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Interaction of Protamine Sulfate With Thrombin

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Protamine sulfate (salmine), a basic protein with a molecular weight of 4,626 ± 109, is a known antiheparin agent which in the absence of heparin demonstrates an anticoagulant activity. To date, much work has been done to elucidate the interaction of heparin with thrombin and its physiologic inhibitor, Antithrombin III (ATIII). Little is known, however, about the mechanism of anticoagulant action of protamine sulfate and its mode of thrombin inactivation. We provide information about the interaction of protamine sulfate with purified, labeled thrombin and ATIII through binding experiments in which protamine is shown to inhibit the inactivation of thrombin by ATIII. Furthermore, we show in clotting assays that protamine sulfate has an inhibitory effect on thrombin in the conversion of fibrinogen to fibrin, and that this inhibition is concentration dependent, partial, and reversible.

Key words: protamine sulfate, inhibitor, thrombin

INTRODUCTION

An ever-increasing number of substances which favor or tend to inhibit or retard some phase of the coagulation process are being recognized. There are even accelerators of inhibitors and inhibitors of accelerators. Antithrombin III (ATIII) is the major physiologic inactivator of most of the serine proteases involved in the ultimate formation of a blood clot [1]. Addition of heparin greatly enhances the rate at which ATIII inhibits FXa and thrombin [2,3]. Protamine sulfate, the principal neutralizing agent for heparin, is indicated in cases of heparin overdose, and is employed in the measurement of heparin in vitro [4–6]. Protamine also fulfills the necessary substrate requirements for plasmin, trypsin, factor Xa, and thrombin, and is used in an assay to measure these enzymes [7].

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In the absence of heparin, anticoagulant properties have been attributed to protamine sulfate both in vivo and in vitro. However, little is known about the nature of this anticoagulant activity. In addition, the inhibitory effect of higher protamine concentrations on the clotting activity of thrombin is not well documented.

This paper presents the results of an investigation of the interaction between protamine and thrombin. The mode of thrombin inactivation by protamine sulfate is studied by radiolabeled ligand experiments and by clotting assays. The effect of protamine on the interaction of heparin and thrombin is also described.

MATERIALS AND METHODS

Reagents

Fibrinogen, lot number 281, prepared by the American National Red Cross Blood Program, Washington, D.C., was reconstituted daily before use at 18 mg/ml in distilled water.

α-Thrombin, preparation number 174, was kindly donated by John Fenton II. Enzymic activity was 2,382 US (N.I.H.) clotting units/mg. The composition was 99.61 percent α-, 0.27 percent β-, and 0.12 percent γ-thrombin. Thrombin dilutions for the assays were prepared in distilled water.

Human antithrombin III (ATIII), lot number 72-062-SL, was provided by the American Red Cross Blood Services Laboratories, Bethesda, MD. The inhibitory ATIII activity was evaluated by incubating varying concentrations of ATIII with a thrombin solution (0.5 unit) for 1 hour at 37°C. The residual thrombin activity was measured by a fibrinogen clotting assay. It was found that 28% of the lyophilized power had ATIII activity. Stock solutions were prepared daily for use by reconstituting .28 mg powder (10 mg ATIII) in 10 ml distilled water.

Protamine sulfate (salmine), mw 4,626 $\pm$ 109 daltons, purchased from Eli Lilly and Co., Indianapolis, IN was used in the assays in concentrations ranging from 10 μM to 700 μM. Heparin sodium prepared from beef lung was obtained from The Upjohn Co., Kalamazoo, MI. Iodine^{125} 2 mCi in 0.1 MNaOH was purchased from New England Nuclear, Boston, MA.

Preparation of Radiolabeled Thrombin

Ten microliters (2 mCi) I^{125} and 100 μl α-thrombin (5 mg/ml in dist. water) were placed in a test tube. 100 μl of chloramine T (100 μg/ml in 0.15 M potassium phosphate buffer pH 7.5) was added and mixed for two minutes on ice. To stop the reaction, 100 μl sodium metabisulfite (240 μg/ml) was added, and the reaction mixture fractionated on a Sephadex G-50 column. Thrombin labeled by this approach was found to retain clotting and amidolytic activities.

Determination of Thrombin Inhibition

ATIII complexed with a xenogeneic antibody raised against human ATIII was used to probe the site of protamine interaction with thrombin. When tested, the anti-ATIII antibody (anti ATIII ab) had been found to be specific against ATIII and to react with ATIII at a site removed from its thrombin binding site [8]. In this study, the anti ATIII ab/ATIII was used to achieve a better separation of bound ATIII-I^{125} thrombin from free I^{125} thrombin.

Control ligand experiments were performed by mixing a I^{125} thrombin solution (20 μg/100 μl in 0.15 M potassium phosphate buffer pH 7.1) with ATIII (anti ATIII ab/ATIII) in excess molar concentration of the labeled enzyme. Incubation times were kept for 45 minutes at 37°C.

To investigate the effect of protamine sulfate, I^{125} thrombin (20 μg/100 μl 0.15 M potassium phosphate buffer pH 7.1) was incubated with a tenfold molar excess of protamine sulfate and/or PMSF for 45 minutes at 37°C. An ATIII solution (anti-ATIII ab/ATIII) was then added to the reaction mixture and incubation times kept for 45 minutes at 37°C. Gel filtration of the mixture was performed on a Sephacryl S-200 2.6 × 70 cm column equilibrated in 0.15 M potassium phosphate buffer pH 7.1.

Functional Assays To Study Thrombin/Protamine Sulfate Interaction

The proteolytic activity of thrombin was examined in two systems, one containing fibrinogen and the other a synthetic tripeptide substrate, S-2238.

In both assay systems, the interaction of thrombin and protamine sulfate was studied by holding the concentration of enzyme and substrate constant and by varying the concentration of protamine sulfate. Incubation times of thrombin and protamine were kept for one hour at 37°C.

In the clotting assays, clotting times of 9 to 10 seconds resulting from the hydrolysis of a 0.2 ml standard fibrinogen solution (18 mg/ml) by thrombin (0.5 unit) were taken as control values.

In the amidolytic assays, a synthetic substrate, S-2238, specific for thrombin, was used. The amount of chromophore release per unit time was the measure of the proteolytic activity of thrombin.

The amidolytic assay was conducted by adding a 0.2 ml sample (enzyme or enzyme-inhibitor complex) to 0.3 ml substrate solution and 0.3 ml Tris-HCl buffer, pH 8.3. Buffer composition was 0.151 M NaCl, 3.3 mM Tris, and 0.17 mM $CaCl_2$.

Determination of the Effect of Heparin on the Interaction of Thrombin and Protamine Sulfate

Experiments were performed in which incubation times were kept for 45 minutes. Concentrations of enzyme (0.5 u), fibrinogen (1.8 mg), and heparin (2.5 u) were held constant. Incremental amounts of protamine sulfate were added to the reaction mixtures. Heparin was first mixed with thrombin for 1 hour followed by addition of protamine sulfate and further incubation for 45 minutes. The medium in the assays was distilled water and the temperature was held at 37°C.

RESULTS

Effects of Protamine Sulfate on the Binding of I^{125} Thrombin by ATIII

The inactivation of thrombin by protamine sulfate is seen in Figure 1. Superimposed for comparison in that figure are three elution profiles of radiolabeled thrombin obtained from different reaction mixtures fractionated under identical chromatographic conditions. In Figure 1, experiment 1, depicted by the solid lines, ATIII (anti ATIII ab/ATIII) was incubated with I^{125} thrombin in excess molar ratio of enzyme to inhibitor. A major peak at the void volume indicates I^{125} thrombin bound by ATIII. Excess free I^{125} thrombin emerges as a peak at the lesser molecular weight elution volume. In Figure 1, experiments 2 and 3, solutions of I^{125} thrombin were

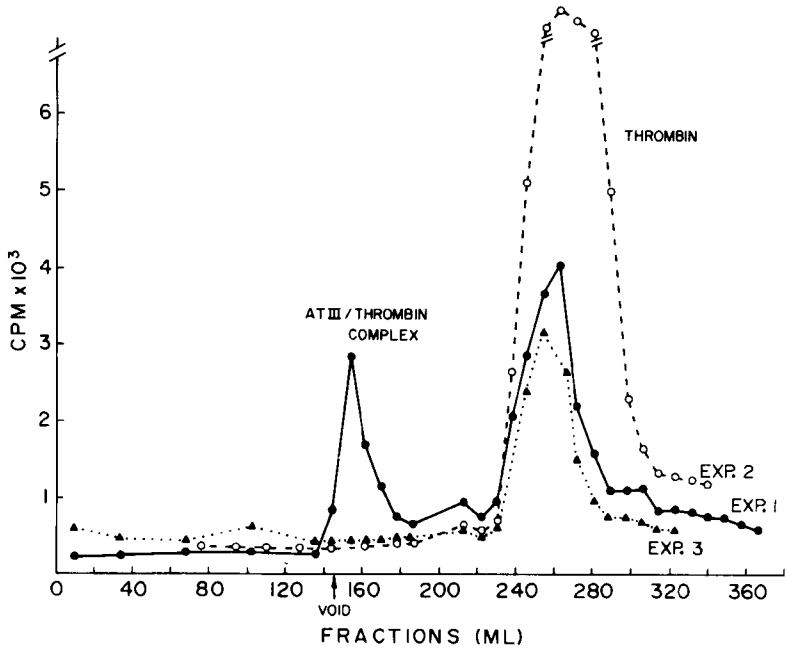

Fig. 1. Gel filtration chromatography to determine binding of I^{125} thrombin by protamine sulfate. Aliquots (20 μg/100 μl in 0.15 M potassium phosphate buffer pH 7.1) of thrombin radiolabeled with I^{125} by the chloramine T method were mixed for 45 minutes at 37°C with a tenfold molar excess of protamine sulfate (experiment 2) and PMSF (experiment 3). At the end of the incubations ATIII was added in excess molar concentration of the labeled enzyme. A control experiment was performed with I^{125} thrombin incubated with anti ATIII ab/ATIII (experiment 1). The samples were separated by filtration on Sephacryl S-200. The radioactivity was determined in a gamma counter.

preincubated with protamine in experiment 2 and with PMSF in experiment 3; PMSF (phenylmethylsulfonylfluoride) is an irreversible seryl protease inhibitor. The result of these two experiments appears on the chromatograms at lower molecular weight. The two peaks represent I^{125} thrombin not bound to ATIII (anti ATIII ab/ATIII). The observation that the peak at high molecular weight in the chromatogram of experiment 1 was suppressed in those from experiments 2 and 3 provides proof that following preincubations of I^{125} thrombin with protamine or PMSF both reagents inhibit ATIII from binding I^{125} thrombin. It also suggests that protamine interacts with thrombin at its active site.

Effect of Protamine on the Clotting Activity of Thrombin

The resulting inactivation of α-thrombin determined by a fibrinogen assay is given in Figure 2. The data show that in the presence of concentrations of protamine sulfate ranging from 10 μM to 200 μM there is a steady inhibition of the clotting activity of α-thrombin. A maximum 90% inhibition of the initial thrombin activity is reached with 200 μM protamine sulfate. The remaining 10% thrombin activity

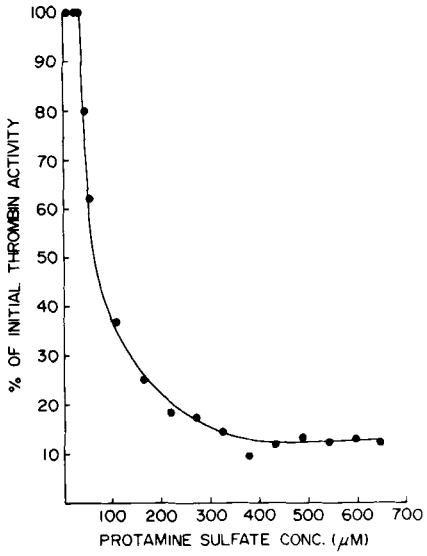

Fig. 2. Study of the effect of protamine sulfate on the clotting activity of thrombin. One hour presubstrate incubation of thrombin (0.5 unit) with varying concentrations of protamine sulfate ranging from 10 μM to 700 μM was performed in 200 μl distilled water. Fibrinogen (1.8 mg/100 μl) was then added and clot formation recorded on a BBL fibrometer.

appears unchanged with increasing concentrations of protamine as high as 700 μM. It is apparent from these results that within a restricted concentration range of 30 μM to 200 μM protamine sulfate there is a steady increase in the inhibition of the clotting activity of α-thrombin.

The graph in Figure 3 illustrates that the observed increase in clotting time is proportional to concentrations of the inhibitor. In this graph, changes in the clotting times can be used to directly determine the amount of protamine sulfate interacting with thrombin in the reaction mixtures. When heparin alone is added to thrombin, a marked inhibition of thrombin's clotting activity is obtained, as has previously been described [9]. However, we demonstrate in Figure 4 that the reversal of heparin's anticoagulant effect is dependent on the concentration of protamine sulfate used in the assay. At low protamine concentrations ranging from 43.2 μM to 108 μM, the antithrombin heparin activity is reversed. At protamine concentrations of 216 μM up to 324 μM, the characteristic protamine sulfate inhibitory activity for thrombin is once again restored.

Effect of Protamine on the Amidolytic Activity of Thrombin

Under the experimental conditions described in the materials and methods section, the residual amidolytic activity of α-thrombin mixed with protamine sulfate was determined by a change in the rate of hydrolysis of S-2238. It was found that the effectiveness of protamine in the inhibition of thrombin was less than 10%, even when molar concentrations of the added reagent were tenfold those of the enzyme. Under identical conditions, the decrease in clotting activity of thrombin was 90% (data not shown).

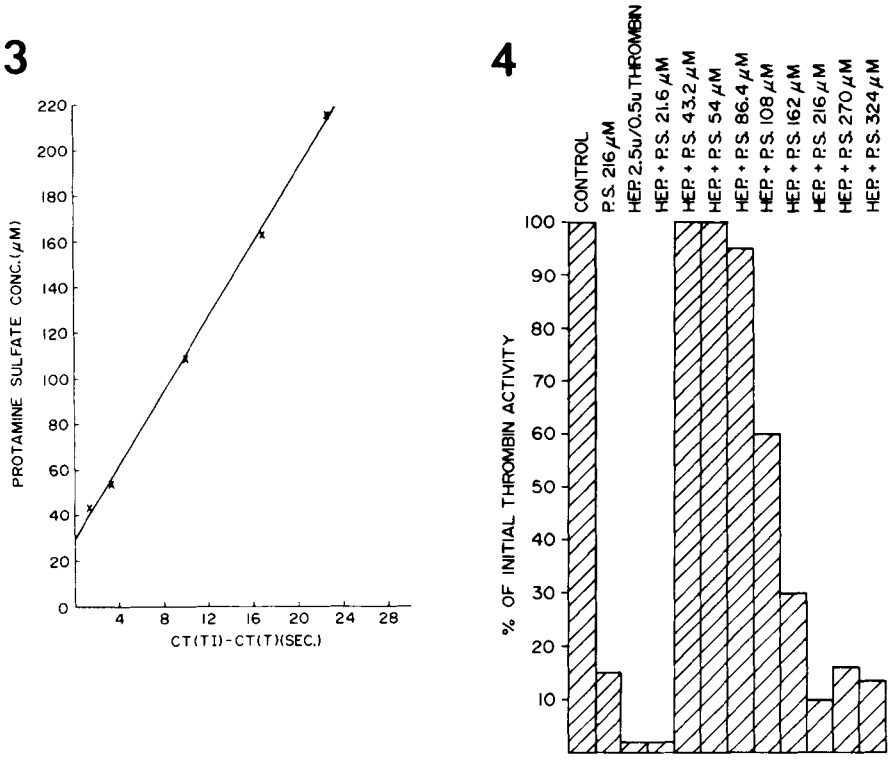

Fig. 3. Protamine sulfate inhibition of the thrombin catalyzed conversion of fibrinogen. The incubations were performed as described for Figure 2. Protamine sulfate concentrations ranged from 30 μM to 200 μM. The data plotted on the ordinate represent the fibrinogen clotting time resulting from the thrombin protamine interaction with fibrinogen CT (T1). The curve represents values that were corrected for a clotting time of 9 to 10 seconds obtained by addition of thrombin (0.5 unit) to fibrinogen (1.8 mg/100 μl) CT(T).

Fig. 4. Effect of protamine sulfate on the heparin mediated inactivation of thrombin. Heparin (2.5 u) was added to thrombin (0.5 u) and incubated for 1 hour followed by addition of concentrations of protamine sulfate ranging from 21.6 μM to 324 μM. The data are plotted as percent of the initial thrombin activity on fibrinogen.

DISCUSSION

The anticoagulant properties that have been attributed to protamine sulfate imply an interaction between the reagent and the enzyme thrombin. From the data presented in this paper in Figure 1, it is evident that in the binding experiments protamine has an inhibitory effect on the interaction of ATIII with thrombin. Whether protamine associates with I^{125} thrombin and thereby prevents its binding by ATIII or whether protamine inhibits the activity of ATIII rather than thrombin is unclear from the results obtained in Figure 1.

However, in the light of recent findings and from the evidence provided from further results presented in our study, it seems unlikely that protamine binds to ATIII.

In a two-dimensional electrophoretic analysis of plasma, ATIII treated with protamine, Okajima et al [10] showed that protamine did not affect the mobility of ATIII on gels, whereas addition of heparin resulted in markedly increasing this mobility. Furthermore, they found that the pattern was returned to the original when protamine was added to the heparinized plasma prior to electrophoresis. In our results shown in Figures 2–4, we demonstrate that in the absence of ATIII there is a definite inhibition of the clotting activity of thrombin by protamine sulfate and that this inhibition is concentration dependent, partial and reversible.

The data presented in our work providing evidence for the thrombin interaction with protamine sulfate is in accordance with previous findings that protamine acts as a substrate for thrombin. Brown et al, in 1973, demonstrated that thrombin is capable of hydrolyzing a peptide bond in protamine. Protamine with the amino-terminal group blocked with dinitrofluorobenzene (DNP) was used to determine the proteolytic activity of thrombin. They reported that thrombin was capable of hydrolyzing protamine at a concentration of 8.3 N.I.H. units/ml at 40 minutes [11]. In another study, the velocity of protamine hydrolysis by plasminlike enzymes was compared. The relative sensitivity for thrombin was reported to be the least effective [7].

It is apparent from the findings reviewed in these two studies that the rate of the thrombin-catalyzed reaction proceeds very slowly. This suggests that prior to the breakdown of the enzyme-substrate complex and the resulting hydrolysis of protamine by thrombin, there is a prolonged intermediate step during which the clotting activity of thrombin is inhibited.

Thus, it would be possible as a result of our work to consider protamine as a competitive inhibitor of the thrombin-fibrinogen interaction. This is in addition to its previously recognized role as a substrate for thrombin. Furthermore, since we show that protamine inhibits the inactivation of thrombin by ATIII, it can be postulated that the mode of neutralization of α-thrombin by protamine is similar to the mechanism by which thrombin is inhibited by ATIII in its interaction with its natural substrate fibrinogen.

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