THE HEPARIN-THROMBIN COMPLEX IN THE MECHANISM OF THROMBIN INACTIVATION BY HEPARIN

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Received May 13,1977

Heparin and thrombin, at very low concentrations, are shown to form a complex with a very high association constant. The heparin-thrombin complex contains an altered thrombin structure, as evidenced by a 70% decrease in the thrombin amidase activity. In the presence of α_2-antithrombin the heparin-thrombin complex can be completely inactivated. It is shown that heparin and α_2-antithrombin do not interact under conditions in which the heparin-thrombin complex is formed and observed. Therefore, it is proposed that the first molecular event in the heparin-mediated thrombin inactivation process in blood is the rapid formation of the heparin-thrombin complex. The complete inactivation of the thrombin in this complex is achieved by further reaction with α_2-antithrombin in blood, the latter reaction probably being the rate limiting step in the thrombin inactivation process.

Knowledge of the mechanism of the heparin-mediated inhibition of thrombin in blood is critical to the understanding and application of heparin as an antithrombotic. In recent years the clear demonstration that heparin action allows thrombin to be effectively inactivated by blood α_2-antithrombin has significantly advanced understanding of the heparin therapeutic mechanism (1,2,3). One of the basic questions remaining unanswered, regarding the mechansim of heparin participation in the α_2-antithrombin-thrombin interaction is whether heparin initially binds α_2-antithrombin to produce a hyperthrombinophilic inhibitor (4,5,6), or whether heparin initially binds directly to thrombin to render the thrombin more susceptible to the large excess of α_2-antithrombin present in blood (7,8,9,10).

In this report it is shown that heparin directly binds thrombin with a large association constant. The data were generated from kinetic analysis of the thrombin hydrolysis of an amide substrate. Recently, we have found that

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ISSN 0006-291X

heparin in plasma will inactivate added thrombin on a mole/mole basis (10). This finding and the present proof of the existence of the heparin-thrombin complex are consistent with the hypothesis that this complex is the first product formed in blood during heparin antithrombotic activity. The excess of α_2-antithrombin in blood, about 3×10^{-6} M (11), then serves as the reagent for final inactivation of the initially formed heparin-thrombin complex.

METHODS. Bovine thrombin was purified from Parke-Davis Topical Thrombin (12,13), and stored frozen in tris (0.06M), NaCl (0.3M), pH 7.4. The thrombin was assayed by plasma clotting times with NIH reference human thrombin lot 3-B and found to be 2200 NIH units/mg (10). The thrombin was homogeneous and free of α_2-antithrombin, as judged by gel electrophoresis (10% polyacrylamide, 0.1 M phosphate, 0.1% sodium dodecyl sulfate, pH 7.4; 7% polyacrylamide, 0.188 M tris-glycine, pH 8.9).

α_2-antithrombin was generously provided by Dr. Milan Wickerhauser of the American National Red Cross Laboratory, Bethesda, lot 51-IIA. This protein was about 95% homogeneous upon gel electrophoresis using the same conditions as above. α_2-antithrombin was dissolved in 0.15 M NaCl, 0.06 M tris, pH 7.4, for use in the experiments discussed herein.

Heparin was Sodium Heparin (Lilly), lot 8LA11A, 160 USP units/mg. Heparin was dissolved in 0.15 M NaCl, 0.06 M tris, pH 7.4, for experimentation.

Benzoylphenylalanylvalylarginyl-p-nitroanilide hydrochloride (BzPheValArgpNA) was purchased from AB Bofors, Sweden. This substrate was dissolved in water for experimentation.

The amidase action of thrombin was measured with BzPheValArgpNA (16.3×10^{-5} M) by continuous monitoring of reaction mixtures at 405 nm with a Varian 635 spectrophotometer equipped with a Varian model 135-1 recorder. The liberated p-nitroaniline has an extinction coefficient of 10,600 liter-cm/mole. The final conditions for all amidase assays in this report were: tris (0.033 M), NaCl (0.095 M), pH 7.4; temperature was 21°. The data were obtained with average deviation of 2% or less.

Calculations with heparin assume that the average molecular weight is about 11,000 (14,15). Bovine thrombin is assumed to have a molecular weight of about 39,000 (16), and is assumed to assay as about 2500 NIH units/mg when pure (10,17,18,19).

RESULTS. The direct binding of thrombin by heparin is shown by the experiment illustrated in Table I. In this experiment BzPheValArgpNA (16.3×10^{-5} M) was hydrolyzed by a fixed concentration of thrombin (1.8×10^{-8}M). The heparin (0.5 ml), at various concentrations, and thrombin (0.1 ml) were incubated at 21° for 60 seconds, then the BzPheValArgpNA (0.5 ml) added, and the reaction mixtures immediately monitored for absorbance at 405 nm.

TABLE I

DIRECT INHIBITION OF THROMBIN BY HEPARIN
BzPheValArgpNA (16.3×10^{-5} M) Hydrolysis Kinetics

Thrombin	Heparin	Heparin/Thrombin	Amidase Inhibition**	Thrombin Bound
1.86×10^{-8} M	0.054×10^{-8} M	0.028 mole/mole	0 %	0 %
1.86 "	0.107 "	0.057 "	3.4	5
1.86 "	0.214 "	0.114 "	6.4	9
1.86 "	0.286 "	0.153 "	8.3	12
1.86 "	0.429 "	0.230 "	16.6	24
1.86 "	0.573 "	0.310 "	23.0	33
1.86 "	0.859 "	0.460 "	34.2	49
2.3* "	1.61 "	0.70 "	46	64
1.86 "	1.72 "	0.92 "	51.3	73
2.3* "	2.58 "	1.12 "	70	100
2.3* "	3.87 "	1.7 "	70	100
1.86 "	all levels up to	25 "	70	100

*Data from similar experimentation using 2.3×10^{-8} M thrombin.

**The control hydrolysis (no heparin) was 0.220 absorbance/minute.

The final heparin concentrations are shown in Table I.

The resulting kinetic data (see Table I) demonstrate that the thrombin amidase reaction is inhibited increasingly as the heparin concentration is increased. The observed inhibition reaches a plateau level of about 70% inhibition. This inhibitory plateau indicates saturation of the thrombin with heparin molecules, and occurs when the heparin concentration has reached (or slightly exceeded) equimolarity with the thrombin. Increasing the heparin to 50×10^{-8} M did not produce additional thrombin amidase inhibition. Therefore, the kinetics conclusively show that heparin does directly bind thrombin, and , at these very low concentrations, the heparin-thrombin complex is formed nearly stoichiometrically. The heparin-thrombin complex retains about 30% of the original thrombin amidase activity, suggesting that the active site on thrombin is not blocked. The marked change in the amidase-related catalytic function of the bound thrombin may result from conformational changes

induced allosterically by the attached heparin molecule.

Calculations from the experimental data, presented in Table I, include three items of data from similar experimentation. Note that 1×10^{-8} M heparin is equivalent to about 0.017 USP units/ml. The third column in Table I shows the increasing heparin/thrombin ratios. The thrombin amidase inhibition observed in each kinetic analysis is shown in the fourth column. Assuming that 70% inhibition is equivalent to 100% thrombin bound, the thrombin bound in each experiment can be calculated (the fifth column). Calculations from data such as these, and from graphical analyses (20, 21), show that the association constant for the formation of the heparin-thrombin complex is very large (apparently, $10^8 - 10^9$ liter/mole). At all heparin concentrations used, the heparin-thrombin complex was formed essentially completely.

When the above experiments were performed adding α_2-antithrombin (0.45×10^{-8} M) together with the heparin, the thrombin active site became completely blocked, as expected, producing 100% amidase inhibition when the heparin/thrombin ratio was 1.0 or greater.

The experiments described above and in Table I were also repeated with the sequence of events altered so that thrombin would be added lastly to a pre-incubated mixture of substrate and α_2-antithrombin and/or heparin.

The altered design was: 0.25 ml heparin (or buffer)
0.25 ml α_2-antithrombin (or buffer)
0.50 ml BzPheValArgpNA
after 60 seconds, 0.10 ml thrombin added and the reaction immediately monitored for absorbance at 405 nm (all operations at 21°).

This procedure will allow time for a hypothetical interaction (complex formation) between heparin and α_2-antithrombin, while there is no chance for formation of a pre-equilibrated heparin-thrombin complex. Thus, the added thrombin (2.3×10^{-8} M) will see, concomitantly, heparin, α_2-antithrombin (or a complex

TABLE II
REVISED THROMBIN AMIDASE ASSAY
(Pre-incubation of heparin and/or α_2-antithrombin)

Pre-incubation Mixture		Thrombin	%Amidase
α_2-antithrombin	heparin	(added last)	Inhibition
4.5×10^{-6} M	0	2.3×10^{-8} M	0
0	25.8×10^{-8} M	2.3×10^{-8} M	15
4.5×10^{-6} M	25.8×10^{-8} M	2.3×10^{-8} M	15
0	129×10^{-8} M	2.3×10^{-8} M	70

BzPheValArgpNA was 16.3×10^{-5} M

between these two), and a large excess of BzPheValArgpNA (16.3×10^{-5} M). The data from these experiments are shown in Table II, in which the final concentrations of the reactants are indicated, along with the resulting inhibition of thrombin amidase activity. The data in Table II show that heparin, at 0.45 USP units/ml (25.8×10^{-8} M), is able to compete with the excess substrate for thrombin binding and does form enough heparin-thrombin complex to cause an observed 15% inhibition of thrombin amidase activity. However, pre-incubation of this same amount of heparin with a physiological concentration of α_2-antithrombin (4.5×10^{-6} M) causes NO enhancement of thrombin inhibition. Therefore, there is no evidence of interaction between heparin and α_2-antithrombin even though they were incubated together, while the heparin-thrombin complex was observed in this system.

DISCUSSION. The fact that a heparin-thrombin complex does form, with very high association constant, between very low concentrations of heparin and thrombin, and under nearly physiological conditions, clearly implicates the heparin-thrombin complex as the initial product formed in the heparin-mediated thrombin inactivation in blood. Such a complex is necessary to explain the

immediate inactivation of thrombin added to heparinized plasma (10). The heparin-thrombin complex, which contains an altered thrombin structure whose amidase properties are markedly changed, also has a markedly changed affinity (greatly increased) for α_2-antithrombin. The final event in the thrombin inactivation in blood is the reaction between the heparin-thrombin complex and α_2-antithrombin to completely block the thrombin active site. This final reaction seems much slower than the rate of formation of the heparin-thrombin complex, according the data in Table II. Hence, it can be postulated that the rate limiting factor in the heparin-mediated thrombin inactivation process in blood is the concentration of α_2-antithrombin in blood. This postulate agrees generally with clinical experience, which suggests that diminished α_2-antithrombin results in resistance to heparin anticoagulation and tends toward thrombosis.

The hypothesis that heparin binds thrombin in blood (rather than binding α_2-antithrombin) has been elaborated by Machovich in a convincing fashion, demonstrating that heparin-thrombin interactions were observed by gel chromatography while α_2-antithrombin-heparin interactions could not be observed (9). Marciniak also failed to observe binding between α_2-antithrombin and heparin with gel chromatographic analysis (22), while Li, et al, using dye binding techniques did observe a heparin-thrombin complex (23).

In this present report proof is presented that a heparin-thrombin complex does form, with very strong binding properties, from concentrations of reactants which must be considered important in vivo. It is also shown that, under conditions in which the heparin-thrombin complex is observed, interaction between α_2-antithrombin and heparin could not be detected. Therefore, it is proposed that in the process of thrombin inactivation in heparinized blood the first molecular event is the formation of the heparin-thrombin complex.

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