Kinetic analysis of the heparin-enhanced plasmin—antithrombin III reaction

Apparent catalytic role of heparin

Raymund MACHOVICH, Pál I. BAUER, Péter ARÁNYI, Éva KECSKÉS, Kálmán G. BÜKI and István HORVÁTH
Second Institute of Biochemistry, Semmelweis University Medical School, Puskin u 9, H-1444 Budapest, Box 262, Hungary

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Inactivation of plasmin by a 3–4-fold molar excess of antithrombin III follows pseudo-first-order kinetics and the apparent rate constants are proportional to the concentration of the inhibitor. Heparin accelerates the inactivation reaction without changing its pseudo-first-order character, and the apparent rate constants are also proportional to the concentration of the polysaccharide. Heparin results in a minimum 20-fold rate enhancement of the reaction between plasmin and antithrombin III when the concentrations of heparin and plasmin are approx. 0.5 μM and 1 μM respectively. Heparin at a molar concentration well below that of plasmin still accelerates the reaction: one molecule of the polysaccharide is able to facilitate the inactivation of about 100 molecules of plasmin. Heparin must bind to plasmin to accelerate the plasmin—antithrombin III reaction, since the modification of four to five lysine residues of the enzyme inhibits the rate-enhancement effect of heparin and the dissociation of heparin—plasmin complex decreases the inactivation rate of plasmin. Increasing the concentration of antithrombin III, at a constant amount of heparin, results in increase of the inactivation rate. By contrast, the effect of increasing the amount of plasmin in the presence of constant amount of heparin and antithrombin III is such that higher plasmin-to-heparin ratios are associated with lower rates of inactivation. It seems, therefore, that to obtain 'optimal' conditions for fast enzyme inactivation, the amount of heparin should be matched to plasmin rather than to antithrombin III. Arrhenius plots of the plasmin—antithrombin III reaction are linear both in the absence and presence of heparin, at concentrations of 1 or 2 μg/ml, over a range of 26 K. Under these experimental conditions, heparin increases activation entropy. The findings show that heparin seems to fulfill some criteria that are characteristic for biological catalysis: binding, reaction-rate enhancement (increasing activation entropy), recycling of heparin (effectiveness of non-stoichiometric amounts of the polysaccharide) and specificity.

Heparin accelerates the inactivation of the serine proteinases of blood coagulation by antithrombin III. The mechanism by which heparin enhances the enzyme—inhibitor reaction is not entirely understood at present. Its capability to bind antithrombin III (Rosenberg & Damus, 1973; Einarssson & Andersson, 1977; Nordenman & Björk, 1978; Piepcorn et al., 1978; Holmer et al., 1979; Villanueva & Danishefsky, 1979), thrombin (Machovich, 1975; Machovich et al., 1975, 1977, 1978, 1980a; Hatton & Regoecri, 1977; Stürzebecher & Markwardt, 1977; Smith, 1977; Griffith, 1979a,b; Griffith et al., 1979) or both (Danishefsky et al., 1977; Machovich & Arányi, 1978; Pomerantz & Owen, 1978; Laurent et al., 1978) have all been proposed as likely mechanisms. In view of this uncertainty, we decided to investigate the heparin-enhanced inactivation of another enzyme of blood coagulation, plasmin. There is some evidence in favour of the plasmin—heparin interaction in the enzyme inactivation by antithrombin III (Stürzebecher & Markwardt, 1977; Hatton & Regoecri, 1977; Smith & Sundboom, 1980; Machovich et al., 1980b). This interaction, however, has...
not yet been studied in detail. Our knowledge about the kinetics of the plasmin inactivation by antithrombin III is also insufficient.

Therefore, in the present work, we have examined the kinetics of plasmin inactivation by antithrombin III and heparin, and have determined the thermodynamic activation parameters. Furthermore, modification of plasmin with pyridoxal 5'-phosphate has been also used to study the interaction of heparin with plasmin.

The results obtained suggest that heparin must bind to the enzyme to accelerate the plasmin–antithrombin III reaction.

**Experimental**

**Materials**

Lysine–Sepharose 4B and pyridoxal 5'-phosphate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. D-Val-Leu-Lys p-nitroanilide dihydrochloride (S-2251) was obtained from Kabi AB, Stockholm, Sweden. Sephadex G-25, streptokinase (Kabikinese) and urokinase were the products of Pharmacia Fine Chemicals (Uppsala, Sweden), Kabi AB, (Stockholm, Sweden) and Koch-Light (Colnbrook, Bucks., U.K.) respectively. Heparin (from bovine intestine, with a specific activity of 165 units/mg) was obtained from G. Richter Pharmaceuticals (Budapest, Hungary): 1 unit is defined as 7.6µg of the International Standard Heparin Preparation (Jaques et al., 1973). Other chemicals were purchased from Reanal Fine Chemicals (Budapest, Hungary).

**Methods**

Plasminogen was purified from human plasma by the method of Deutsch & Mertz (1970), desalted, freeze-dried and stored at −18°C. (This preparation contained approx. 60% Glu- and 40% Lys-plasminogen.) Immediately before experimentation, plasminogen was activated by streptokinase (10µg/mg of plasminogen) at 37°C for 30 min in 0.1M-sodium phosphate buffer, pH 7.4, containing 0.1mM-6-aminohexanoic acid. Thereafter, plasmin was gel-filtered on Sephadex G-25, equilibrated with 0.1M-sodium phosphate buffer, pH 7.4. The specific activity of the plasmin preparation was 7.8 units/mg determined with Cagtest (Kabi AB) as a standard.

[One proteolytic unit was arbitrarily taken as the amount of enzyme producing an increase of 450µg of acid-soluble tyrosine in a medium of 4% casein in 1h (Remmert & Cohen, 1949).] In some experiments, plasmin was isolated from citrated human plasma by activation with urokinase on Sepharose–lysine as detailed by Hatton & Regoecci (1977).

Antithrombin III, purified from human plasma (Wickerhauser et al., 1979), was the product of the American Red Cross Fractionation Center, Bethesda, MD, U.S.A. The final product of the inhibitor protein, over 95% purity, was freeze-dried and before experimentation was dissolved in 0.15M-NaCl containing 0.01M-sodium phosphate, pH 7.0. No contaminants were detected in the preparation by immunoelectrophoresis against an antiserum raised to whole human serum.

Protein concentrations were determined as described by Lowry et al. (1951), with human serum albumin as a standard.

Molar concentrations of plasmin, antithrombin III and heparin were calculated by using mol.wtS. 76500 (Sodetz et al., 1972), 65000 (Miller-Andersson et al., 1974) and 11000 (Hilborn & Anastassiadis, 1971) respectively.

**Modification of plasmin with pyridoxal 5'-phosphate.** A portion (0.1ml) of pyridoxal 5'-phosphate (4mg/ml) was added to 1ml of plasmin (1mg) solution. After 8 min incubation at 22°C in the dark, 0.1ml of NaBH₄ (10mg/ml) was pipetted into it. After incubation for 1 more min, 0.1ml of lysine (4mg/ml) was added and the reaction mixture placed immediately on ice. In the reaction mixture, every reactant was dissolved in 0.1M-triethanolamine, at pH 8.0. Thereafter, it was dialysed overnight at 0°C against 3 litres of 0.1M-sodium phosphate buffer, pH 7.4, containing 1mM-6-aminohexanoic acid. The bound pyridoxal 5'-phosphate was determined by absorbance at 322nm, a molar absorption coefficient of 9000M⁻¹cm⁻¹ (Griffith, 1979a) being used. As a control, the same procedure was performed without pyridoxal 5'-phosphate addition.

**Assay of plasmin activity.** A portion (0.5ml) of D-Val-Leu-Lys p-nitroanilide (0.1mg) dissolved in 0.05M-sodium phosphate buffer, pH 7.4, was preincubated at 22°C for 30s. Thereafter, 0.05ml of plasmin solution was pipetted and the change in absorbance at 405nm was monitored with a Beckman Model 25 spectrophotometer.

**Measurement of plasmin inactivation.** Distilled water (0.1ml), heparin (0.1ml; dissolved in 0.1M-NaCl) and plasmin (0.1ml) were preincubated for 1 min at the appropriate temperature. Thereafter 0.1ml of antithrombin III solution was added and the reaction mixture incubated at 0–37°C for 0–60 min. At various times, 0.05ml portions were withdrawn and assayed for plasmin activity as described above.

Pseudo-first-order rate constants for plasmin inactivation were calculated by the method of least squares.

Activation enthalpy and entropy, as well as free energy was calculated from the rate constants (Tanford, 1970) as:

\[ k = A e^{-\Delta H^*/RT + \Delta S^*/R} \]

where \( H \) is activation enthalpy, \( S \) is activation entropy, \( R \) is the gas constant. For \( A \), the value \( RT/ Nh \)
was used, where $N$ is Avogadro's number, $h$ is Plank's constant and $T$ is absolute temperature.

**Results**

*Catalyst-like function of heparin in the plasmin–antithrombin III reaction*

Inactivation of plasmin by severalfold molar excess of antithrombin III followed pseudo-first-order kinetics and the apparent rate constants were proportional to the concentration of the inhibitor (Fig. 1). Heparin, at a molar concentration well below that of enzyme, accelerated the inactivation reaction without changing its pseudo-first-order character, and the apparent rate constants were also proportional to the concentration of the polysaccharide (Fig. 2). Under our experimental conditions, heparin caused an approx. 3.4-fold rate enhancement of the reaction when the molar concentrations of the reactants were: antithrombin III, 4.6 $\mu$M; plasmin, 0.99 $\mu$M; heparin, 91 nM. In another series of experiments (Fig. 2, inset) 11 nM-heparin was found to be sufficient to accelerate the inactivation of 1 $\mu$M-plasmin by excess antithrombin III. The semilogarithmic plot was linear down to about 35% remaining plasmin activity, with no sign of a fraction of plasmin being inactivated via a mechanism different from that of the inactivation of the rest. The heparin-to-plasmin molar ratio was about 1:100 in this case.

*Dependence of the rate of the plasmin–antithrombin III–heparin reaction on the relative concentrations*

Plasmin inactivation was measured in the presence of heparin at various concentration of antithrombin III or plasmin.

In the absence of heparin, the apparent rate constants were proportional to the antithrombin III concentration in the range used, in agreement with the result obtained above. The rate of inactivation of plasmin by antithrombin III increased with the inhibitor concentration in the presence of heparin too (results not shown). By contrast, increasing the

**Fig. 1. Dependence of apparent first-order rate constant of the plasmin–antithrombin III reaction on the inhibitor concentration**

Plasmin (75 $\mu$g of protein/ml) was incubated in 25 mM-sodium phosphate buffer, pH 7.4, containing 37 mM- NaCl, at 16°C in the presence of various concentrations of antithrombin III. Remaining enzyme activity was determined at different times for estimation of the rate constants as described in the Experimental section.

**Fig. 2. Time course of plasmin inactivation by antithrombin III in the presence of heparin**

Plasmin (75 $\mu$g of protein/ml) and antithrombin III (300 $\mu$g of protein/ml) in 25 mM-sodium phosphate buffer, pH 7.4, containing 37.5 mM- NaCl and various concentrations of heparin was incubated at 16°C for 0–10 min. Remaining plasmin activity was determined as described in the Experimental section. In the inset, heparin (µg/ml) was added as follows: O, none; ▲, 0.125; ■, 5.
concentration of plasmin relative to heparin resulted in a decreased inactivation rate (Table 1).

**Characterization of heparin–plasmin binding**

**Effect of ionic strength.** The ionic bonds participating in the plasmin–heparin interaction are apparently easier to disrupt than those involved in the antithrombin III–heparin interaction. Thus, the plasmin–heparin complex was dissociable in solution containing slightly more than 0.25 M-salt (Machovich et al., 1980b), whereas the antithrombin III–heparin complex required 1 mM-NaCl for dissociation (Miller-Andersson et al., 1974). Therefore it was hoped that the significance of the plasmin–heparin interaction in the inactivation reaction of the enzyme by antithrombin III could be judged by studying the effect of ionic strength, similarly to the case of the thrombin–antithrombin III–heparin system (Machovich et al., 1979). As shown in Table 2, the inactivation of plasmin by antithrombin III is not significantly dependent on ionic strength in the absence of heparin. On the other hand, the rate-enhancing effect of heparin was manifold in the presence of 25 mM-salt but barely significant in more concentrated NaCl solutions.

**Chemical modification of plasmin.** The apparent role of ionic interactions in the plasmin–heparin complex, the chemical structure of heparin and assumed analogy with the thrombin–heparin interaction, led us to suspect that lysine side chains of plasmin might be involved in the binding of the polysaccharide to the enzyme. We tried to block lysine residues with pyridoxal 5’-phosphate, a reagent that proved most useful in the case of thrombin (Griffith, 1979a).

When four to five lysine residues (if N-terminal amino acids were not involved in the reaction) of plasmin were modified, the interaction of enzyme by antithrombin III was not affected (Fig. 3, inset). However, the rate of inactivation of modified plasmin in the presence of antithrombin III was less influenced by heparin than that of the control plasmin (Fig. 3). This finding supports also the pivotal role of the heparin–plasmin interaction in the inactivation of the enzyme by inhibitor.

**Thermodynamic characterization of the plasmin–heparin–antithrombin III reaction**

Plasmin inactivation by antithrombin III was measured in the range 4–30°C. The actual values of the rate of inactivation were slightly dependent on plasmin concentration and specific activity. They showed also batch-to-batch variation.

In the presence of heparin at low concentration (1 μg/ml) the rate of inactivation increased at any temperature and the time course still followed pseudo-first-order kinetics within the measurable range. Arrhenius plots of the plasmin–antithrombin III reaction were linear in both the absence and presence of heparin over a range of almost 26 K (Fig. 4). Furthermore, the plots are parallel to each other, with ΔH° = 43 KJ·mol⁻¹. Absolute values of activation entropy as well as free energy are somewhat arbitrary. Substituting RT/Nh for A (see under ‘Methods’), we have ΔG° = 8.9 KJ·mol⁻¹, ΔS° = −152 J·mol⁻¹·K⁻¹ at 15°C for the plasmin–antithrombin III reaction. Variation of ΔS° with heparin concentration can be reliably derived from the data. Heparin (1 μg/ml) increases activation

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**Table 1. Influence of various plasmin concentrations on the heparin-enhanced plasmin–antithrombin III reaction rate**

Plasmin (at the concentrations shown) and antithrombin III (300 μg of protein/ml) were incubated at 16°C in the presence of 25 mM-sodium phosphate buffer, pH 7.4, containing 25 mM-NaCl, for 0–10 min. The rate constants were determined as described in the Experimental section.

<table>
<thead>
<tr>
<th>Heparin added (μg/ml)</th>
<th>Plasmin (μg/ml)</th>
<th>k (inactivation rate) (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>0.57</td>
</tr>
</tbody>
</table>

**Table 2. Effect of NaCl on the heparin enhancement of the plasmin–antithrombin III reaction**

Plasmin (75 μg of protein/ml) and antithrombin III (350 μg of protein/ml) dissolved in 25 mM-sodium phosphate buffer, pH 7.4, were incubated in the presence of different concentrations of NaCl and heparin. After incubation at 16°C for various times, portions were taken and determined for remaining plasmin activity. Pseudo-first-order rate constants were calculated as described in the Experimental section.

<table>
<thead>
<tr>
<th>Heparin added (μg/ml)</th>
<th>NaCl (mm)</th>
<th>k (inactivation rate) (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>25</td>
<td>0.090</td>
</tr>
<tr>
<td>0.5</td>
<td>75</td>
<td>0.086</td>
</tr>
<tr>
<td>1.0</td>
<td>125</td>
<td>0.080</td>
</tr>
<tr>
<td>2.0</td>
<td>0.86</td>
<td>0.113</td>
</tr>
<tr>
<td>&gt;1.0</td>
<td>0.36</td>
<td>0.17</td>
</tr>
</tbody>
</table>

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3. Fig. 2 reveals the negative effect of heparin (250 μg of protein/ml) on plasmin activity in a thrombin-heparin system (Machovich & Arányi, 1978; Machovich et al., 1979). To test this interesting possibility, we wanted to check if the heparin was exhausted in the reaction or not, and therefore the following experiments were done. An incubation mixture was set up by mixing heparin, plasmin and antithrombin III, and was supplemented at regular intervals by further additions of the same amount of freshly mixed enzyme and inhibitor but no heparin. The results shown in Table 3 reveal no apparent loss of heparin activity, even after five ‘turns’ of complete inactivation of fresh plasmin.

Discussion

It is generally believed that heparin accelerates the inactivation of enzymes of blood coagulation by interacting with antithrombin III. Namely, heparin acts as if it were a co-inhibitor of the antithrombin III (‘inhibitor-fitting’ hypothesis). Several other results, however, suggest the primary importance of the heparin binding to the serine protease of blood coagulation (‘enzyme-fitting’ hypothesis). The present findings support the ‘enzyme-fitting’ hypothesis, i.e. the enhancing effect of heparin on the plasmin inactivation by antithrombin III correlates with the binding of heparin to the enzyme. In this respect, dependence of the heparin-enhanced plasmin inactivation on ionic strength and on the chemical modification of certain plasmin side chains are particularly relevant. In these cases, experimental conditions that could influence the enzyme-heparin interaction without affecting the interaction between heparin and antithrombin III substantially decreased the effect of the polysaccharide on the reaction between the enzyme and its inhibitor.

Minute amounts of heparin were effective in the reaction. Kinetics proved that one molecule of heparin could enhance the inactivation as much as about 100 molecules of plasmin, or perhaps many more, considering that only about one-fifth of the heterogeneous heparin preparation was active (Rosenberg et al., 1978). Besides, heparin activity...
could be recovered without loss after the reaction between plasmin and antithrombin III. Activation parameters of the enzyme–inhibitor reaction were also determined. Heparin apparently influenced only activation entropy.

These facts could be visualized by assuming that heparin exerts an ordering function on the reactants. After the heparin binds plasmin, and antithrombin III formed an inactive complex, heparin could be released and become available for other plasmin and antithrombin III molecules.

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References

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Table 3. Availability of residual heparin activity in the plasmin–antithrombin III reaction

<table>
<thead>
<tr>
<th>Additives</th>
<th>No. of plasmin/antithrombin supplements</th>
<th>Percentage of enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin (in recycling)</td>
<td>...</td>
<td>0 1 2 3 4 No heparin</td>
</tr>
<tr>
<td>Heparin added (in dilution)</td>
<td></td>
<td>0 18 32 40 50 —</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 19 30 52 71 100</td>
</tr>
</tbody>
</table>

First sample (62.5 µg of plasmin/ml, 86 µg of antithrombin III/1 ml, 6.25 µg of heparin/ml, 25 mM-sodium phosphate buffer, pH 7.4, 25 mM-6-aminohepanoic acid and 87.5 mM-NaCl) incubated for 5 min at room temperature, was mixed with an equivalent volume of supplement containing the same additives as first sample but no heparin (first supplement). After 5 min incubation, a portion of the reaction mixture was mixed again with equivalent volume of a fresh supplement. Thereafter these procedures were repeated several times. During the 5 min incubation periods, 0.1 ml portions of reaction mixture were taken exactly at 3 min for determination of remaining plasmin activity as described in the Experimental section. In the control assay (second line), the first sample did not contain heparin, but to the first, second, third and fourth supplements, 6.25, 3.12, 1.56 and 0.78 µg of heparin/ml was added respectively. Values (means of the two determinations) are percentages of the enzyme activity obtained after incubation of plasmin and antithrombin III for 3 min without heparin.

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