which has potent anticoagulant properties (22, 23).

Kinetic studies with purified inhibitors indicate that α_1PI is the major in vitro plasma inhibitor of Factor X_a (24). The present studies were undertaken to examine the in vivo regulation of Factor X_a. The distribution of ^{125}I-Factor X_a among the plasma proteinase inhibitors in human plasma was studied using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and IgG-(antiproteinase inhibitor)-Sepharose beads. These results were compared with those obtained using mouse plasma, and the in vivo regulation of Factor X_a then was studied using our previously described mouse model. Human and mouse plasma proteinase inhibitors including α_2M, ATIII, and α_1PI have been shown to bind proteinases and clear from the circulation via hepatic receptor systems without distinction attributable to crossing species lines (25–29).

The possibility of proteinase transfer, from either α_1PI or ATIII to α_2M, as described by Beatty et al. (30) was examined, as well as the possible involvement of the endothelium in the regulation of Factor X_a.

METHODS

Reagents. Diisopropylfluorophosphate, Russell's viper venom (RVV), bovine serum albumin, Sephacryl S-200, and Sepharose 4B CL were purchased from the Sigma Chemical Co., St. Louis, MO. ^{125}Iodine, carrier-free, and lactoperoxidase, coupled to Sepharose, were obtained from New England Nuclear, Boston, MA and P-L Biochemicals, Inc., Milwaukee, WI, respectively. Iodo-beads were obtained from Pierce Chemical Co., Rockford, IL. The Factor X_a substrate, *N*-benzoyl-L-isoleucyl-L-glutamyl-L-glycyl-L-arginine-*p*-nitroanilide hydrochloride (and its methyl ester), S-2222, and the thrombin substrate H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide, S-2238, were purchased from the Kabi Co., Greenwich, CT. The IgG fractions of goat antisera to human α_2M, α_1PI, and ATIII were obtained from Atlantic Antibodies, Scarborough, ME. Ultrogel AcA-22 was purchased from LKB Instruments, Rockville, MD. Electrophoresis reagents were purchased from Bio-Rad Laboratories, Richmond, CA. All other reagents were of the best commercial grade available.

Proteins. Human Factor X was purified to homogeneity from plasma according to the method of Miletich et al. (31). The Factor X-activating fraction of Russell's viper venom (RVV-X) was purified as described by Schiffman et al. (32) and coupled to Sepharose 4B CL according to the method of Porath et al. (33). Factor X was activated by incubating 50 μg with 100 μl RVV-X-Sepharose in the presence of 10 mM $CaCl_2$ at 37°C for 3–5 min. Activation was at least 95% complete, as assessed by both the hydrolysis of S-2222 (34) and by polyacrylamide gel electrophoresis in the presence of SDS and 2-mercaptoethanol. α_2M, ATIII, and α_1PI were purified to homogeneity from human plasma as previously described (25–27).

Trypsin was purchased from Worthington Biochemicals Corp., Freehold, NJ, and was 70% active, as determined by active site titration (35). Trypsin complexes of α_2M, α_1PI, and ATIII were prepared by incubating equimolar amounts of active trypsin and proteinase inhibitor at room temperature for 10 min. Human α-thrombin (sp act 2,700 units/mg), diisopropylphosphoryl (DIP)-thrombin and DIP-Factor X_a were prepared as previously described (26, 36).

Protein concentrations. The concentrations of purified proteins were calculated using the following extinction coefficients and molecular weights: Factor X, A 1%/1 cm, 280 nm = 11.6, 49,000 and 17,000 for the heavy and light chains, respectively (31); Factor X_a, 34,000 and 17,000 for the heavy and light chains, respectively (37); human α_2M, A 1%/1 cm, 280 nm = 8.93, 718,000 (38); mouse α_2M 720,000 (28); human ATIII, A 1%/1 cm, 280 nm = 5.6, 56,000 (39); mouse ATIII, 63,000 (26); human α_1PI A 1%/1 cm, 280 nm = 5.3, 53,000 (40); mouse α_1PI, 64,000 (41).

Protein radiolabeling. Human α_2M, α_1PI, and ATIII were radiolabeled with ^{125}I using the solid state lactoperoxidase method (42). Human Factor X and bovine albumin were radiolabeled with ^{125}I using Iodo-beads, in 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4. Typically, 100 μg of Factor X or albumin was incubated with 1 mCi ^{125}I and two beads at room temperature for 30 min. Radioactivity was measured in a gamma counter (model AW14-120 Scientific Products, Inc., Div. American Hospital Supply Corp., McGraw Park, IL). Proteins were labeled to specific radioactivities of 1,000–2,000 cpm/ng and assayed for activity. α_2M was assayed by the method of Ganrot (43). α_1PI was assayed as described by Dietz et al. (44) by measuring the inhibition of the hydrolysis of α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) by trypsin. ATIII activity was assayed as the inhibition of the thrombin-catalyzed hydrolysis of the chromogenic substrate, S-2238 (45). Factor X was assayed as described above. In all cases, proteins retained at least 95% activity following radiolabeling.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis of purified protein samples was performed using 5 and 7.5% acrylamide slab gels, as described by Wyckoff et al. (46). Samples were denatured in 1% SDS and 1% 2-mercaptoethanol at 95°C for 2 min. SDS-polyacrylamide gel electrophoresis of plasma samples was performed on 5% slab gels according to the method of Weber and Osborn (47). 25-μl plasma samples were denatured in 75 μl 6% SDS at 95°C for 2 min. After drying, gel lanes containing radiolabeled proteins were sliced into sections 3 mm long in the direction of migration and the radioactivity in each slice measured in a gamma counter. Protein content was then calculated using the previously determined specific radioactivities of the preparations. Gel profiles were plotted and relative peak areas obtained by cutting and weighing the peaks.

Reaction of factor X_a with purified plasma proteinase inhibitors. Complexes of ^{125}I-Factor X_a wth human α_2M, α_1PI, and ATIII were prepared by reacting ^{125}I-Factor X_a with each inhibitor for 10 min at room temperature at proteinase inhibitor excess. The complexes of α_1PI and ATIII with ^{125}I-Factor X_a were then purified by chromatography on Sephacryl S-200. The complexes of α_2M with ^{125}I-Factor X_a were purified by chromatography on Ultrogel AcA-22. The purified complexes were denatured in 6% SDS and subjected to gel electrophoresis according to the method of Weber and Osborn (47) as described above to assess the degree of covalent binding of ^{125}I-Factor X_a to each inhibitor, and to establish reference markers for the in vitro experiments with mouse and human plasma and the in vivo clearance studies.

IgG-(antiproteinase inhibitor)-Sepharose beads. The IgG fraction of goat anti-human α_2M, α_1PI, or ATIII (20 mg each) was coupled to 5 ml of CNBr-activated Sepharose 4B CL (33, 48). The beads were characterized by incubating 50 μl of the resultant IgG-Sepharose preparations with 1–500 μg

of ^{125}I-labeled α_2M, α_1PI, and ATIII or the corresponding ^{125}I-proteinase inhibitor-trypsin complex in 500 μl of 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4, 60 mg/ml bovine serum albumin at 37°C for 2 h with constant mixing in a series of saturation curves. The tubes were then centrifuged in an Eppendorf microcentrifuge. The supernatants were removed and the beads washed three times with cold Tris buffer (1 ml each). The bottoms of the tubes were cut off, and the radioactivity content in beads, supernatants, and washes was determined. Under the conditions chosen for the plasma experiments described below, 80–90% of the applied ^{125}I-labeled proteinase inhibitors bound to the corresponding IgG-Sepharose, with ~1.5% nonspecific binding as determined by incubating the noncorresponding inhibitors with a given IgG-Sepharose preparation. The binding of the proteinase inhibitors to their corresponding IgG-Sepharoses was virtually unaffected by formation of trypsin complexes before incubation with the beads. ^{125}I-Factor X_a (5 μg) was also incubated with the IgG-Sepharoses; the maximum binding of this ligand to any of the IgG-Sepharose preparations was 3%.

Mouse plasma. Mouse plasma for in vitro studies was obtained by incising anesthetized mice in the midline, and cannulating the inferior vena cava. Blood was drawn into syringes containing 1/10 vol of 3.8% sodium citrate, and centrifuged immediately. The plasma was drawn off and used in experiments within 1 h of bleeding.

In vitro *plasma studies.* Citrated human or mouse plasma (250 μl) was incubated with 1 μg ^{125}I-Factor X_a at 37°C. Samples (25 μl) were removed at various times and either incubated with IgG-Sepharose beads in 500 μl of 50 mM Tris-HCl, 0.15 M NaCl, 60 mg/ml albumin, pH 7.4 as described above, or denatured immediately for SDS gel electrophoresis.

Plasma elimination studies. Plasma elimination studies of ^{125}I-Factor X_a alone or in the presence of unlabeled proteins were performed using CD-1 female mice as previously described (29). Studies were performed either with or without the precipitation of sampled blood in 7.5% trichloroacetic acid with no significant difference in results. To examine the distribution of Factor X_a between the plasma and the cellular elements of blood, ^{125}I-Factor X_a was injected and 600 μl of blood was collected after 5 min into 1/10 vol 3.8% sodium citrate. The sampled blood was centrifuged at 10,000 *g* for 10 min at 4°C to pellet the cellular elements, and the supernatant plasma was removed with a pipette. The cellular pellet was washed three times with 1 ml cold 0.050 M Hepes, 0.15 M NaCl, pH 7.4, and the radioactivity content of the plasma, washes, and the cellular pellet was determined. In some studies, duplicate samples were taken at each time point. One sample was counted in a gamma counter, the other was drawn into 1/10 vol of 3.8% sodium citrate and centrifuged immediately at 4°C. The plasma was then denatured immediately for SDS gel electrophoresis. In general, studies were performed a minimum of four times.

Tissue distribution studies. Organ distribution studies were performed as previously described (29). In some studies aorta and inferior vena cava were removed en bloc and counted.

RESULTS

Reaction of Factor X_a with purified plasma proteinase inhibitors. Complexes prepared with ^{125}I-Factor X_a and either α_1PI or ATIII were 100% covalent, as assessed by SDS gel electrophoresis (data not shown). The degree of covalent binding of proteins to α_2M varies with the proteinase (49). Complexes of α_2M prepared with two different Factor X_a preparations were examined by gel electrophoresis on multiple occasions consistently demonstrating essentially 100% covalent binding of ^{125}I-Factor X_a to α_2M (Fig. 1). The small radioactivity peak at slice 34 represents free Factor X_a, which was noncovalently bound to α_2M.

In vitro distribution of Factor X_a among the proteinase inhibitors in human plasma. The time course of inactivation of ^{125}I-Factor X_a in human plasma was studied using SDS-polyacrylamide gel electrophoresis. The distribution of radioactivity was unchanged from 1 to 20 min of incubation. This result suggests that Factor X_a does not transfer from one inhibitor to another in contrast to porcine trypsin (30). The 5-min sample is shown as a representative distribution (Fig. 2 *A*). The arrows indicate the positions of reference standards of proteinase inhibitor-^{125}I-Factor X_a complexes and free ^{125}I-Factor X_a. Peak I represents α_2M-^{125}I-Factor X_a complexes. Peak II consists of both α_1PI-^{125}I-Factor X_a and ATIII-^{125}I-Factor X_a complexes, as these two species differ by only 3,000 M_r, and are not resolvable using this gel technique. Peak III represents free ^{125}I-Factor X_a. The areas under these peaks correspond to the relative amounts of ^{125}I-Factor X_a in each species (Table I). The vast majority of ^{125}I-Factor X_a is bound to α_1PI and/or ATIII, with much less bound to α_2M. To distinguish between binding of ^{125}I-Factor X_a to α_1PI and ATIII, the following studies were performed.

IgG-(antiproteinase inhibitor)-Sepharose bead studies. Samples of human plasma, after 10-min incubation with ^{125}I-Factor X_a were mixed with goat IgG anti-human α_2M, α_1PI, or ATIII covalently linked to Sepharose. The results are shown in Table II. These studies indicate that α_1PI is the principal inhibitor of Factor X_a in vitro, a result consistent with the kinetic data of

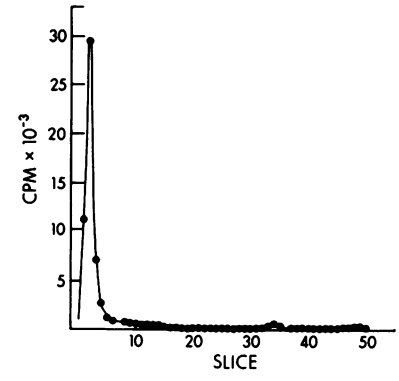

FIGURE 1 SDS-polyacrylamide gel electrophoresis of α_2M-Factor X_a. Electrophoresis performed as in Methods with α_2M-^{125}I-Factor X_a complexes purified by gel filtration chromatography on Ultrogel AcA-22 before electrophoresis.

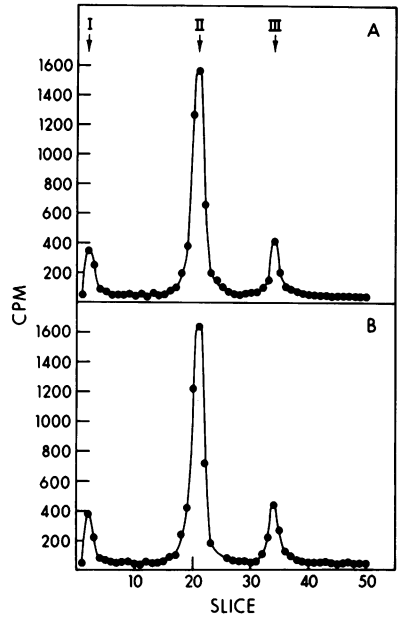

FIGURE 2 In vitro distribution of ^{125}I-Factor X_a in human and mouse plasma. SDS-polyacrylamide gel electrophoresis of 1 μg ^{125}I-Factor X_a incubated with 250 μl citrated human (*A*) or mouse plasma (*B*) for 5 min. Arrows indicate the positions of reference samples of proteinase inhibitor complexed, or free ^{125}I-Factor X_a. Peak I represents α_2M-^{125}I-Factor X_a complexes. Peak II consists of both α_1PI-^{125}I-Factor X_a and ATIII-^{125}I-Factor X_a complexes, as these two species differ by only 3,000 M_r, and are not resolvable by this gel technique. Peak III represents free ^{125}I-Factor X_a. Approximately 10,000 cpm were loaded onto each lane and run as described in Methods.

Ellis et al. (24). The data are also consistent with the SDS-polyacrylamide gel electrophoresis experiments described above, since the total Factor X_a bound to ATIII and α_1PI yields a virtually identical ratio for α_2M:α_1PI/ATIII of 1:6.8.

TABLE II
In Vitro Distribution of ^{125}I-Factor X_a in Human Plasma Determined by Adsorption by IgG-Sepharose

Plasma	Antiserum to α_2M	Antiserum to α_1PI	Antiserum to ATIII	Free Factor X_a
Total (%)	10.9	53.4	20.7	15.0
Ratio of bound Factor X_a	1.00	4.90	1.90	—
Ratio of bound factor X_a as determined by Ellis et al. (24)*	1.00	4.64	2.08	—

* The data of Ellis et al. (24) is based on in vitro kinetic studies using purified proteinase inhibitors. The second order rate constants obtained in this manner were then multiplied by the plasma concentrations of the proteinase inhibitors to assess their relative effectiveness as Factor X_a inhibitors in plasma (24).

In vitro distribution of Factor X_a among the proteinase inhibitors in mouse plasma. Before studying the in vivo catabolism of Factor X_a in mouse, the distribution of human Factor X_a in mouse plasma in vitro was examined using SDS-polyacrylamide gel electrophoresis after various times of incubation. There was no significant change in the distribution of Factor X_a between 1- and 20-min incubation with mouse plasma, and the 5-min sample is shown as a representative distribution (Fig. 2 *B*), for comparison to human plasma (Fig. 2 *A*). The relative amounts of Factor X_a bound to α_2M and α_1PI/ATIII are shown in Table III. Again, the vast majority of Factor X_a was found in the α_1PI/ATIII peak with much less Factor X_a bound to α_2M, essentially identical to the distribution seen in human plasma.

Plasma elimination of Factor X_a. The clearance curve of ^{125}I-Factor X_a is biphasic (Fig. 3), with an initial rapid disappearance of protein followed by a

TABLE I
In Vitro Distribution of ^{125}I-Factor X_a in Human Plasma Determined by SDS-Polyacrylamide Gel Electrophoresis

	Proteinase inhibitor-bound Factor X_a		
	α_2M	α_1PI/ATIII	Free Factor X_a
Factor X_a in peak (%)	11.0	75.0	14.0
Molar ratio of factor X_a in peak	1.0	6.82	1.30
Bound Factor X_a (%)	12.8	87.2	—
Ratio of bound Factor X_a	1.00	6.82	—

TABLE III
In Vitro Distribution of ^{125}I-Factor X_a in Mouse Plasma Determined by SDS-Polyacrylamide Gel Electrophoresis

	Proteinase inhibitor-bound Factor X_a		
	α_2M	α_1PI/ATIII	Free Factor X_a
Factor X_a in peak (%)	10.7	73.5	15.8
Ratio of Factor X_a in peak	1.00	6.87	1.48
Bound Factor X_a (%)	12.7	87.3	—
Ratio of bound Factor X_a	1.00	6.87	—

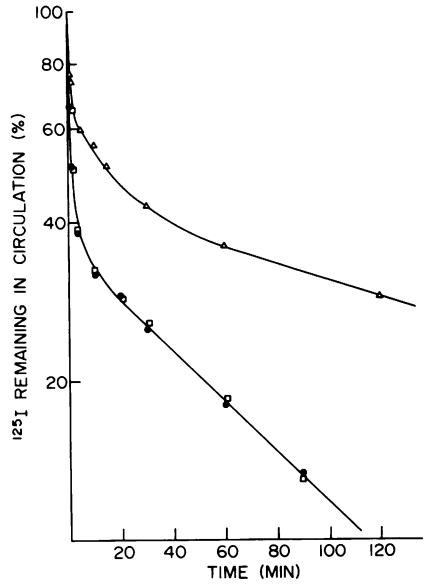

FIGURE 3 Clearance from the circulation of intravenously injected Factor X_a. ^{125}I-Labeled protein was injected into mice and blood samples were collected at intervals. ^{125}I-Factor X_a (●). The clearance of ^{125}I-Factor X_a in the presence of a 2,000-fold molar excess of unlabeled α_1PI-trypsin (□) and a 1,000-fold molar excess of α_2M-trypsin (△).

slow phase of longer duration. To further characterize the clearance of Factor X_a, competition experiments with unlabeled proteinase inhibitor-proteinase complexes were performed. The clearance pathways for proteinase complexes of α_2M, ATIII, and α_1PI have been characterized in mouse (25–27). Because proteinase complexes of ATIII and α_1PI are cleared by the same hepatocyte receptor (27), competition experiments were performed only with α_2M-trypsin and α_1PI-trypsin. Even at 2,000-fold molar excess, α_1PI-trypsin failed to inhibit the clearance of Factor X_a, which is inconsistent with clearance of Factor X_a in complexes with either α_1PI or ATIII. In contrast, α_2M-trypsin at 1,000-fold molar excess did inhibit the clearance of ^{125}I-Factor X_a (Fig. 3). It appears, however, that only the later phase of clearance is inhibited by α_2M-trypsin, indicating that the early clearance phase involves some other mechanism. The possibility that the cellular elements of blood are involved in the catabolism of Factor X_a was investigated by injecting ^{125}I-Factor X_a into a mouse, collecting blood into 1/10 vol sodium citrate and separating the plasma and cellular elements as described in Methods, and determining the radioactivity content of each. The cellular pellet contained 0.3% of the recovered radioactivity with the remaining 99.7% in the plasma.

The possibility that Factor X_a binds to endothelial cell-binding sites, analogous to the related proteinase thrombin (20), was investigated using DIP-^{125}I-Factor X_a. The inactivated Factor X_a cannot bind to the plasma proteinase inhibitors, but might bind to endothelial-binding sites, since proteinase activity would not be required for this interaction (20). The clearance of DIP-^{125}I-Factor X_a (Fig. 4) is extremely rapid, and apparently first order ($t_{1/2}$ = 2.5 min). The clearance of DIP-^{125}I-Factor X_a is dramatically slowed in the presence of a 1,000-fold molar excess of DIP-thrombin.

Organ distribution of ^{125}I-Factor X_a and DIP-^{125}I-Factor X_a. Radiolabeled Factor X_a and DIP-Factor X_a were injected intravenously. The organ distributions of radioactivity at 3 and 15 min are shown in Table IV. Both Factor X_a and DIP-Factor X_a are found primarily in lung, kidney, and liver, with a greater amount in lung at 3 min than at 15 min.

Organ distribution studies were then performed 30 s after injecting either ^{125}I-Factor X_a, α_2M, or albumin. The recovery of radioactivity in the various organs as well as aorta and inferior vena cava was compared (Table V). These studies demonstrated that ~10-fold more radioactivity was bound to the vessel wall when ^{125}I-Factor X_a was injected than was bound when either ^{125}I-albumin or α_2M were injected. These latter proteins were chosen for comparison since neither should bind to the vessel wall.

In vivo distribution of ^{125}I-labeled Factor X_a among the plasma proteinase inhibitors. Plasma samples obtained 15 s, 2 min, and 20 min after the injection of

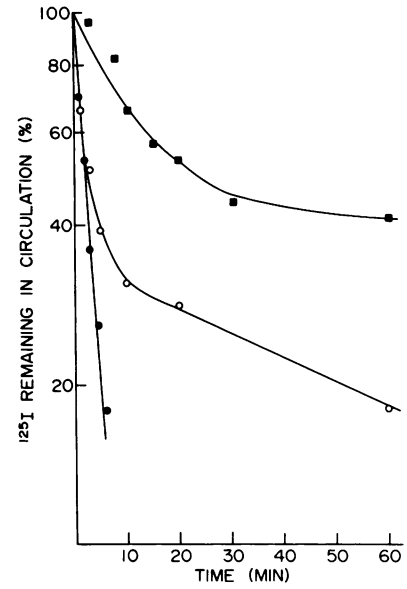

FIGURE 4 Clearance studies of intravenously injected DIP-^{125}I-Factor X_a. Clearance studies as described in Fig. 3. ^{125}I-DIP-Factor X_a (●). The clearance of ^{125}I-Factor X_a is shown for comparison (○). Clearance of ^{125}I-DIP-Factor X_a in the presence of 1,000-fold molar excess of DIP-thrombin (■).

TABLE IV
Organ Distribution of ^{125}I-Factor X_a and DIP-^{125}I-Factor X_a

	Active Factor X_a		DIP-Factor X_a	
	3 min	15 min	3 min	15 min
	% injected dose			
Heart	1.5	1	0.5	0.5
Lung	11	6	20	12.5
Spleen	3	3	2	2
Kidneys	24	22	23	28
Liver	60	68	54.5	57

^{125}I-Factor X_a were subjected to SDS-polyacrylamide gel electrophoresis (Fig. 5). The 2-min sample is shown as a representative distribution. The reference peak positions are identical to those in Fig. 1. The relative amounts of ^{125}I-Factor X_a in the various species are shown in Table VI. The distribution of Factor X_a in vivo differs markedly from that observed in vitro, since by 2 min in vivo, 90% of the bound Factor X_a was complexed to α_2M.

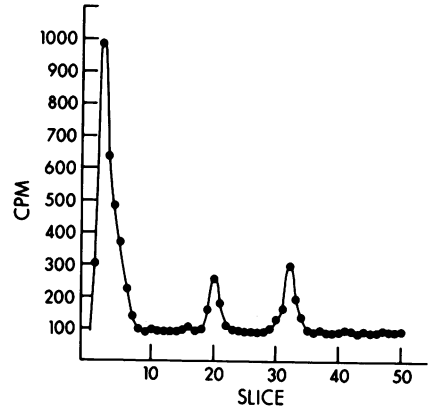

FIGURE 5 In vivo distribution of ^{125}I-Factor X_a among the plasma proteinase inhibitors. SDS-polyacrylamide gel electrophoresis of blood samples obtained at 2 min after injection of ^{125}I-Factor X_a. The reference peak positions are identical to those in Fig. 2.

DISCUSSION

These studies were undertaken to elucidate the catabolism of Factor X_a, which occupies a central position in the coagulation cascade. The control of Factor X_a activity may, therefore, play a crucial role in the in vivo regulation of coagulation. Ellis et al. (24) used purified plasma proteinase inhibitors to study the kinetics of inactivation of Factor X_a. They concluded that α_1PI was the major plasma proteinase inhibitor of Factor X_a, based on second order rate constants and plasma inhibitor concentrations. No experiments were performed with mixtures of the inhibitors to confirm these calculations and to rule out the possibility of transfer of Factor X_a from α_1PI to α_2M. Such transfer occurs with porcine, but not bovine, trypsin (30). Nevertheless, their result was confirmed in human plasma by SDS-polyacrylamide gel electrophoresis and IgG-(antiproteinase inhibitor)-Sepharose, since we obtained very similar ratios of inhibition, 4.9:1.9:1 (α_1PI/ATIII/α_2M). The in vitro distribution of Factor X_a among the proteinase inhibitors in mouse plasma was very similar to that obtained in human plasma. Clearance studies with mice were then performed to determine whether this result holds in vivo. The clearance of Factor X_a in mice was rapid and biphasic. The clearance pathways for proteinase complexes of α_2M, ATIII, and α_1PI have been well studied (25–27), and it has been found that proteinase complexes of ATIII and α_1PI are cleared by the same receptor in hepatocytes (27). Therefore, to elucidate the role of these proteinase inhibitors in the clearance of Factor X_a, competition experiments were performed with large molar excesses of unlabeled α_2M-trypsin and α_1PI-trypsin. Only α_2M-trypsin slowed the clearance of Factor X_a, suggesting that in vivo α_2M,

TABLE V
In Vivo Binding of ^{125}I-Factor X_a, Albumin, and α_2-M to Aorta and Vena Cava[*]

Protein	^{125}I-Radioactivity
	(%)
Factor X_a	5
Albumin	0.6
α_2M	0.5

[*] Comparable ^{125}I-radioactivity was injected into mice in each study and the animals autopsied 30 s after injection. The percent recovered in the tissues was then calculated.

TABLE VI
In Vivo Distribution of ^{125}I-Factor X_a among the Plasma

	Factor X_a bound to proteinase inhibitors	
Time	α_2M	α_1PI/ATIII
	%	
15 s	62	38
2 min	90	10
20 min	97	3

and not α_1PI or ATIII, is the primary plasma inhibitor of Factor X_a. α_2M-trypsin only inhibited the late phase of Factor X_a plasma clearance, indicating that the early clearance of Factor X_a occurs via a different mechanism. The distribution of Factor X_a among the plasma proteinase inhibitors in vivo was also examined using SDS-polyacrylamide gel electrophoresis, demonstrating that 90% of the Factor X_a bound to inhibitors is complexed to α_2M by 2 min. This is in contrast to the result obtained in vitro, where only 12.7% of the bound Factor X_a is present on α_2M after 10-min incubation in mouse plasma. The early mechanism of clearance of Factor X_a may therefore be responsible for the altered specificity for proteinase inhibitors seen in vivo.

The possibility that the cellular elements of blood may be involved in the catabolism of Factor X_a was examined by separating the plasma and the cellular elements from an in vivo blood sample by centrifugation. The plasma fraction contained 99.7% of the recovered radioactivity, indicating that the cellular elements of blood, such as platelets and leukocytes, are not involved in the regulation of Factor X_a.

The possibility that the early clearance of Factor X_a may be due to endothelial cell binding was examined by studying the clearance of DIP-Factor X_a. Lollar and Owen (20) have shown that DIP-thrombin is cleared more rapidly from the circulation than thrombin, and that the binding of DIP-thrombin to endothelium is saturable, using competition studies with large molar excesses of unlabeled DIP-thrombin. This result was confirmed in mice by our laboratory (23, 26). Similarly, the clearance of DIP-Factor X_a, which cannot bind to the plasma proteinase inhibitors, is more rapid than Factor X_a ($t_{1/2}$ of 2.5 vs. 3 min) and this clearance can be greatly diminished in the presence of a large molar excess of unlabeled DIP-thrombin. These studies suggest that Factor X_a binds to thrombin-binding sites on the endothelial surface. Organ distribution studies with Factor X_a and DIP-Factor X_a demonstrated that both ligands are concentrated mainly in three organs—lung, kidney, and liver, with a greater fraction of the ligand in lung at earlier times. These results are similar to those obtained with thrombin and DIP-thrombin (22, 23, 26), where 80% of the injected DIP-thrombin was found in the lung, the first vascular bed available to the injected ligand, at 3 min. It appears that the affinity of Factor X_a for these endothelial thrombin-binding sites is lower than that of thrombin, since only 20% of the DIP-Factor X_a is removed in the first pass through the lung, and more is available to distribute in other vascular beds throughout the body. That Factor X_a does bind to endothelium was directly demonstrated by autopsy studies performed 30 s after injecting ^{125}I-Factor X_a. In these studies, a significant fraction of the radioactivity was recovered in the aorta and inferior vena cava. Organ distribution studies at later times show a higher percentage of Factor X_a as compared with DIP-Factor X_a in the liver, consistent with the hepatic clearance of proteinase inhibitor-proteinase complexes (25–27).

These data indicate that the clearance of Factor X_a occurs by a two step mechanism. The first step involves the binding of Factor X_a to thrombin-binding sites on the endothelial surface. The binding alters the affinity of Factor X_a for the plasma proteinase inhibitors, and α_2M becomes the primary in vivo inhibitor of this proteinase, whereas α_1PI is the primary in vitro inhibitor of Factor X_a. A similar alteration in specificity for plasma proteinase inhibitors by proteinase bound to a macromolecule has been described previously (29). The primary plasma inhibitor of plasmin is α_2-antiplasmin, but α_2M is the principal inhibitor of plasmin in activator complexes with streptokinase (29). Similarly, the binding of thrombin to thrombomodulin on the endothelium alters the substrate specificity of thrombin since the cleavage of fibrinogen and Factor V by thrombin bound to thrombomodulin, is drastically reduced (50). However, thrombin bound to thrombomodulin activates protein C at least 100-fold faster than does free thrombin (51).

The identification of the shared endothelial cell-binding site for thrombin and Factor X_a, and the possibility that the substrate specificity of Factor X_a is altered on binding, analogous to the change in proteinase inhibitor specificity, are currently under investigation.

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