Electrostatic interactions in the heparin-enhanced reaction between human thrombin and antithrombin

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Binding of heparin to thrombin is monitored by means of an aqueous two-phase partition system, and binding of heparin to antithrombin is monitored by means of heparin-induced enhancement of the intrinsic fluorescence of the protein. Both types of binding are studied at various electrolyte compositions of the medium. Heparin is displaced from thrombin at lower concentrations of electrolyte than those necessary for its displacement from antithrombin. K^+ is more efficient than Na^+ , which is again more efficient than Li^+ in displacing heparin from these proteins. The kinetics of the reaction between thrombin and antithrombin in the presence of heparin were studied by using an assay where synthetic peptide substrate is present in the reaction mixture during the reaction between proteinase and inhibitor. The kinetics are studied at various electrolyte compositions of the medium and the results are compared with those obtained from the binding studies performed under similar conditions. The results are consistent with a model where binding of heparin to antithrombin causes enhancement of the reaction rate, and where this enhancement is abolished again when additional binding of heparin to thrombin takes place on further addition of heparin.

Antithrombin is a plasma protein (M_r 60000–65000) that is present in the blood in micromolar concentrations. It is generally assumed that the primary function of this protein is to work as a tight-binding inhibitor of thrombin, but it is also known to inhibit most other serine proteinases of the coagulation process (see, e.g., Jackson & Nemer-son, 1980). It also inhibits trypsin (Wong *et al.*, 1982), plasmin (Machovich *et al.*, 1981) and urokinase (Clemmensen, 1978), but this may not have any physiological significance. A characteristic feature of the antithrombin–proteinase reaction is the ability of heparin to enhance the reaction rate. This effect of heparin is thought to serve as an ‘*in-vitro*’ model for the regulatory role in thrombosis and haemostasis of certain mucopolysaccharides located on the luminal surface of vascular endothelial cells (see, e.g., Lindahl & Höök, 1978). However, the exact mechanism of the heparin effect is still a matter of controversy. Some investigators have proposed that heparin acts primarily by its binding to antithrombin, and that interaction with heparin induces a more active conformation of the antithrombin molecule (Rosenberg & Damus, 1973;

Jordan *et al.*, 1980*a,b*; Olson *et al.*, 1981). Others claim that heparin binds preferentially to serine proteinases of the haemostatic system and that this interaction is significant to the heparin enhancement effect (Machovich, 1975; Griffith, 1979*a,b*). The physico-chemical properties of heparin are those of a polyanion, and the interactions with various proteins are likely to be of electrostatic nature. The importance of such interactions in the heparin-enhanced reaction between thrombin and antithrombin is in fact indicated by several observations on the effect of various electrolytes on the reaction rate (Machovich *et al.*, 1979; Griffith *et al.*, 1980; Long & Williamson, 1982), and also by studies on the ionic-strength-dependency of the binding of thrombin and antithrombin to heparin (Nordenman & Björk, 1977, 1978, 1980, 1981).

The effect of electrolyte composition on these interactions is examined in further detail in the present study, and the results are interpreted in terms of current mechanisms proposed for the heparin enhancement of antithrombin inhibition.

Materials and methods

Chemicals

Phe-Pip-Arg-Nan·2HCl (S-2238) was from Kabi A.B., Stockholm, Sweden, and *p*-nitrophenyl *p*-

Abbreviations used: Phe-Pip-Arg-Nan·2HCl, D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide dihydrochloride; Mops, 4-morpholinepropanesulphonic acid; SDS, sodium dodecyl sulphate.

guanidinobenzoate was from ICN Pharmaceuticals, Cleveland, OH, U.S.A.

Poly(ethylene glycol) (M_r 6000) was from Merck, Hohenbrun, Germany, and Dextran T 500 (M_r 500000) was from Pharmacia Fine Chemicals, Uppsala, Sweden. Lithium heparinate (Heparin-I) was from Sigma Chemical Co., St. Louis, MO, U.S.A., and high-affinity heparin (Heparin-II) prepared by affinity chromatography on antithrombin-Sepharose was a gift from Dr. L.-O. Andersson (Kabi A.B.).

Proteins

Highly purified human thrombin [618 NIH (National Institutes of Health) units/mg] was a gift from Dr. W. Lawson, New York State Department of Health, Albany, NY, U.S.A., to Dr. I. Clemmensen of this laboratory. Human antithrombin was a gift from Dr. L.-O. Andersson (Kabi A.B.). It was further purified on heparin-Sepharose and DEAE-Sephadex A-50 (Pharmacia) essentially as described by Miller-Andersson *et al.* (1974) and dialysed against 20 mM-Tris/Mops, pH 7.4. Both proteins were homogeneous as judged by SDS/polyacrylamide-gel electrophoresis. Crossed immunoelectrophoresis of purified antithrombin against anti-(human serum) (Clemmensen, 1978) revealed no impurities.

The active-site concentration of thrombin was determined with *p*-nitrophenyl *p*-guanidinobenzoate as described by Chase & Shaw (1969), and the concentration of antithrombin was determined by titration of known quantities of thrombin as described by Jesty (1979).

Heparin binding to thrombin

This was studied by means of an aqueous two-phase system. The general principle of such systems and the application to measurements of interaction between macromolecules have been described by Albertsson (1971, 1978). The two-phase system used in the present study contained 7.0% (w/w) dextran, 4.4% (w/w) poly(ethylene glycol) and 20 mM-Tris/Mops buffer, pH 7.4. Various concentrations of electrolyte (LiCl, NaCl or KCl) were added to this system. Thrombin and heparin were mixed into the two-phase system by using a Whirlimixer, and the two phases were separated by centrifugation at 2000 *g* for 5 min at room temperature. The concentration of thrombin in the top phase and in the bottom phase was determined by measuring the activity of 100 μ l samples added to 2.0 ml of a mixture containing 0.1 M-NaCl/0.05 M-Tris/HCl, pH 7.4, and 0.13 μ M-Phe-Pip-Arg-Nan·2HCl. The proteolytic activity of thrombin was not affected by the addition of heparin, poly(ethylene glycol) or dextran at the concentrations attained in the activity assay.

Heparin binding to antithrombin

This was monitored by means of heparin enhancement of intrinsic tryptophan-residue fluorescence as described by Nordenman *et al.* (1978). Fluorescence was measured by means of a Perkin-Elmer 512 double-beam fluorescence spectrophotometer. Human antithrombin was excited at 280 nm and the emission measured at 340 nm. The band-pass for both excitation and emission was 10 nm.

Kinetics of the reaction between thrombin and antithrombin in the presence of heparin

This was measured by mixing thrombin into a solution containing both inhibitor and substrate, essentially as described by Griffith (1982). This method takes advantage of the competition for the active site between substrate and inhibitor. In the presence of substrate, only a fraction of the enzyme is unprotected and free to react with inhibitor, and this slows down the reaction rate. At a suitable high concentration of substrate (Phe-Pip-Arg-Nan·2HCl) and inhibitor, the reaction can be monitored as a pseudo-first-order reaction in an ordinary spectrophotometer. The kinetic theory and the application of the method to monitor the reaction between an enzyme and a practically irreversible inhibitor has been described in several studies (see, e.g., Main & Dauterman, 1963; Hollaway *et al.*, 1980; Petersen & Clemmensen, 1981). The reaction was monitored at 410 nm with a Beckman Acta M VI spectrophotometer operating in the derivative mode (dA/dt) as described previously (Petersen & Clemmensen, 1981).

Results

Interaction between thrombin and heparin measured in an aqueous two-phase partition system

The effect of heparin concentration on the partition coefficient (K_T) of thrombin in two-phase systems of various electrolyte composition is shown in Fig. 1. Thrombin alone is approximately equally distributed in the two phases, and the partition is only slightly affected by the electrolyte composition. In the presence of excess heparin the partition pattern of thrombin is changed. This is an indication of thrombin-heparin interaction. The partition in the presence of heparin depends strongly on the electrolyte composition.

At relatively low concentrations of electrolyte an effect on the partition is observed between approx. 20 nM- and 200 nM-heparin (assuming M_r 12 500), indicating a dissociation constant in this range. The effect of heparin on the partition of thrombin disappears at high salt concentration (0.3 M-NaCl). Under these conditions the partition is unaffected by addition of heparin, even at high concentrations (the

Fig. 1. Effect of heparin concentration on the partition of thrombin in dextran/poly(ethylene glycol) two-phase systems of various electrolyte composition

The partition coefficient for thrombin, K_T , in the two phases is calculated as the ratio between catalytic activities of samples withdrawn from the top phase and the bottom phase, measured as described in the text. The two-phase system contained: 4.4% (w/w) dextran, 7.0% (w/w) poly(ethylene glycol), 20 mM-Tris/Mops, pH 7.4, and various electrolytes (○, 0.1 M-LiCl; □, 0.1 M-NaCl; ●, 0.1 M-KCl; △, 0.3 M-NaCl). In one series of experiments (▲) the buffer was 60 mM-sodium phosphate, pH 7.4, not Tris/Mops. To each two-phase system was added 24 nM-thrombin and various concentrations of Heparin-I.

Fig. 2. Effect of LiCl and NaCl concentration on the partition of thrombin in the dextran/poly(ethylene glycol) two-phase system in the presence and absence of heparin

The partition coefficient for thrombin, K_T , was determined as described in Fig. 1. Open symbols show the effect of NaCl concentration in the absence (△) and presence (○) of 12 mg of Heparin-II/litre. Closed symbols show the effect of LiCl concentration in the absence (▲) and in the presence (●) of 12 mg of Heparin-II/litre. Other conditions were as described in Fig. 1.

highest concentration tested was 200 mg of heparin/litre).

Dissociation of thrombin from heparin is the most likely explanation for this behaviour, although in theory the possibility exists, that the heparin-thrombin complex could accidentally have the same partition coefficient as free thrombin at 0.3 M-NaCl.

The effect of electrolyte concentration on the interaction between thrombin and heparin is further investigated in Fig. 2, which shows the partition coefficient of thrombin in the presence and absence of heparin at various concentrations of LiCl and NaCl. The results shown are those obtained with high-affinity heparin (Heparin-II) but the partition is indistinguishable from that obtained with heparin that has not been purified according to its antithrombin-binding capability (Heparin-I). Fig. 2 shows that the complex appears to be dissociated by NaCl at a concentration of about 0.3 M, and that much higher concentrations of LiCl are needed to obtain the same effect.

Interaction between heparin and antithrombin

The aqueous two-phase system may be used also to study this interaction. However, the effect of heparin on antithrombin partition, although present, is relatively small under conditions similar to those

used to study the interaction between heparin and thrombin. For this reason, and also because it appears to monitor the complex-formation more directly, the antithrombin-intrinsic-fluorescence method described by Nordenman *et al.* (1978) was used. Much higher concentrations of NaCl than used in case of thrombin binding have to be added before dissociation of the heparin-antithrombin complex is fully accomplished. In accordance with previous measurements (Nordenman *et al.*, 1978; Olson & Shore, 1981) the fluorescence enhancement is more pronounced, and higher concentrations of salt are needed for the dissociation of high-affinity heparin (Heparin-II) than for Heparin-I.

Kinetics of the heparin-enhanced reaction between thrombin and antithrombin

Fig. 3 shows the time course of antithrombin inhibition of thrombin in the presence of substrate. The decline in enzyme activity is exponential, as indicated by the semilogarithmic plot of the results inserted in Fig. 3.

Fig. 4(a) shows the effect on the apparent first-order constant, k' , of varying the concentration of inhibitor, and Fig. 4(b) shows the effect of varying the concentration of substrate. The first-order constant is proportional to the inhibitor concentration and inversely proportional to the substrate concentration. The method is only valid over a

Fig. 3. Progressive inhibition of thrombin by antithrombin in the presence of heparin and synthetic tripeptide substrate

Buffer (1.0ml) containing 30nM-thrombin was mixed in a flow cell with an equal volume of buffer containing 0.6 μM-antithrombin, 52mg of Heparin II/litre and 0.24mM-Phe-Pip-Arg-Nan · 2HCl. The rate of nitroaniline production measured at 410nm was recorded by using the spectrophotometer in the dA/dt mode. The experiment was performed in 0.1M-NaCl/0.05M-Tris/HCl, pH 7.4, at 25°C. A semilogarithmic plot of the results is inset.

Fig. 4. Effect of inhibitor and substrate concentration on the heparin-enhanced reaction between thrombin and antithrombin

(a) The apparent first-order constant, ' k' ', for the inactivation of thrombin is plotted as a function of the antithrombin concentration ($[AT]$). (b) $1/k'$ is plotted as a function of the concentration of synthetic tripeptide substrate ($[S]$). The final reaction mixture contained 15 nM-thrombin and 11 mg of heparin/litre. A substrate concentration of 0.2mM was used in (a) and an inhibitor concentration of 0.19 μM was used in (b). Other conditions were as described in Fig. 3.

limited range of substrate concentrations, depending on the actual concentrations of inhibitor and proteinase. At low concentrations of substrate this

Fig. 5. Effect of heparin concentration on the reaction between thrombin and antithrombin

The apparent first-order constant, ' k' ', for the inactivation of thrombin is plotted as a function of log (heparin concentration). Δ , Heparin-I; $\circ$, Heparin-II (high-affinity heparin). Other conditions were as described in Fig. 3.

may be depleted at a rate comparable with the reaction with inhibitor, and at a very high concentration of substrate the inhibition of thrombin may not go to completion. Incomplete inhibition of thrombin under the conditions described in Fig. 4(b) was observed at concentrations of substrate higher than approx. 0.4 mM.

Effect of heparin concentration on inhibition kinetics

Fig. 5 shows the effect of heparin concentration on the reaction between thrombin and antithrombin in the presence of 0.15 mM-Phe-Pip-Arg-Nan · 2HCl. In accordance with previous studies on the kinetics (see, e.g., Griffith, 1979b; Jordan *et al.*, 1980b) the plot is described by a bell-shaped curve.

The second-order constant for the reaction may be extrapolated from the observed pseudo-first-order constant by using eqn. (1) (Main & Dauterman, 1963; Hollaway *et al.*, 1980; Petersen & Clemmensen, 1981; Griffith, 1982):

$$k'_{\text{obs.}} = k_1 \frac{K_m}{K_m + [S]} [AT] \quad (1)$$

where $[S]$ and $[AT]$ are the concentrations of substrate and antithrombin, k_1 is the second-order constant and K_m is the Michaelis constant of thrombin for the chromogenic peptide substrate. Under the conditions of the experiments, K_m is 2 μM (results not shown), and accordingly a maximal rate constant of $3 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ is obtained with high-affinity heparin (Heparin-II).

Fig. 6. Effect of NaCl concentration on the heparin-enhanced reaction between thrombin and antithrombin. The reaction mixture contained 15 nM-thrombin, 0.75 μ M-antithrombin, 0.2 mM-tripeptide substrate, 10 mg of Heparin-I/litre (Δ) or 11 mg of Heparin-II/litre (O), various concentrations of NaCl, and 20 mM-Tris/Mops, pH 7.4. Other conditions were as described in Fig. 3.

This is approx. 5 times faster than the maximal value obtained with the Heparin-I preparation that had not been purified according to its antithrombin-binding capability.

Effect of electrolyte composition on inhibition kinetics

Fig. 6 shows the effect on NaCl on the heparin-enhanced reaction between thrombin and antithrombin. The apparent first-order constant, k' , measured in the presence of substrate is plotted as a function of NaCl concentration with two different heparin preparations. At relatively low ionic strength the rate increases almost proportionally to the NaCl concentration in accordance with the results obtained by Griffith *et al.* (1980). However, a plateau is reached at about 0.2 M-NaCl and the rate decreases when the NaCl concentration is increased further beyond this level. According to eqn. (1) an effect of $[\text{NaCl}]$ on K_m is also likely to affect the observed rate constant k' , for thrombin inactivation; however, preliminary results on the effect of $[\text{NaCl}]$ on thrombin kinetics suggest that such effects on K_m are relatively small and cannot account for the strong effect of $[\text{NaCl}]$ on k' shown in Fig. 6. When Na^+ in the medium is replaced by Li^+ or K^+ , the rate of the heparin-enhanced reaction between thrombin and antithrombin increases in the sequence: $\text{LiCl} < \text{NaCl} < \text{KCl}$ when the experiments are performed at low ionic strength (Griffith *et al.*, 1980). This is also

Table 1. Effect of electrolyte composition on k' . The apparent first-order constant, k' , was measured under the conditions described in Fig. 6. In addition to alkali-metal chlorides the buffer contained 20 mM-Tris/Mops, pH 7.4.

Electrolyte	Concn. (mM)	...	k' (s^{-1})	
			90	300
LiCl			0.013	0.033
NaCl			0.024	0.012
KCl			0.029	0.003

Fig. 7. Effect of heparin concentration on the antithrombin fluorescence enhancement, and on the reaction between thrombin and antithrombin at 0.3 M-NaCl (Δ). The enhancement in fluorescence (ΔF) in arbitrary units on addition of Heparin-II to 0.25 μ M-antithrombin is plotted as a function of the heparin concentration on a logarithmic scale. (O) The apparent rate constant, k' , for the inactivation of thrombin is plotted against the same scale. The reaction mixture contained 15 nM-thrombin, 0.25 μ M-antithrombin, and 0.15 mM-synthetic tripeptide substrate. The experiments were performed in 0.3 M-NaCl/20 mM-Tris/Mops, pH 7.4.

confirmed with the rate assay used in the present study; however, at high ionic strength (10.3) the effect is exactly reversed (Table 1).

Effect of heparin concentration on complex-formation and inhibition kinetics at high ionic strength

The results presented in Figs. 1 and 2 suggest that the interaction between thrombin and heparin at 0.3 M-NaCl is negligible even with high concentrations of heparin added. This is not the case for the interaction between antithrombin and heparin (Nordenman & Björk, 1981). Fig. 7 shows the change in intrinsic fluorescence of antithrombin as a function of the heparin concentration at 0.3 M-NaCl. Also shown is the effect of heparin concentration on the reaction between thrombin and antithrombin at the same concentration of electrolyte. This profile is

different from the kinetic profile obtained at low ionic strength (Fig. 5) in that no decrease in rate is observed at high concentrations of heparin.

Discussion

The kinetics of the heparin-enhanced inhibition of thrombin were measured in the presence of tripeptide substrate. The results presented in Figs. 3 and 4 suggest that the kinetics can be described by a simple model that assumes competition between substrate and inhibitor for the active site and also assumes a practically irreversible reaction between proteinase and inhibitor. It is an advantage of the 'substrate' method that antithrombin can be added in large molar excess over thrombin such that the presence of thrombin is of little or no significance for antithrombin binding to heparin. The results obtained on the effect of heparin concentration on the inhibition kinetics at physiological salt concentrations (Fig. 5) are similar to those obtained by other methods (Griffith, 1979*b*; Jordan *et al.*, 1980*b*). The kinetic plot is bell-shaped with an ascending limb at low heparin concentrations and a descending limb at high concentrations. The results suggest that the maximal rate enhancement obtained with high-affinity heparin is about 5-fold higher than the maximal value obtained with heparin that has not been separated according to its antithrombin-binding capability. The interpretation of such results have been complicated by the fact that binding of both thrombin and antithrombin takes place over a relatively narrow heparin concentration range and by the fact that the values for the dissociation constants determined by different methods vary widely. It is therefore important to obtain information about the interactions of these proteins with heparin from binding studies using independent methods.

Partition of thrombin in an aqueous two-phase system appears to be a useful monitor of thrombin-heparin interaction (Fig. 1). The partition of thrombin alone in various electrolytes indicates a small net charge, whereas the partition of its complex with heparin indicates a high net charge.

The partition of charged macromolecules in a two-phase partition system is very sensitive to the electrolyte composition. This effect is caused by a Donnan potential imposed across the phase border due to a difference in affinity between electrolyte cations and anions for the two phases (see Albertsson, 1971). The effect of the Donnan potential on the partition of the macromolecule is proportional to its charge, and the effect of electrolyte composition on the partition pattern of heparin is similar to that of other polyanions such as, e.g., DNA, RNA and phosvitin (L. C. Petersen, unpublished work with [³⁵S]heparin). The binding curve (partition coeffi-

cient plotted against heparin concentration) (Fig. 1) is more complex than can be accounted for by means of the single-site models that have previously been applied for a quantitative determination of binding constants in more simple cases of macromolecular electrostatic interaction (see, e.g., Petersen, 1978; Petersen & Cox, 1980). This is apparent from the biphasic character of the binding curve, which is most pronounced in the LiCl-containing two-phase system.

Heparin binds univalent cations in the sequence: $K^+ > Na^+ > Li^+$ (see, e.g., Scott, 1973). Sulphate ester groups on heparin are likely to promote this preference sequence to the polyanion (Prue *et al.*, 1974). The observations made on the displacement of antithrombin (Griffith *et al.*, 1980) and of thrombin (Fig. 2) from heparin by univalent cations are fully consistent with this sequence. The binding studies (Fig. 2) also confirm previous experience from affinity chromatography (Nordenman & Björk, 1978, 1980, 1981) that thrombin is displaced from heparin at considerably lower concentrations of NaCl than is antithrombin. This is particularly true with high-affinity heparin (Nordenman & Björk, 1981).

Studies on the effect of electrolytes on heparin binding provide a framework that can be used for an evaluation of the various models proposed to account for the kinetics of thrombin inhibition. Changes in electrolyte composition with known consequences for the binding of heparin should be accounted for in the kinetics.

Binding studies suggest that heparin binds to thrombin and antithrombin with increasing affinity as the ionic strength of the medium is lowered. It is also expected that the series: K^+, Na^+, Li^+ increases the affinity for heparin at a given ionic strength of the medium. The effect on the kinetics of such changes in the binding properties is investigated in Fig. 6 and Table 1. The model proposed by Rosenberg & Damus (1973) and by Jordan *et al.* (1980*a,b*) seems to offer the most simple explanation for the results obtained. This model assumes that the heparin-enhanced rate is determined by the reaction between free thrombin and the heparin-antithrombin complex. At low ionic strength, increased heparin binding results in a decreased rate; according to the model this is due to a decrease in the concentration of free thrombin. A major effect on the concentration of the heparin-antithrombin complex is unlikely, since essentially all antithrombin is bound to heparin under these conditions.

A basic difference between the kinetics at low and high ionic strength is indicated by the bell-shaped curve in Fig. 6. Such difference in kinetics is also indicated by the experiments with Li^+, Na^+ and K^+ (Table 1). At high ionic strength, increased heparin

binding results in an increased rate. The change from one pattern to another seems to be roughly correlated with the dissociation of thrombin observed from the binding studies (Fig. 2).

Additional strong support for the model discussed above comes from the results shown in Fig. 7. These experiments are performed at 0.3 M-NaCl, where binding studies suggest that the binding of thrombin to heparin is negligible, whereas the interaction between antithrombin and high-affinity heparin is still considerable. Such a simplification in the binding properties is also reflected in the kinetic profile of the rate as a function of the heparin concentration. The decrease in reaction rate at very high concentrations of heparin observed at lower salt concentrations as a descending limb (Fig. 5) is not observed at 0.3 M-NaCl (Fig. 7). It is notable that a considerable heparin enhancement is still observed at high ionic strength, even though the binding of thrombin to heparin appears to be negligible under these conditions, and it is also notable that the rate of inhibition is approximately proportional to the concentration of the heparin-antithrombin complex. These observations are fully consistent with the proposal of the involvement of an antithrombin-heparin complex in the heparin-enhanced thrombin inhibition, and further suggest that the heparin-thrombin complex does not participate as an active reactant. Complex-formation between heparin and thrombin rather tends to prevent this enhancement.

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