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Inhibition of Factor XIa by Antithrombin III

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ABSTRACT: The inactivation of human factor XIa by human antithrombin III was studied under pseudo-first-order reaction conditions (excess antithrombin III) both in the absence and in the presence of heparin. The time course of inactivation was followed by using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. After electrophoresis, proteins were blotted onto nitrocellulose and stained either for glycoprotein or for antithrombin that links the contact phase to the intrinsic factor IX activation. Factor XI, is composed of two heavy chains (80,000 molecular weight) held together by (a) disulfide bond(s). Factor XI, is converted to factor XI, by the cleavage of an internal peptide bond in both precursor chains. Factor XI, is composed of two heavy chains (M, 50,000) and two light chains (M, 33,000) and contains one active site at each light chain (Bouma et al., 1977; Fujikawa et al., 1986).

It is the only known enzyme participating in blood coagulation that contains two active sites.

Four plasma protease inhibitors, α1-antitrypsin, antithrombin III, C1 inhibitor, and α2-antiplasmin, have been reported to inactivate human factor XIa. In plasma, α1-antitrypsin is thought to be the main factor XIa inhibitor followed by antithrombin III (Scott et al., 1982a). However, the inactivation by antithrombin III can be accelerated in the presence of heparin (Damas et al., 1973). Scott et al. reported a 4-fold enhancement while Beeler et al. reported a 40-fold acceleration of the inactivation at saturating heparin concentrations (Scott et al., 1982b; Beeler et al., 1986).

The stoichiometry of the complex formed between factor XIa and antithrombin III has shown to be 1 mol of factor XIa to 2 mol of inhibitor (Kurachi & Davie, 1977), indicating that both active sites interact with antithrombin III. Thus, the presence of an intermediate, factor XIa complexed with one antithrombin III, formed during the inactivation of factor XIa is likely to be expected but has not been demonstrated yet.

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The kinetics of the inactivation of both active sites of factor XI\textsubscript{a} as well as the mutual interactions of factor XI\textsubscript{a}, antithrombin III, and heparin remain also to be established.

This study was undertaken to explore the kinetics of the inactivation of factor XI\textsubscript{a} by antithrombin III in the presence and absence of heparin. A model is suggested in which the two active sites of factor XI\textsubscript{a} are inhibited independent of each other by antithrombin III and in which the enhancement of the inactivation due to the presence of heparin is correlated to the binding of antithrombin III to heparin.

**Materials and Methods**

**Materials.** Chromogenic substrates pyro-Glu-Pro-Arg-p-nitroanilide (pNA) (S2366) and H-d-Pro-Phe-Arg-pNA (S2302) were purchased from AB Kabi Diagnostica, Sweden. Rabbit anti-human antithrombin III, concanavalin A, and horseradish peroxidase were obtained from Sigma Chemical Co., St. Louis, MO. Nitrocellulose membrane was from Bio-Rad Laboratories, Richmond, CA. 3,3'-Diaminobenzidine tetrahydrochloride was purchased from Fluka AG, Switzerland. Swine anti-rabbit IgG horseradish peroxidase conjugated antibodies were obtained from Nordic, Tilburg, Holland. All reagents used were of the highest grade commercially available.

**Proteins.** Human factor XI was purified according to Bouma et al. (1983). Human factor XII was isolated as described by Griffin and Cochrane (1976). Human antithrombin III was purified as described by Thaler and Schmer (1975). \( \beta \)-Factor XII\textsubscript{a} was prepared from purified factor XII as described by Fujikawa and McMullen (1983). Human serum albumin (Sigma) was further purified on a concanavalin A-Sepharose column to remove glycoprotein impurities. Factor XI, factor XII, and antithrombin III preparations were homogeneous and pure as determined by gel electrophoresis in the presence of sodium dodecyl sulfate on 10% gels according to Laemmli (1970). The specific activities determined in a clotting assay for factors XI and XII were 211 and 74 units/mg, respectively, assuming 1 unit to be present per milliliter of normal human plasma. All proteins were stored at \(-70^\circ C\) after dialysis against 50 mM Tris-HCl and 150 mM NaCl at pH 8.0.

**Preparation of human factor XI\textsubscript{a} from human factor XI using human \( \beta \)-factor XII\textsubscript{a} was performed as described earlier (Soons et al., 1986). Factor XI\textsubscript{a} was separated from \( \beta \)-factor XII\textsubscript{a} on a DEAE-Sephadex column (1.5 \( \times \) 11.5 cm) at 4 \( ^\circ C \) in 50 mM Tris-HCl and 150 mM NaCl at pH 8.0.

Amidolytic activity of human factor XI\textsubscript{a} was measured using the chromogenic substrate S2366 in a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 20 mM EDTA, and 0.5 mg/mL human serum albumin, pH 7.9. The kinetic parameters for the hydrolysis of the chromogenic substrate S2366 by human factor XI\textsubscript{a} were \( k_{\text{cat}} = 0.42 \text{mM/s} \) and \( k_{\text{m}} = 758 \text{s}^{-1} \).

**Protein concentrations** were routinely determined according to Bradford (1976). Factor XI\textsubscript{a} concentrations were expressed as 80,000 molecular weight subunits (van der Graaf et al., 1983). Antithrombin III concentration was measured by employing an \( E_{\text{280nm}} \) (1%) of 5.7 (Kurachi & Davie, 1977) and by a titration against a known concentration of human thrombin.

**Gel Electrophoretic Analysis.** Proteins were subjected to gel electrophoresis in the presence of sodium dodecyl sulfate on 5% slab gels as described by Laemmli (1970) and subsequently transferred onto nitrocellulose sheets as described by Towbin et al. (1979). The blots were soaked in 0.1% bovine serum albumin and 0.05% Tween-20 in phosphate-buffered saline. Immunological detection of antithrombin III and antithrombin III-protease complexes was performed essentially as described by Towbin et al. (1979) with rabbit anti-human antithrombin III and using swine anti-rabbit IgG conjugated with horseradish peroxidase as second antibodies. Glycoprotein staining was performed as described by Clegg (1982) by incubating the blots with concanavalin A (50 \( \mu \text{g/mL} \)) in phosphate-buffered saline containing 1 mM CaCl\(_2\), 0.1 mM MgCl\(_2\), 0.1 mM MnCl\(_2\), 0.1% bovine serum albumin, and 0.05% Tween-20. Horseradish peroxidase (10 \( \mu \text{g/mL} \)) in the same buffer was used as indicator. Staining of the blots was achieved by soaking in a freshly prepared and filtered solution of diaminobenzidine tetrahydrochloride (0.5 mg/mL) and 0.01% \( \text{H}_2\text{O}_2 \) in 0.05 M Tris-HCl, pH 7.5.

**Fluorescence studies** were performed with an SLM/Aminco SPF-500 C spectrophotometer at 25 \( ^\circ C \). The excitation and emission wavelengths were 285 and 345 nm (band-passes of 2 and 5 nm, respectively). The interaction of heparin with antithrombin III was measured by changes in the intrinsic fluorescence of antithrombin III. Small aliquots of heparin were added in succession to a solution of antithrombin III (1 and 2 \( \mu \text{M} \)) in 50 mM Tris-HCl/100 mM NaCl, pH 7.5. After each addition, the intrinsic fluorescence intensity of antithrombin III was determined. The data were plotted, and the titer was measured from the point of intersection of lines drawn through the ascending limbs and the plateaus of the plots.

**Kinetic Data Analysis.** The disappearance of factor XI\textsubscript{a} amidolytic activity appeared to follow pseudo-first-order reaction kinetics both in the absence and in the presence of heparin over the whole time course of inactivation. Since inactivation was only complete upon covalent binding of two antithrombin III molecules to each factor XI\textsubscript{a} dimer, it appears that the reaction of a catalytic site of factor XI\textsubscript{a} occurs in a random fashion and is not influenced by the fact of whether or not the other active site in the dimer has already been occupied by another antithrombin III (AT-III) molecule (also see Results). Therefore, the inactivation can be described as

\[
\text{XI}_\text{a} + \text{AT-III} \xrightarrow{k_1} \text{XI}_\text{a}-\text{AT-III} \tag{I}
\]

in which \( \text{XI}_\text{a} \) is the concentration of factor XI\textsubscript{a} expressed per \( M_\text{r} \), 80,000 subunit. The rate constant \( k_1 \) can then be obtained as described in the legend to Figure 1.

The stimulation of the inhibition of factor XI\textsubscript{a} by antithrombin III due to the presence of heparin was assumed to be due to the binding of antithrombin III to heparin. In that case, the reaction is given as

\[
\text{XI}_\text{a} + \text{AT-III} \xrightarrow{k_1} \text{XI}_\text{a}-\text{AT-III} \tag{II}
\]

\[
\text{XI}_\text{a} + \text{AT-III} \xrightarrow{k_2} \text{XI}_\text{a}-\text{AT-III} \tag{III}
\]

in which \( \text{XI}_\text{a} \) is again the concentration of factor XI\textsubscript{a} expressed per \( M_\text{r} \), 80,000 subunit and in which AT-III\textsubscript{a} and AT-III\textsubscript{b} are the concentrations of free antithrombin III and of antithrombin III bound to heparin, respectively. \( k_1 \) and \( k_2 \) are the rate constants of inactivation by free antithrombin III and by the antithrombin III–heparin complex. Assuming rapid binding equilibrium, the rate equation can be written as

\[
d[\text{XI}_\text{a}] /dt = -(k_1[\text{AT-III}_1] + k_2[\text{AT-III}_b])[\text{XI}_\text{a}] \tag{IV}
\]

and the slope of a pseudo-first-order plot will equal \(-k_1[\text{AT-III}_1] + k_2[\text{AT-III}_b] \). \( k_1 \) and \( k_2 \) can be independently
In vitro, amidolytic activity present in the absence of antithrombin was determined by measuring the residual amidolytic activity remaining. The residual amidolytic activity was expressed as the percentage of the original amidolytic activity remaining was determined by measurement of the rate of change in absorbance at 405-500 nm on an Aminco DW2a spectrophotometer set in the dual-wavelength mode. The residual amidolytic activity was expressed as the percentage of the original amidolytic activity present in the absence of antithrombin III taken as 100%.

Panel A shows the pseudo-first-order rate constant obtained from the slopes of the plots shown in panel A as a function of the concentration of antithrombin III present.

**FIGURE 1: Inactivation of human factor XI by human antithrombin III in the absence of heparin.** Factor XI was incubated at 37 °C in 50 mM Tris (pH 7.5 at 37 °C), 175 mM NaCl, 20 mM EDTA, and 0.5 mg/mL human serum albumin. After 5 min, reaction was started by adding varying amounts of antithrombin III. Final concentrations were 8 nM factor XI and antithrombin III in micromolar as indicated in the figure. (Panel A) At the time points indicated, aliquots were removed from the reaction mixture and diluted 20-fold in the above-mentioned buffer containing 0.29 mM S2366. The amidolytic activity remaining was determined by measurement of the rate of change in absorbance at 405-500 nm on an Aminco DW2a spectrophotometer set in the dual-wavelength mode. The residual amidolytic activity was expressed as the percentage of the original amidolytic activity present in the absence of antithrombin III taken as 100%. Panel B shows the pseudo-first-order rate constant obtained from the slopes of the plots shown in panel A as a function of the concentration of antithrombin III present.

**FIGURE 2: Inactivation of human factor XI by human antithrombin III in the presence of heparin.** Factor XI (8 nM) and heparin (0.1 mg/mL) were preincubated at 37 °C in 50 mM Tris (pH 7.5 at 37 °C), 175 mM NaCl, 20 mM EDTA, and 0.5 mg/mL human serum albumin. After 5 min, reaction was started with the addition of antithrombin III to result in a final concentration of 64 nM. At the time points indicated in the figure, aliquots were withdrawn from the reaction mixture for the determination of the amidolytic activity remaining (panel A) and for gel electrophoresis under nonreducing conditions on 5% slab gels (panels B and C). (Panel A) From the residual amidolytic activity present in the sample, the pseudo-first-order plot was constructed as described in the legend to Figure 1. The obtained pseudo-first-order reaction rate constant was 0.13 mg/mL min. (Panel B) A thrombin-protein complex was blotted onto nitrocellulose sheets and stained for glycoprotein using concanavalin A. (Panel C) A second blot was stained for antithrombin III antigen using antibodies against human antithrombin III. For further experimental details, see Materials and Methods.
occupied has never been shown, and no attempts have been made yet to correlate the occurrence of the various reaction products with the disappearance of factor XIa amidolytic activity. Therefore, during the time course of factor XIa inactivation, samples were withdrawn from the reaction mixture and subjected to sodium dodecyl sulfate (SDS)-5% polyacrylamide gel electrophoresis under nonreducing conditions. The protein bands were blotted onto nitrocellulose sheets and subsequently stained for glycoprotein using concanavalin A (Figure 2B). Since factor XIa and antithrombin III are glycoproteins, both were stained by using this method. Factor XIa was present as a single band, and antithrombin III migrated at the dye front of the gel (lane C, Figure 2B). Free antithrombin III was not visible on the blots because it migrates at the dye front together with an excess of carrier protein (human serum albumin) that interferes with the staining of the free antithrombin III. The glycoprotein pattern on the blot showed three protein bands, which changed in intensity during the inactivation of factor XIa. The protein band representing factor XIa decreased in intensity during the inactivation, and after 10 min, no factor XIa was visible. Concomitantly, two slower migrating bands appeared. Both these bands stained positive for antithrombin III antigen using rabbit anti-human antithrombin III antibodies (Figure 2C). Free factor XIa was not visible on this blot. These results demonstrate the appearance and disappearance of the intermediate (in which only one active site is occupied in factor XIa) during the time course of the reaction. This intermediate, which represents the faster migrating band, rapidly appeared and reached an optimum between 2 and 15 min after which it slowly decreased. The slowest migrating band, representing the final reaction product (one factor XIa and two antithrombin III molecules), gradually increased during the time course of factor XIa inactivation. From the data represented in Figure 2A–C, it is clear that only the final reaction product had no amidolytic activity. However, although factor XIa rapidly disappeared within 10 min and substantial amounts of intermediate were visible on the gels, the reaction remained first order throughout the whole time course of inactivation (Figure 2A). This strongly suggests that both active sites in factor XIa are inactivated with the same rate constant independent of each other (i.e., irrespective of whether or not the other site is already occupied). In such a model, the distribution of the various reaction products (i.e., XIa, XIa-AT-III, and XIa2AT-III) at each given time interval can be calculated by using the rate constant determined from the pseudo-first-order plot (see also legend to Figure 3). Figure 3 shows the result of such a calculation for the experiment described in Figure 2. As can be seen, the appearance and disappearance of the various reaction products as calculated correlate quite well with the distribution of these products as seen on the blots in Figure 2.

The inactivation of factor XIa by antithrombin III in the presence of heparin is a three-component reaction. To investigate the mutual interactions, the concentration of each of the three components was varied while the two others were kept constant. Figure 4A shows that the pseudo-first-order reaction rate constant increased, when factor XIa (8 nM) and antithrombin III (0.1 μM) were titrated with heparin (0.067–10 μM) and reached a plateau at a heparin concentration of approximately 5 μM. The second-order reaction rate constant at saturating heparin concentrations was calculated to be 26.7 × 10^3 M^−1 s^−1. Figure 4B shows that the pseudo-first-order reaction rate constant also increased, when at a constant concentration of factor XIa (8 nM) and heparin (0.3 μM) the concentration of antithrombin III was increased (0.05–1.0 μM). The rate constant did not reach a plateau (closed circles). This is due to the fact that the rate constant of factor XIa inhibition in the absence of heparin (closed triangles) was not negligible. The pseudo-first-order reaction rate constant did not change (k = 0.15 min^−1) when heparin (0.17 μM) and antithrombin III (1.0 μM) were titrated with factor XIa (0.1–200 nM). These data suggest that the rate of inactivation of factor XIa by antithrombin III in the presence of heparin is a direct measure for the binding of antithrombin III to heparin and that this binding is not influenced by factor XIa. That is the case for the data presented in Figure 5 in which the data of the experiment presented in Figure 4 were used to obtain the binding data of antithrombin III to heparin. For this, the data were analyzed as described under Materials.
and Methods using rate constants of $10^3 \text{M}^{-1} \text{s}^{-1}$ for the inhibition of factor XI$_a$ by free antithrombin III and of $26.7 \times 10^3 \text{M}^{-1} \text{s}^{-1}$ for heparin-bound antithrombin III. Figure 5 shows a plot calculated from the data shown in Figure 4B. As can be seen, a straight line was obtained from which a $K_d$ of 142 nM was determined. The amount of sites present on heparin was calculated to be 0.42 mol of antithrombin III per mole of heparin assuming a molecular weight of 15000 for the heparin.

**Discussion**

The results presented in this paper give insight in the way via which the two active sites in factor XI$_a$ are inhibited by antithrombin III. In agreement with earlier studies (Kurachi & Davie, 1977; Scott et al., 1982a), it was found that the final reaction product has no amydolistic activity and consists of a complex of factor XI$_a$ to which two antithrombin III molecules become attached. However, the experiments presented in Figure 2 also clearly demonstrate the occurrence of the intermediate consisting of one factor XI$_a$ with one antithrombin III molecule as a transient product during the time course of the reaction. To our knowledge, this is the first time that this intermediate has been unequivocally demonstrated using gel electrophoretic techniques. The intermediate appears amydolistic active since even when the band representing free factor XI$_a$ has disappeared there is still considerable amydolistic activity present in the reaction mixture. However, in agreement with the literature (Scott et al., 1982b), it was found that both in the absence and in the presence of heparin the disappearance of factor XI$_a$ amydolistic activity cannot be distinguished from pseudo-first-order kinetics over the whole time course of the reaction. This is most likely explained by a mechanism in which both active sites of factor XI$_a$ interact with antithrombin III in random order (i.e., independent of each other) with the same rate constant. The changes in the distribution of the reaction products visible on the gels during the time course of the reaction correlate well with changes calculated according to such a model (cf. Figures 2 and 3).

Simulation experiments showed that, as soon as the two rate constants would differ by a factor of 2 or more, a deviation of pseudo-first-order kinetics would have been readily observable (data not shown).

The kinetic data obtained in the presence of heparin can be used to determine the binding parameters of antithrombin III binding to heparin (cf. Figures 4 and 5). The $K_d$ of 142 nM determined is in good agreement with that reported in the literature (Griffith, 1982). The stoichiometry of the interaction of antithrombin III with heparin was found to be 0.42 mol of antithrombin III/mol of heparin assuming an average molecular weight of 15000 for the heparin used. This is somewhat lower than that reported in the literature (Nesheim et al., 1986). The stoichiometry of the interaction was confirmed for the used reactants by independent measurement using the fluorescence technique described by Nesheim et al. (1986) (data not shown). Therefore, it seems justified to conclude that the stimulation of the reaction by heparin is due to the binding of antithrombin III to heparin and since the inactivation remains first order for all the concentrations of factor XI$_a$ tested (0.1 -200 nM) the binding of antithrombin III to heparin is apparently not influenced by factor XI$_a$ and/or by the reaction products formed. It has been shown that factor XI$_a$ binds to heparin-Sepharose (osterud & Rapaport, 1977). Therefore, it can be expected that factor XI$_a$ may interact with heparin under our experimental conditions. At the moment, we do not know whether binding of factor XI$_a$ to heparin influences the rate of factor XI$_a$ inactivation by antithrombin III. If such is the case, however, the fact that the reaction is saturable with heparin and the fact that the kinetics are first order in factor XI$_a$, indicate then either that all factor XI$_a$ has to be bound or that no factor XI$_a$ is bound at all to heparin under our conditions.

The rate constants determined were $10^3 \text{M}^{-1} \text{s}^{-1}$ in the absence of heparin and $26.7 \times 10^3 \text{M}^{-1} \text{s}^{-1}$ in the presence of saturating amounts of heparin. This rate enhancement induced by heparin is in good agreement with the value reported by Beeler et al. (1986). However, the actual values of rate constants reported here are considerably higher than earlier reported values (Beeler et al., 1986; Scott et al., 1982b). Apart from differences in experimental conditions, a possible explanation for these discrepancies is that in the present study factor XI activated by $\beta$-factor XI$_a$ was used whereas in the previous studies trypsin-activated factor XI has been used. Similar differences have been reported for the enzymatic activity of factor XI$_a$ in the activation of factor IX (Walsh et al., 1984; Soons et al., 1986). Beeler et al. have argued that under certain conditions antithrombin III may become a physiologically significant inhibitor of factor XI$_a$ (Beeler et al., 1986). The finding that the rates of inactivation of $\beta$-factor XI$_a$ activated factor XI are even higher than the values reported by these authors can be regarded to support this concept. It should be stressed, however, that the conditions in whole plasma are so much more complicated than those in purified systems that much more will need to be done to gain insight in the biological importance of the regulation of factor XI$_a$ inactivation by antithrombin III.

Finally, we would like to emphasize that our data pertain to the mechanism of the interaction of the two active sites in factor XI$_a$ with its macromolecular substrate antithrombin III. The intriguing question remains whether the remaining active site in a factor XI$_a$ molecule, complexed with one antithrombin III, can also interact with factor IX the same way as free factor XI$_a$ or whether in this case changes occur in the mechanism of activation of factor IX by factor XI$_a$.

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Acceptor–Donor Relationships in the Transglutaminase-Mediated Cross-Linking of Lens β-Crystallin Subunits†

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ABSTRACT: Following the isolation of the Nα-(γ-glutamyl)lysine-containing polymers from human cataracts, our efforts were directed to induce such cross-links experimentally in rabbit lens, and evidence was obtained for the selective reactivities of certain β-crystallin subunits in this transglutaminase-catalyzed event. In the present work, we examined the enzymatic cross-linking of purified crystallins individually (α, βH, βL, and γ) and in combinations, with particular emphasis on forming the approximately 55K dimer. This species was the primary product in the cross-linking of βH-crystallins; βL also reacted with transglutaminase. Neither α- nor γ-crystallins formed appreciable amounts of cross-linked structures with transglutaminase. Dansylcadaverine, known to compete against the reactive lysines of proteins in forming Nα-(γ-glutamyl)lysine cross-bridges, was shown to inhibit the generation of dimeric and higher ordered oligomers from βH and βL. The fluorescent amine specifically labeled only two subunits in βH (29–30K and 26K) and one in βL (26K), identifying these substrates as possessing transglutaminase-reactive endo-γ-glutamylamin residues. An antiserum to bovine PBp recognized the 23K subunit of rabbit β-crystallins and also the 55K dimer, suggesting that the 23K protein participates as a lysine donor in generating the cross-linked dimer with transglutaminase. Inasmuch as the same antiserum reacts with a ~50K material reported to appear in increasing amounts with age in human lens, the results lend added support to the physiological significance of transglutaminase in the aging of lens.

Isolating Nα-(γ-glutamyl)lysine peptides from polymers which are characteristically present only in cataractous specimens called attention to the role of transglutaminase in lens (Lorand et al., 1981). Previous work focused on reactions catalyzed by the intrinsic enzyme, activated from its latent form by adding Ca2+ to homogenates of rabbit lens or to the whole organ. Using the enzyme-directed incorporation of amines ([14C]putrescine or N-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide (dansylcadaverine)1 as a strategy for identifying acceptor proteins [see Lorand and Conrad (1984)], it could be shown that, among all the crystallin components

1 Abbreviations: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; Mr, relative molecular weight; K, X106; dansylcadaverine, N-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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