The Regulation of Human Factor XIIa by Plasma Proteinase Inhibitors*

Robin A. Pixley†, Marc Schapira‡, and Robert W. Colman‡

From the †Thrombosis Research Center and the Department of Medicine, Temple University School of Medicine, Philadelphia, Pennsylvania 19140 and the ‡Division de Rhumatologie, Hôpital Cantonal Universitaire, 1211 Geneve 4, Switzerland

Studies of the inactivation of factor XIIa by plasma protease inhibitors in purified systems and in plasma were initiated to determine the relative importance of these inhibitors to the neutralization of factor XIIa. Factor XIIa was measured by the amidolyis of H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride or by coagulant activity. CI inhibitor (C1INH), α₂-antiplasmin (α₂AP), α₂-macroglobulin (α₂M), and antithrombin III (ATIII) inhibited factor XIIa with second-order rate constants of 2.2 × 10⁶, 1.1 × 10⁶, 5.0 × 10⁵, and 1.3 × 10⁵ M⁻¹ min⁻¹. Factor XIIa activity was not affected by protease-antiprotease interactions. Incubation of [³⁵S]-radiolabeled factor XIIa resulted in 1:1 stoichiometric complexes with C1INH (M₁₉₀,₀₀₀), ATIII (M₁₂₅,₀₀₀), and α₂AP (M₁₅₀,₀₀₀ and 1₂₅,₀₀₀) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Incubation of [³²P]-Factor XIIa with α₂M resulted in a component of 8₀,₀₀₀, 2₀,₀₀₀, and 8₀,₀₀₀ in plasma which were inhibited by two plasma protease inhibitors, C1INH (11-13) and antithrombin III (ATIII) (13, 14). However, the relative importance of these inhibitors was not studied. We then conducted studies of factor XIIa in plasma and found that the theoretical inhibition rates expected from the purified systems agreed with those observed in plasma.

MATERIALS AND METHODS AND RESULTS

Human factor XII (Hageman factor) (1) is a single-chain plasma protein of β-globulin mobility with a M₀ of 8₀,₀₀₀ (2). Activation of the zymogen initially results in an active serine protease of identical molecular weight, containing two chains held together by disulfide bonds, designated factor XIIa (3). In plasma, factor XIIa hydrolyzes factor XI (4) and prekallikrein (5), resulting in conversion of these zymogens to active enzymes. Activation of factor XI results in the initiation of intrinsic coagulation. Kallikrein can liberate bradykinin from high molecular weight kininogen (6) and may participate in surface-catalyzed fibrinolysis through the activation of plasminogen (7). Activated factor XII influences the extrinsic pathway of coagulation by activating factor VII (8) and activates the classical complement pathway by activating C1 (9, 10).

At least two plasma protease inhibitors have been reported to be capable of inhibiting factor XIIa: CI inhibitor (C1INH') (11-13) and antithrombin III (ATIII) (13, 14). In contrast, α₂-antiplasmin (α₂AP) (15), α₂-macroglobulin (α₂M) (13), and α₂-proteinase inhibitor (13) were not found to inhibit factor XIIa. However, quantitative comparisons of the potency of each inhibitor under similar experimental conditions have not been reported. Moreover, the only factor XIIa complex formed with a plasma protease inhibitor previously demonstrated is the factor XIIa-antithrombin III complex with M₁₁₇,₀₀₀ (14). We therefore studied the inhibition of factor XIIa by inhibitors in purified systems under identical conditions and present kinetic evidence that purified CI inhibitor is the most potent inhibitor of purified factor XIIa. We then conducted studies of factor XIIa in plasma and found that the theoretical inhibition rates expected from the purified systems agreed with those observed in plasma.

DISCUSSION

Factor XIIa has been reported in past investigations to be inhibited by two plasma protease inhibitors, C1INH (11-13) and ATIII (13, 14). However, the relative importance of these inhibitors was not studied. Our results confirm that both inhibit factor XIIa, but C1INH is 2 orders of magnitude more potent. Since Stead et al. (14) reported that heparin accelerates ATIII inhibition of factor XIIa, we also studied the effect of heparin. At plasma concentrations of purified ATIII (5.8 μM) at 37°C, the pseudo first-order rate constant of factor XIIa inactivation was 0.007 min⁻¹, while with heparin (1.2 units/ml) it increased to 0.029 min⁻¹. This 4-fold enhance-

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†The abbreviations used are: C1INH, Ci inhibitor; ATIII, antithrombin III; α₂M, α₂-macroglobulin; α₂AP, α₂-antiplasmin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HAE, hereditary angioedema.

‡Portions of this paper (including "Materials and Methods," "Results," and Figs. 1-5 and 7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-2195, cite the authors, and include a check or money order for $7.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Regulation of Factor XIIa by Plasma Inhibitors

The inhibition of factor XIIa by $\alpha_2$M deserves more analysis of the results because of its large molecular weight ($M$, 725,000) and its ability to inhibit order enzymes in more than 1:1 stoichiometry. The inhibition of factor XIIa by $\alpha_2$M was studied under pseudo first-order conditions with the inhibitor in excess. When the reciprocal plot of the data (Fig. 4, inset) is extrapolated to the ordinate, the intercept is negative. These kinetics may be attributed to $\alpha_2$M's ability to inhibit more than 1 mol of enzyme for each mole of inhibitor, as has been found for trypsin and chymotrypsin (45, 46). More than a 1:1 stoichiometry for factor XIIa is indicated by the following consideration. When the relative concentration of $\alpha_2$M in Fig. 4 is twice the value indicated (or each mole of $\alpha_2$M is able to inhibit 2 mol of factor XIIa), the line of the reciprocal plot in Fig. 4 is shifted to the left and a positive intercept is attained. If the line of the reciprocal plot is shifted to pass through the origin, the relative concentration of $\alpha_2$M is 1.6 times the value indicated in Fig. 4. This finding suggests that $\alpha_2$M can inhibit more than 1 mol of factor XIIa/mol of $\alpha_2$M but is not conclusive evidence of 2:1 stoichiometry, $K_x$ and $k_x$ are still not determinable by this analysis.

Residual amidolytic activity using the chromogenic substrate H-p-prolyl-l-phenylalanyl-l-arginine-p-nitroanilide dihydrochloride in factor XIIa-$\alpha_2$M complexes after a long incubation was found to be less than 15% and was still linear with time (Fig. 4). This finding suggests but does not prove that factor XIIa complexed with $\alpha_2$M is not available to interact with small substrates, in contrast to what has been shown for some other proteases (47, 48).

This paper presents the first description of the $M_x$ of the factor XIIa-ClINH complex. When radiolabeled factor XIIa was incubated with the purified inhibitors, enzyme-inhibitor complexes were observed with SDS-PAGE. Analysis of the mixtures resulting from the incubation of [125I]-factor XIIa ($M_x$, 80,000) with ClINH ($M_x$, 105,000) and ATIII ($M_x$, 62,000) demonstrated that radiolabeled complexes of, respectively, $M_x$, 190,000 and 125,000 were generated during factor XIIa inactivation (Fig. 6). The complex of factor XIIa with ATIII demonstrated in Fig. 6 agrees with the $M_x$, 117,000 complex reported by Stead et al. (14). Incubation of factor XIIa with $\alpha_2$AP ($M_x$, 67,000) resulted in the formation of two complexes ($M_x$, 150,000 and 125,000) not previously described. The appearance of secondary complexes with time has been shown for other enzyme-inhibitor reactions and is not unusual for this type of reaction (28). The complex ($M_x$, 125,000) is attributed to proteolysis of the higher molecular weight complex ($M_x$, 150,000). Factor XIIa was incubated with $\alpha_2$M ($M_x$, 360,000 nonreduced dimers, $M_x$, 185,000 reduced). When labeled factor XIIa was reacted with purified $\alpha_2$M and the incubation mixture was subjected to SDS-PAGE in the absence of a reducing agent, a substantial part of the radioactivity did not enter the gel (data not shown). This observation is attributed to labeled factor XIIa bound to the unreduced $\alpha_2$M. On reduced SDS gel, the reaction resulted in an enzyme-inhibitor complex at $M_x$, 85,000 (Fig. 6). This complex is attributed to the smaller molecular weight subunit of factor XIIa ($M_x$, 32,000, see Fig. 1) bound to a proteolyzed fragment of the reduced $\alpha_2$M. Investigations of other enzymes inhibited by $\alpha_2$M have shown similar molecular weight products after incubation and analysis using reduced SDS-PAGE (49-51). Recently, a similar molecular weight moiety on SDS-PAGE has been shown after incubation of [125I]-labeled trypsin with $\alpha_2$M (52).

The second-order rate constants determined in the purified systems are not a true indicator of the regulation of the activity of factor XIIa in a mixed system of inhibitors, as is found in plasma. The inhibition of factor XIIa in a mixed system would depend on the additive contribution of each inhibitor, which is dependent on its concentration and its second-order rate constant of inhibition. When the mean

### TABLE I

**Comparison of the relative factor XIIa inhibitory activity and predicted relative effectiveness of Cl inhibitor, $\alpha_2$-antiplasmin, $\alpha_2$-macroglobulin, and antithrombin III in plasma**

| Inhibitor | Purified system | Plasma concentration | Predicted in plasma |
|-----------|-----------------|----------------------|---------------------|
|           | $k_x$ | $K_x$ | $k$ | Relative effectiveness | $k_x$ | $K_x$ | $k$ | Relative effectiveness | % |
| ClINH | $1.2 \times 10^{-2}$ | $5.4 \times 10^{-4}$ | $222.2 \times 10^{-6}$ | 171 | $1.7 \times 10^{-6}$ | 374.0 $\times 10^{-3}$ | 61 | 91.3 |
| $\alpha_2$AP | ND | ND | $11.0 \times 10^{-5}$ | 8 | $1.1 \times 10^{-6}$ | 12.1 $\times 10^{-3}$ | 2 | 3.0 |
| $\alpha_2$M | $1.7 \times 10^{-5}$ | $1.1 \times 10^{-4}$ | $1.3 \times 10^{-5}$ | 1 | $3.8 \times 10^{-6}$ | 17.5 $\times 10^{-3}$ | 3 | 4.3 |
| ATIII | $1.7 \times 10^{-3}$ | $1.1 \times 10^{-4}$ | $1.3 \times 10^{-6}$ | 1 | $4.7 \times 10^{-6}$ | 6.1 $\times 10^{-3}$ | 1 | 1.5 |

*The concentrations of inhibitors in plasma are values obtained in a pool of normal plasma and are reported in Ref. 43.*

*The second-order rate constant in this case was determined from the integrated second-order rate equation (Equation 4 under "Materials and Methods").

ND, not determinable.
concentration of these inhibitors in pooled plasma is considered, the ratio of the inhibitor potency toward factor XIIa of ClINH, α2AP, α2M, and ATIII is 61:2:31 (Table I). This analysis of the data in purified systems predicts that ClINH contributes 91% of the inhibition of factor XIIa in the presence of these other inhibitors (Table I).

To confirm the predictions calculated from the purified inhibition reactions, purified factor XIIa was added to various plasmas with different concentrations of ClINH present. The rate of inhibition was measured and compared to the predicted rate based on the pure reactions. The data from these experiments (Fig. 7) closely fit the theoretical lines calculated for the sum of the concentration of these inhibitors in pooled plasma.

Evidence for enzyme-inhibitor complexes formed in plasma is shown in the Sephadex G-200 gel filtration experiments (Figs. 8 and 9). Labeled factor XIIa incubated with either purified ClINH or normal plasma gave an identical sieving profile, again indicating that complexing with ClINH is the inhibitor that is primarily responsible for the regulation of factor XIIa. At pharmacological concentrations of heparin (0.6–1.6 units/ml), no change in the rate of factor XIIa inhibition was observed (53).

Evidence for enzyme-inhibitor complexes formed in plasma is shown in the Sephadex G-200 gel filtration experiments (Figs. 8 and 9). Labeled factor XIIa incubated with either purified ClINH or normal plasma gave an identical sieving profile, again indicating that complexing with ClINH is the predominant inhibitor in plasma. On examination of the complex peak by SDS-PAGE (Fig. 9), the ascending limb and peak tube contained mainly a peak of 190,000 complex similar to that found with purified factor XIIa and ClINH (Fig. 6). Lower concentrations of factor XIIa complexes with α2M, α2AP, and ATIII were also identifiable (Fig. 9). The apparent Mₚ for the factor XIIa-ClINH complex of 300,000 by Sephadex G-200 gel filtration (Fig. 8) exceeds the predicted value of 190,000 as observed for the complex by SDS-PAGE (Figs. 6 and 9). ClINH has been reported to be a molecule with high asymmetry and thus a high frictional ratio on a sieving column. On denaturation in SDS, this asymmetry is lost, which results in an apparent molecular weight that is lower by PAGE. Similar observations have been reported for other enzyme-inhibitor complexes (44, 55).
plasma.

served by SDS-PAGE radioautography. gel).

have been observed in a recent study of the inhibition of prekallikrein activation during abdominal and laryngeal attacks of HAE (58), consisting of a decrease in functional Ci inhibitor, gives rise to the disease hereditary angioedema factor XIIa in plasma.

vulnerable to even small degrees of factor XII activation.

inactivate Ci, patients with HAE are known to have

of kallikrein, high molecular weight kininogen, was noted.

Ci inhibitor and

such a reaction would form bradykinin which might contrib-

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Regulation of Factor XIIa by Plasma Inhibitors

Supplementary Material

The Regulation of Plasma Factor XII by Plasma Proteinase Inhibitors

by Athos F. Fieschi, Marc Schrumpf and Robert M. Calman

Methods and Materials

Reagents: Prothrombin (Merck), human albumin (Sigma), and BGG (Bayer, Leverkusen, Germany) were obtained from the respective companies. Human Factor XII was a generous gift from Dr. P. D. Arkin, Wellcome Research Laboratories, Beckenham, Kent, England. Cinacalcet (Bayer) and human plasminogen were purchased from Calbiochem, La Jolla, CA. Human plasma obtained from healthy volunteers was purchased from Biologics, Inc., Madison, WI. Human Factor XII was produced in the laboratory by recombinant DNA technology. 

Enzyme Assays: Factor XIIa activity was assayed fluorometrically by generating the zymogen into the active enzyme. The reaction was stopped by the addition of 20 mM Tris-HCl, pH 7.6, and the absorbance at 405 nm was measured. The factor XIIa activity was inhibited by 5 mM EDTA and 1 mM PMSF. The inhibition was reversed by 100 mM DFP. 

Results: The kinetic properties of Factor XIIa were determined in the presence of the following inhibitors: 2 mM EDTA, 1 mM PMSF, 5 mM DFP, 1 mM PMSF and 10 mM EDTA, and 10 mM EDTA alone. The inhibition of Factor XIIa by 2 mM EDTA was completely reversed by the addition of 100 mM DFP. The inhibition by 1 mM PMSF was partially reversed by the addition of 100 mM DFP. The inhibition by 5 mM DFP was completely reversed by the addition of 100 mM DFP. 

Discussion: The results of this study indicate that the inhibition of Factor XIIa by these inhibitors is due to the reversible inhibition of the active site of Factor XIIa. The inhibition by 2 mM EDTA is due to the formation of a metalloproteinase complex. The inhibition by 1 mM PMSF is due to the formation of a metalloproteinase complex. The inhibition by 5 mM DFP is due to the formation of a metalloproteinase complex. 

Conclusion: The inhibition of Factor XIIa by these inhibitors is due to the reversible inhibition of the active site of Factor XIIa. These results suggest that the inhibition of Factor XIIa by these inhibitors is due to the reversible inhibition of the active site of Factor XIIa. 

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Regulation of Factor XIIa by Plasma Inhibitors

The inhibition of factor XII by UN also followed pseudo first-order inhibition kinetics (Figure 2) with a k determined to be 0.0661 M⁻¹ min⁻¹. The inhibition was 25 times weaker than C1 INH but 2 times stronger than ATIII (Table 1). If the reciprocal plot is extrapolated, it intercepts with the ordinate at negative values of k which are not determinable. Therefore, the k and NH were not determinable. These results are consistent with the concept that factor XII is inhibited by each molecule of inhibitor where more than one molecule of enzyme may be able to be inactivated by each molecule of inhibitor (see Discussion). Factor XIIa was not inhibited by UN at high concentrations (17 µM data not shown). The inhibition of factor XII by UN was determined using an integrator form of the second-order rate equation (see Methods) and the results are shown in Figure 5. The second-order rate constant was 1.1×10⁻⁵ M⁻¹ min⁻¹. The value of k determined by this method was equal to the value for k as determined by pseudo first-order kinetics. The k data indicates that UN is 20-fold weaker than C1 INH in its ability to inhibit factor XIIa.

FIGURE 1: Purified human factor XIIa. SDS PAGE of 10 µg of factor XIIa using a 10% acrylamide running gel and 5% stacking gel according to Lowell. Left lane contains the ENR Red. Right lane contains the NR Red. The numbers on the left are the Mr of the proteins based on the Mr of the standards (see Methods) on the right.

FIGURE 2: Inactivation of factor XIIa by C1 INH. Factor XIIa, 0.17 µM was incubated with various concentrations of C1 INH at 37°C in polypropylene Eppendorf tubes. Incubation buffer was 0.02 M Tris, 0.1 M NaCl, pH 7.8. Aliquots were removed at appropriate intervals and assayed for residual factor XIIa activity using the chromogenic substrate, B-2800. The decrease in activity follows first-order kinetics as described in Methods. Each point is the mean of 2 determinations done in duplicate. The line graph was generated by an inverse plot to a second-order rate equation

FIGURE 3: Inactivation of factor XIIa by ATIII. Factor XIIa, 0.11 µM was incubated with various concentrations of ATIII. Methodology and assay is the same as in Figure 2. Each point is the mean of 2 determinations done in duplicate.

FIGURE 4: Inactivation of factor XIIa by UN. Factor XIIa, 0.15–0.18 µM was incubated with various concentrations of UN. Methodology and assay is the same as in Figure 2. Each point is the mean of 2 determinations done in duplicate.

FIGURE 5: Inactivation of factor XIIa by UN. Factor XIIa, 0.21–0.25 µM was incubated with various concentrations of UN under the same condition as described in Figure 2. Aliquots of the incubation were withdrawn at various time points and assayed for residual factor XIIa activity. The data indicated that UN was 20-fold weaker than C1 INH in its ability to inhibit factor XIIa.

Reactions of radiolabeled factor XIIa with purified inhibitors. In order to confirm that complex formation occurred between factor XIIa and inhibitor during the inhibition reaction, 125I-labeled factor XIIa was incubated with each inhibitor and the resulting complexes were analyzed by immunoblotting of SDS PAGE. This method was used to confirm the presence of factor XIIa and inhibitor in the reaction mixture. The lower molecular weight complexes were then digested with a 5% trypsin in 50 mM NH₄HCO₃ buffer at pH 8.0. The resulting tryptic peptides were separated by SDS PAGE and stained with Coomassie Brilliant Blue R-250.

The results of this experiment demonstrated that complex formation occurred between factor XIIa and each inhibitor in the reaction mixture. The lower molecular weight complexes were then digested with a 5% trypsin in 50 mM NH₄HCO₃ buffer at pH 8.0. The resulting tryptic peptides were separated by SDS PAGE and stained with Coomassie Brilliant Blue R-250.

Comparison of the second-order rate constants of the inhibitors of factor XIIa. The kinetic data derived from the reactions using the purified proteins is summarized and compared in Table 1. The values of k for each inhibitor indicate that C1 INH is 26.4, and 111 times better inhibitor of factor XII than UN, AT III, and ATIII respectively. C1 INH was the most effective inhibitor tested.

To further confirm the ability of C1 INH and the second-order rate constant, k, for each inhibitor indicates that C1 INH is significantly better inhibitor of factor XIIa than UN, AT III, and AT III respectively. C1 INH was the most effective inhibitor tested.

The inhibition of factor XIIa in plasma. The inhibition of factor XIIa was studied in plasma containing various concentrations of C1 INH (Table 1). The inhibitor concentration in each plasma was determined using a chromogenic assay (see Methods). The inhibitor activity was determined using the chromogenic assay. The inhibitor concentration in each plasma was determined using a chromogenic assay (see Methods). The inhibitor activity was determined using the chromogenic assay.
 Regulation of Factor XIIa by Plasma Inhibitors

To observe the formation of enzyme-inhibitor complexes of factor XIIa reacting with the intrinsic inhibitors in plasma, 125I-labeled factor XIIa was incubated with purified CIIRN or in plasma and subjected to gel filtration on Sephadex G-200. The Ns in these experiments were determined by a calibration curve constructed with normal plasma. Three peaks were identified by absorbance at 280 nm (A) and by gel filtration (B). The first peak identified at 125000 Da correlated with peak 1 (red triangles), the second peak identified at 100000 Da correlated with peak 2 (black circles), and the third peak identified at 90000 Da correlated with peak 3 (black squares). The absorption at 280 nm of the plasma was determined by the absorbance at 280 nm (A) and the second peak identified by gel filtration (B). The elution profile of factor XIIa from normal plasma is shown in Figure 1.

FIGURE 1: Activation of factor XIIa in plasma. Enzyme activity was assayed with chromogenic substrate. The plasma contained 0.1% (v/v) Nla. Fifty microliters of factor XIIa was incubated with plasma at a final concentration of 0.09% to 0.35% (v/v) to observe if factor XIIa was formed. Each point in the graph was determined for each time point (A) and adjusted for the effect of Nla on the rate of inhibition and estimation of the contribution of factor XIIa from the plasma. Factor XIIa-deficient plasma (▵), Factor XII-deficient plasma (■), Factor XII-deficient plasma (●), Factor XII-deficient plasma (□), Factor XII-deficient plasma (▲), Factor XII-deficient plasma (△), Factor XII-deficient plasma (♦). For data analysis, the line of the theoretical Elab corrected for dilution.