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- Lottenberg, R., Hall, J. A., Fenton, J. W., II, & Jackson, C. M. (1982) *Thromb. Res.* 28, 313-332.
- Lundblad, R. L., Brown, W. V., Mann, K. G., & Roberts, H. R. (1980) *Chemistry and Biology of Heparin*, Elsevier/North-Holland, New York.
- Morrison, J. F. (1982) *Trends Biochem. Sci. (Pers. Ed.)* 7, 102-105.
- Morrison, J. F., & Stone, S. R. (1985) *Comments Mol. Cell. Biophys.* 2, 347-368.
- Morrison, J. F., & Walsh, C. T. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol.* (in press).
- Okamoto, S., Kinjo, K., Hijikata, A., Kikumoto, R., Tamao, Y., Ohkubo, K., & Tonomura, S. (1980) *J. Med. Chem.* 23, 827-830.
- Olson, S. T., & Shore, J. D. (1982) *J. Biol. Chem.* 257, 14891-14895.
- Rosenberg, R. D. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 36, 10-18.
- Segel, I. H. (1975) *Enzyme Kinetics*, pp 196-198, Wiley, New York.
- Simon, T. L. (1980) in *Chemistry and Biology of Heparin* (Lundblad, R. L., Brown, W. V., Mann, K. G., & Roberts, H. R., Eds.) pp 597-614, Elsevier/North-Holland, New York.
- Sonder, S. A., & Fenton, J. W., II (1984) *Biochemistry* 23, 1818-1823.
- Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry* 25, 4622-4628.
- Tollefsen, D. M., Feagler, J. R., & Majerus, P. M. (1974) *J. Biol. Chem.* 249, 2646-2651.
- Walker, B., Wikstrom, P., & Shaw, E. (1985) *Biochem. J.* 230, 645-650.
- Walsmann, P., & Markwardt, F. (1981) *Pharmazie* 36, 653-660.

Inhibition of Factor XI_a by Antithrombin III

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ABSTRACT: The inactivation of human factor XI_a 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 inhibition 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 III using antibodies against antithrombin III. Concomitant with factor XI_a inactivation, two new slower migrating bands, one of which represented the intermediate complex consisting of one antithrombin III complexed with factor XI_a, appeared as a transient band. Complete inactivation resulted in a single band representing the complex of factor XI_a with two antithrombin III molecules. Quantitative analysis of the time course of inactivation was accomplished by measurement of the disappearance of factor XI_a amidolytic activity toward the chromogenic substrate S2366. Pseudo-first-order reaction kinetics were observed throughout. The rate constant of inactivation was found to be $10^3 \text{ M}^{-1} \text{ s}^{-1}$ 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. From the kinetic data, a binding constant (K_d) of $0.14 \mu\text{M}$ was inferred for the binding of antithrombin III to heparin. The time course of inactivation and the distribution of the reaction products observed upon gel electrophoresis are best explained assuming a mechanism of inactivation in which the two active sites present in factor XI_a are inhibited in random order (i.e., independent of each other) with the same rate constant of inhibition.

Factor XI is a coagulation protein present in plasma in a precursor form (Bouma et al., 1977). It is the (pro)enzyme that links the contact phase to the intrinsic factor IX activation. Four proteins appear to be involved in contact activation reactions: factor XII, factor XI, prekallikrein, and high molecular kininogen (Cochrane & Griffin, 1982). Active factor XII with high molecular weight kininogen as a cofactor can convert factor XI to an active form (Griffin & Cochrane, 1979), which subsequently is able to activate factor IX by hydrolyzing two internal peptide bonds (Di Scipio et al., 1978).

Factor XI is a dimeric molecule which contains two identical protein chains (80 000 molecular weight) held together by (a) disulfide bond(s). Factor XI is converted to factor XI_a by the cleavage of an internal peptide bond in both precursor chains. Factor XI_a is composed of two heavy chains (M_r 50 000) and two light chains (M_r 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, α_1 -antitrypsin, antithrombin III, C₁ inhibitor, and α_2 -antiplasmin, have been reported to inactivate human factor XI_a. In plasma, α_1 -antitrypsin is thought to be the main factor XI_a inhibitor followed by antithrombin III (Scott et al., 1982a). However, the inactivation by antithrombin III can be accelerated in the presence of heparin (Damus 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 XI_a and antithrombin III has been shown to be 1 mol of factor XI_a to 2 mol of inhibitor (Kurachi & Davie, 1977), indicating that both active sites interact with antithrombin III. Thus, the presence of an intermediate, factor XI_a complexed with one antithrombin III, formed during the inactivation of factor XI_a 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_a as well as the mutual interactions of factor XI_a, antithrombin III, and heparin remain also to be established.

This study was undertaken to explore the kinetics of the inactivation of factor XI_a by antithrombin III in the presence and absence of heparin. A model is suggested in which the two active sites of factor XI_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). β -Factor XII_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 °C after dialysis against 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)/175 mM NaCl, pH 7.9, for human antithrombin III and 4 mM sodium acetate, 2 mM acetic acid, 0.15 M NaCl, and 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 5.0, for human factors XI and XII.

Heparin was crude porcine intestinal mucosal heparin (Organon). It had a mean molecular weight of 15 000 and an anti-X_a and anti-thrombin activity of 175 units/mg.

Preparation of human factor XI_a from human factor XI using human β -factor XII_a was performed as described earlier (Soons et al., 1986). Factor XI_a was separated from β -factor XII_a on a DEAE-Sephadex column (1.5 × 11.5 cm) at 4 °C in 50 mM Tris-HCl and 150 mM NaCl at pH 8.0.

Amidolytic activity of human factor XI_a was measured by 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 of the hydrolysis of the chromogenic substrate S2366 by human factor XI_a were $K_m = 0.42$ mM and $k_{cat} = 758$ s⁻¹.

Protein concentrations were routinely determined according to Bradford (1976). Factor XI_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_{280nm} (1%) of 5.7 (Kurachi & Davie, 1977) and by a titration against a known concentration 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 μ g/mL) in phosphate-buffered saline containing 1 mM CaCl₂, 0.1 mM MgCl₂, 0.1 mM MnCl₂, 0.1% bovine serum albumin, and 0.05% Tween-20. Horseradish peroxidase (10 μ 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% H₂O₂ in 0.05 M Tris-HCl, pH 7.5.

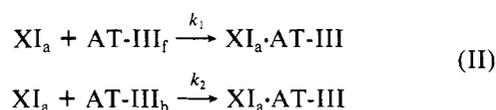
Fluorescence studies were performed with an SLM/Aminco SPF-500 C spectrophotometer at 25 °C. The excitation and emission wavelengths were 285 and 345 nm (band-passes of 2 and 5 nm, respectively). The interaction of heparin with antithrombin III was measured by changes in the intrinsic fluorescence of antithrombin III. Small aliquots of heparin were added in succession to a solution of antithrombin III (1 and 2 μ 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_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_a dimer, it appears that the reaction of a catalytic site of factor XI_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



in which XI_a is the concentration of factor XI_a expressed per M_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_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



in which XI_a is again the concentration of factor XI_a expressed per M_r 80 000 subunit and in which AT-III_f and AT-III_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

$$d[XI_a]/dt = -(k_1[AT-III_f] + k_2[AT-III_b])[XI_a] \quad (III)$$

and the slope of a pseudo-first-order plot will equal $-(k_1[AT-III_f] + k_2[AT-III_b])$. k_1 and k_2 can be independently

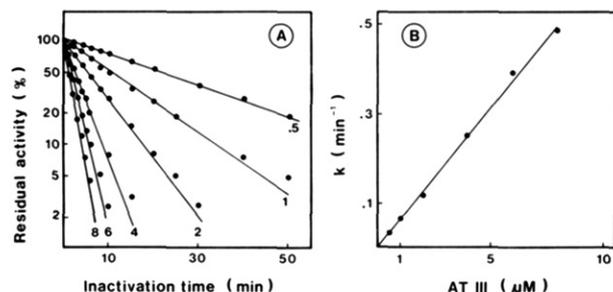


FIGURE 1: Inactivation of human factor XI_a by human antithrombin III in the absence of heparin. Factor XI_a was incubated at 37 °C in 50 mM Tris (pH 7.5 at 37 °C), 175 mM NaCl, 20 mM EDTA, and 0.5 mg/mL human serum albumin. After 5 min, reaction was started by adding varying amounts of antithrombin III. Final concentrations were 8 nM factor XI_a 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.

determined. Measurement of the reaction in the absence of heparin (all antithrombin III is free) yields k_1 and in the presence of saturating amounts of heparin (all antithrombin III is bound) yields k_2 . Thus, from the observed rates of factor XI_a inactivation, the binding of antithrombin III to heparin can be determined. It should be stressed that in the above equation it is tacitly assumed that the binding equilibrium of antithrombin III binding to heparin is not influenced by factor XI_a or by the produced factor XI_a–antithrombin III complexes. Since the reaction kinetics appear to remain first order in factor XI_a over a wide range of factor XI_a concentrations (0.1–200 nM), this assumption appears to hold.

Binding of Antithrombin III to Heparin. The binding parameters (K_d and amount of binding sites) of antithrombin III binding to heparin were determined from the kinetic data of factor XI_a inactivation at varying antithrombin III and

heparin concentrations. At a given heparin concentration, the rate of factor XI_a inhibition was determined at varying antithrombin III concentrations. At each concentration of antithrombin III, the amounts of antithrombin III bound and free were calculated from the slope of the pseudo-first-order plot of inactivation using the experimentally determined k_1 and k_2 as described above. The binding constant K_d is given as

$$K_d = [\text{AT-III}_f][\text{S}_f]/[\text{AT-III}_b] \quad (\text{IV})$$

and this can be rearranged to

$$1/[\text{AT-III}_b] = (K_d/S_{\text{tot}})(1/[\text{AT-III}_f]) + 1/S_{\text{tot}} \quad (\text{V})$$

Thus, the binding constant K_d and the total amount of binding sites present (S_{tot}) at the given heparin concentration can be determined from a plot of $1/[\text{AT-III}_b]$ vs. $1/[\text{AT-III}_f]$.

RESULTS

The inhibition of human factor XI_a by human antithrombin III was studied in the present and absence of heparin by measurement of the disappearance of factor XI_a amidolytic activity toward the chromogenic substrate S2366. The reaction was pseudo first order in factor XI_a since a semilogarithmic plot of the residual amidolytic activity vs. time yielded a straight line (Figure 1A). The apparent first-order rate constant obtained from the slope of these lines was directly proportional to the amount of antithrombin III present (Figure 1B). Therefore, in agreement with the literature (Scott et al., 1982a), the inactivation was found to be second order, and the rate constant obtained was $10^3 \text{ M}^{-1} \text{ s}^{-1}$. Figure 2A shows that in the presence of heparin (0.1 mg/mL) the reaction was also pseudo first order but the rate of inactivation was increased some 30-fold. At saturating amounts of heparin (see also below), the second-order rate constant of inactivation was $26.7 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$.

It has been shown that factor XI_a consists of two identical subunits of 80000 each which both contain an active site. The stoichiometry of the interaction of factor XI_a with antithrombin III has been shown to be 1:2 (Kurachi & Davie, 1977), indicating that both active sites become occupied. However, to our knowledge, the intermediate in which one active site is

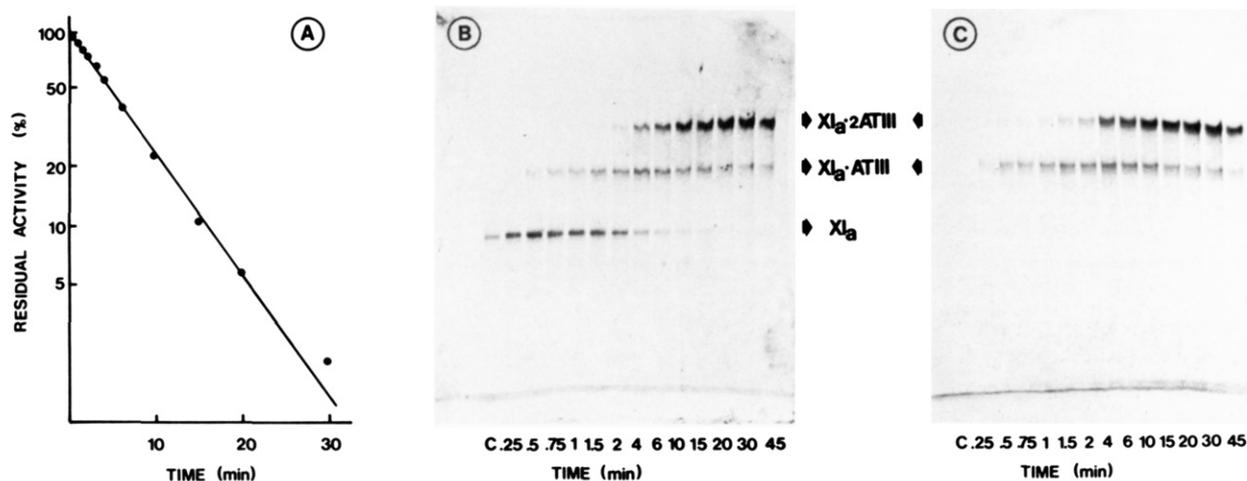


FIGURE 2: Inactivation of human factor XI_a by human antithrombin III in the presence of heparin. Factor XI_a (8 nM) and heparin (0.1 mg/mL) were preincubated at 37 °C in 50 mM Tris (pH 7.5 at 37 °C), 175 mM NaCl, 20 mM EDTA, and 0.5 mg/mL human serum albumin. After 5 min, reaction was started with the 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 min^{-1} . (Panel B) After gel electrophoresis, protein 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 XI_a amidolytic activity. Therefore, during the time course of factor XI_a 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 XI_a and antithrombin III are glycoproteins, both were stained by using this method. Factor XI_a 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 XI_a. The protein band, representing factor XI_a, decreased in intensity during the inactivation, and after 10 min, no factor XI_a 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 XI_a 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 XI_a) 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 XI_a and two antithrombin III molecules), gradually increased during the time course of factor XI_a 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 XI_a 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 XI_a 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., XI_a, XI_a·AT-III, and XI_a·2AT-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 XI_a 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 XI_a (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 $26.7 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. Figure 4B shows that the pseudo-first-order reaction rate constant also increased, when at a constant concentration of factor XI_a (8 nM) and heparin

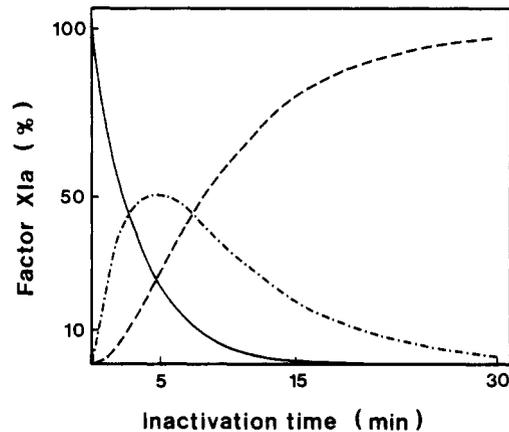


FIGURE 3: Calculated time course of appearance and disappearance of reaction products during the inactivation of factor XI_a by antithrombin III in the presence of heparin. The time course of the changes in the concentration of factor XI_a (—), factor XI_a complexed with one antithrombin III (---), and the final reaction product, factor XI_a complexed with two antithrombin III molecules (-.-), was calculated for the experiment presented in Figure 2. For the calculation, it was assumed that (1) both active sites had the same amidolytic activity, (2) both active sites were independent of each other inhibited by antithrombin III with the same measured pseudo-first-order reaction rate constant (k), and (3) the factor XI_a dimer disappeared with a rate constant of $2k$. With these assumptions, the amount of factor XI_a decreased in time according to Ce^{-2kt} while factor XI_a complexed with one or two antithrombin III's increased in time according to $2C(e^{-kt} - e^{-2kt})$ and $C(1 - 2e^{-kt} + e^{-2kt})$, respectively (Moore, 1972). The constant C was the amount of factor XI_a present at the beginning and was taken as 100% and k was 0.13 min^{-1} as obtained from the data of Figure 2.

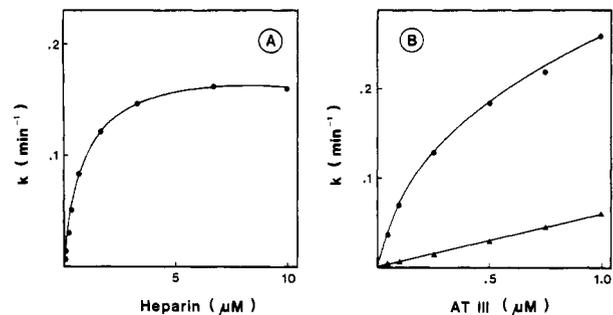


FIGURE 4: Titration experiments. Panel A shows the changes in the pseudo-first-order reaction rate constant, k , when factor XI_a (8 nM) and antithrombin III (100 nM) were titrated with heparin (range: 0.067–10 μM). The pseudo-first-order reaction rate constants were obtained as described in the legend to Figure 1, and at least seven time points were used. Panel B shows the changes in the pseudo-first-order reaction rate constant, k , when factor XI_a (8 nM) and heparin (0.3 μM) were titrated with antithrombin III (range: 0.05–1.0 μM) (closed circles). This experiment was also performed in the absence of heparin (closed triangles).

(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 XI_a inhibition in the absence of heparin (closed triangles) was not negligible. The pseudo-first-order reaction rate constant did not change ($k = 0.15 \text{ min}^{-1}$) when heparin (0.17 μM) and antithrombin III (1.0 μM) were titrated with factor XI_a (0.1–200 nM). These data suggest that the rate of inactivation of factor XI_a 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 XI_a. That this is indeed the case is illustrated 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

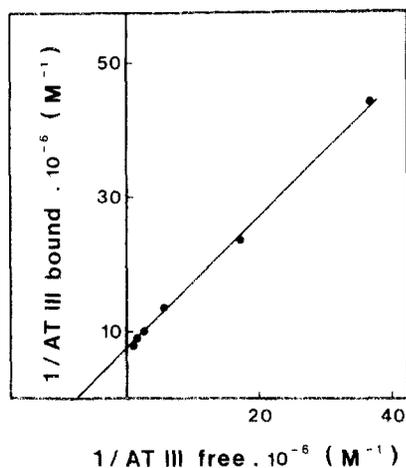


FIGURE 5: Replot of the data of Figure 4B according to the model presented in eq II. For each total antithrombin III concentration, the concentrations of bound and free antithrombin III were calculated as described under Materials and Methods, and their reciprocals were plotted against each other. The binding parameters of the antithrombin III-heparin interaction were a K_d of 142 nM and 0.42 antithrombin III binding site per mole of heparin according to eq V.

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 15 000 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 amidolytic 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 amidolytically active since even when the band representing free factor XI_a has disappeared there is still considerable amidolytic 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 amidolytic 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 15 000 for the heparin used. This is somewhat lower than 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 (østerud & 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 β -factor XII_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 β -factor XII_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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REFERENCES

- Beeler, D. L., Marcum, J. A., Schiffman, S., & Rosenberg, R. D. (1986) *Blood* 67, 1488.
- Bouma, B. N., & Griffin, J. H. (1977) *J. Biol. Chem.* 252, 6432.
- Bouma, B. N., Vlooswijk, R. A. A., & Griffin, J. H. (1983) *Blood* 62, 1123.
- Bradford, M. M. (1976) *Anal. Biochem.* 72, 248.
- Clegg, J. C. S. (1982) *Anal. Biochem.* 127, 389.
- Cochrane, C. G., & Griffin, J. H. (1982) *Adv. Immunol.* 33, 241.
- Damus, P. S., Hicks, M., & Rosenberg, R. D. (1973) *Nature (London)* 246, 355.
- Di Scipio, R. G., Kurachi, K., & Davie, E. W. (1978) *J. Clin. Invest.* 61, 1528.
- Fujikawa, K., & McMullen, B. A. (1983) *J. Biol. Chem.* 258, 10924.
- Fujikawa, K., Chung, D. W., Hendrickson, L. E., & Davie, E. W. (1986) *Biochemistry* 25, 2417.
- Griffin, J. H., & Cochrane, C. G. (1976) *Methods Enzymol.* 45, 56.
- Griffin, J. H., & Cochrane, C. G. (1979) *Semin. Thromb. Hemostasis* 5, 254.
- Griffith, M. J. (1982) *J. Biol. Chem.* 257, 13899.
- Kurachi, K., & Davie, E. W. (1977) *Biochemistry* 16, 5831.
- Laemmli, U. K. (1970) *Nature (London)* 227, 680.
- Moore, W. J. (1972) *Physical Chemistry*, p 345, Prentice-Hall, London.
- Nesheim, M., Blackburn, M. K., Lawler, C. M., & Mann, K. G. (1986) *J. Biol. Chem.* 261, 3214.
- Østerud, B., & Rapaport, S. I. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 5260.
- Scott, C. F., Schapira, M., James, H. L., Cohen, A. B., & Colman, R. W. (1982a) *J. Clin. Invest.* 69, 844.
- Scott, C. F., Schapira, M., & Colman, R. W. (1982b) *Blood* 60, 940.
- Soons, H., Janssen-Claessen, T., Hemker, H. C., & Tans, G. (1986) *Blood* 68, 140.
- Thaler, E., & Schmer, G. (1975) *Br. J. Haematol.* 31, 233.
- Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 4350.
- van der Graaf, F., Greengard, J. S., Bouma, B. N., Kerbiriou, D. M., & Griffin, J. H. (1983) *J. Biol. Chem.* 258, 9669.
- Walsh, P. N., Bradford, H., Sinha, D., Piperno, J. R., & Tuszyński, G. P. (1984) *J. Clin. Invest.* 73, 1392.

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

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ABSTRACT: Following the isolation of the *N*^ε-(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 , β_H , β_L , 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 β_H -crystallins; β_L 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 *N*^ε-(γ -glutamyl)lysine cross-bridges, was shown to inhibit the generation of dimeric and higher ordered oligomers from β_H and β_L . The fluorescent amine specifically labeled only two subunits in β_H (~29-30K and ~26K) and one in β_L (~26K), identifying these substrates as possessing transglutaminase-reactive endo- γ -glutamyl residues. An antiserum to bovine β Bp 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 *N*^ε-(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 Ca^{2+} 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

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¹ Abbreviations: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; *M*_r, relative molecular weight; K, $\times 10^3$; dansylcadaverine, *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.