HEPARIN REACTS STOICHIOMETRICALLY WITH THROMBIN DURING THROMBIN INHIBITION IN HUMAN PLASMA

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The thrombin-induced clotting of human plasma is not inhibited by endogenous protease inhibitors (α_2-antithrombin, α_2-macroglobulin, α_1-antitrypsin) even though these inhibitors are present in huge molar excess. However, when heparin is added into the plasma clotting system, the thrombin is instantaneously and stoichiometrically inhibited. Each mole of added heparin causes the immediate inhibition of exactly one mole of thrombin. When a 1:1 molar ratio of heparin:thrombin exists, 100% inhibition of the thrombin occurs. Obviously, heparin interacts with thrombin in a 1:1 molar fashion. We postulate that a 1:1 thrombin-heparin complex reacts rapidly with the excessive amount of α_2-antithrombin in plasma (normally 2×10^{-6} M) to result in the inactive termolecular compound:(α_2-antithrombin:thrombin:heparin). Therefore, at normal α_2-antithrombin levels, the required therapeutic heparin level will be equal to the molar thrombin challenge in the patient.

Heparin currently represents the most important drug entity available which can inhibit the thrombin induced thrombosis process. The mechanism of the inhibitory action of heparin in blood is, therefore, of vital significance. The mechanistic theory which is now widely accepted describes the role of heparin in plasma as merely a catalyst for the otherwise sluggish formation of a 1:1 molar complex between α_2-antithrombin (a plasma protease inhibitor) and thrombin (1,2,3). In vitro, α_2-antithrombin has also been shown to inhibit the activated coagulation factors IXa, Xa, XIa, XIIa, and the enzyme plasmin (4). Since heparin greatly enhances all of these inhibitions in vitro, the role of heparin has been described, again, as an accelerator, or catalyst (4,5).

Our experiments, described herein, show that heparin, when added to human plasma behaves as a stoichiometric reactant in the inhibition of thrombin and does not behave as a catalyst. This observation may prove to be of critical importance in the

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understanding of the antithrombotic effect produced by heparin clinically. For, according to our hypothesis, unless the heparin blood level reaches stoichiometry with the thrombin challenge in the patient, complete thrombin inhibition will not occur.

Methods. Quantitation of thrombin in solution was performed by the well-established technique of relating thrombin concentration to coagulation kinetics. Human citrated plasma (Dade CNP) was the source of clotting substrate. Bovine thrombin (Parke-Davis) was used either: a) crude reconstituted solution (100 NIH units per mg protein); or b) purified by ion exchange chromatography (6) to 1750 NIH units per mg, free of α_2-antithrombin by electrophoresis). The analytical clotting mixtures consisted of:

0.1 ml human plasma
0.1 ml tris/NaCl buffer (with or without heparin)
0.1 thrombin in tris/NaCl buffer (about 6 NIH units per ml)

The buffer was 0.06 M tris, 0.3 M NaCl, pH 7.4 (alternatively, the buffer contained 0.15 M NaCl).

The clotting times of the mixtures were measured with a Fibrometer from Bioquest, in duplicate, at 37°, allowing 1 minute equilibration before the addition of thrombin. A standard curve was carefully constructed with NIH human thrombin reference lot No. 3-B, plotting thrombin concentration (NIH units per ml) versus clotting time (seconds). The reproducibility of the measurements was ± 2% for clotting times of 50 seconds or lower, and was ± 5% for clotting times of 80 seconds or longer. The same standard data were produced by any vial of Dade CNP plasma within the lot used; therefore, the plasma could be used as a standard substrate for the assay of unknown thrombin concentrations.

The inhibition of thrombin by heparin was studied by including sodium heparin (Upjohn lot 117AT C2, or Eli Lilly lot 8LA11A) in the tris/NaCl buffer. Each heparin assayed about 160 USP units/mg. The analytical mixture contained 0.1 ml plasma, 0.1 ml heparin, and 0.1 ml thrombin, exactly as above; the measured clotting time of the mixture would yield the amount of free (uninhibited) thrombin remaining in the mixture and hence, the amount of thrombin inhibited could be calculated, knowing the initial thrombin concentration.

Thrombin concentrations were converted from NIH units per ml to molar values by assuming that an active molecule of bovine thrombin will behave as though there are 2500 NIH units per mg thrombin; this is the most reasonable value from a consolidation of available data (7-10). That this is a valid calculation is shown by the identical data obtained from crude thrombin and from very pure thrombin. Heparin concentrations were converted from USP units per ml to molar values by assuming a molecular weight of 11,000 (11,12).

Results. In Table I is shown the ability of heparin to inhibit thrombin in a series of human plasma clotting mixtures. The clotting times of the mixtures of human plasma, heparin, and

TABLE I

HEPARIN INHIBITION OF THROMBIN IN HUMAN PLASMA

Clotting Time of Assay (Seconds)	Thrombin Challenge (moles/l)	Heparin USP (units/ml)	Heparin (moles/l)	Thrombin Inhibited (moles/l)	Molar Ratio of Heparin: Thrombin (mole/mole)	Molar Ratio of Thrombin Inhibited to Thrombin Challenge (mole/mole)
32.3	1.96×10^{-8}	None	None	None	0	0 (Control)
34.6	1.96×10^{-8}	0.00333	0.189×10^{-8}	0.157×10^{-8}	0.096	0.08 (8% Inhibition)
34.8	1.96×10^{-8}	0.00416	0.236×10^{-8}	0.176×10^{-8}	0.120	0.09 (9% ")
41.0	1.96×10^{-8}	0.00833	0.474×10^{-8}	0.431×10^{-8}	0.242	0.22 (22% ")
46.8	1.96×10^{-8}	0.01266	0.719×10^{-8}	0.607×10^{-8}	0.367	0.31 (31% ")
83.6	1.96×10^{-8}	0.01666	0.946×10^{-8}	1.04×10^{-8}	0.482	0.53 (53% ")
131.3	1.96×10^{-8}	0.02500	1.42×10^{-8}	1.41×10^{-8}	0.724	0.72 (72% ")
>700 (no clot)	1.96×10^{-8}	0.03333	1.89×10^{-8}	1.96×10^{-8}	0.964	1.00 (100% ")

Thrombin was 1.92 NIH units/ml final (100 units/mg); 39,000 gm per mole (8).
Heparin was Upjohn Sodium Heparin
Assay details in Methods.

added thrombin were measured as described above. The added thrombin (or thrombin challenge) in each mixture was 1.92 NIH units per ml or 1.96×10^{-8} M thrombin. The amounts of heparin in the mixtures varied from 0.00333 USP units per ml (0.189×10^{-8} M heparin) to 0.0333 USP units per ml (1.89×10^{-8} M heparin). At this highest level there was one mole of heparin for each mole of thrombin, and at this point 100% of the thrombin was inhibited (infinite clotting time). The number of moles of thrombin inhibited at each level of heparin is equal to the number of moles of heparin added. Reference to the last two columns of Table I show that the fractional molar ratio of heparin/thrombin equals the fraction of thrombin inhibited. If the molar ratio of heparin/thrombin becomes as high as 1.0 then all of the enzyme is inhibited--of course, higher heparin/thrombin ratios maintained the system as incoagulable. Clearly, these data demonstrate a 1:1 molar stoichiometry between heparin and inhibited thrombin.

The same experimental results were obtained using a different commercial heparin and using a highly purified thrombin (1750 NIH units per mg). Table II presents these data, obtained with the same experimental design as above. In this particular experiment (Table II) the thrombin challenge in the clotting mixtures was 2.12 NIH units per ml (2.16×10^{-8} M thrombin). Here, again, the number of moles of thrombin inhibited in each clotting mixture is equal to the number of moles of heparin added to each mixture. The experiments described in Tables I and II were repeated a number of times with the same experimental results being obtained, using: crude thrombin versus purified thrombin (1750 units per mg and free of α_2-antithrombin); Upjohn versus Lilly sodium heparin; preincubation of heparin with

TABLE II

HEPARIN INHIBITION OF THROMBIN IN HUMAN PLASMA

Thrombin Challenge (NIH units/ml)	Thrombin Challenge (moles/l)	Heparin (USP units/ml)	Heparin (mole/l)	Thrombin Inhibited (moles/l)	Molar Ratio of Heparin : Thrombin Challenge (mole/mole)	Molar Ratio of Thrombin Inhibited to Thrombin Challenge (mole/mole)
2.12	2.16×10^{-8}	0.00250	0.142×10^{-8}	0.093×10^{-8}	0.066	0.043 (4.3% Inhibition)
2.12	2.16×10^{-8}	0.00333	0.189×10^{-8}	0.173×10^{-8}	0.087	0.080 (8.0% ")
2.12	2.16×10^{-8}	0.00416	0.236×10^{-8}	0.205×10^{-8}	0.109	0.095 (9.5% ")
2.12	2.16×10^{-8}	0.00832	0.474×10^{-8}	0.326×10^{-8}	0.219	0.151 (15.1% ")
2.12	2.16×10^{-8}	0.0166	0.946×10^{-8}	0.821×10^{-8}	0.438	0.380 (38.0% ")
2.12	2.16×10^{-8}	0.0250	1.42×10^{-8}	1.40×10^{-8}	0.657	0.646 (64.6% ")
2.12	2.16×10^{-8}	0.0333	1.89×10^{-8}	$>1.72 \times 10^{-8}$*	0.875	>.8* (>80%* ")

Bovine thrombin was 1750 units/mg; 39,000 gm per mole (8).

Heparin was Eli Lilly and Company Sodium Heparin

Assay details in Methods.

* Assay clotting time was greater than 200 seconds and was not recorded.

plasma versus preincubation of heparin with thrombin; 0.15 M NaCl versus 0.3 M NaCl ionic strength. In all cases there was the same stoichiometric relationship between heparin and thrombin, with 100% thrombin inhibition requiring simply a 1:1 molar ratio of heparin:thrombin.

Discussion. The data presented above demonstrate that the role of heparin in the inhibition of thrombin in human plasma is definitely not that of a catalyst, but rather, heparin participates as a stoichiometric reactant in the inhibition process. The inhibited thrombin molecule must be pictured as being bound with one molecule of heparin. It seems very likely that a termolecular compound is actually formed in plasma with one of the excess plasma protease inhibitors (α_2-antithrombin, α_2-macroglobulin, or α_1-antitrypsin), such that the ternary complex would be: (inhibitor:thrombin:heparin). It has been shown that α_2-antithrombin binds stoichiometrically with thrombin, although slowly, in vitro, and that the binding reaction is made instantaneous by adding "small amounts" of heparin (3,13), the heparin being termed an accelerator or catalyst (1,4). However, upon examination of the experimental conditions used (3,13), it is clear that heparin was included in the reaction mixtures in molar excesses and not in "small" or "catalytic" amounts when the instantaneous combinations of α_2-antithrombin with thrombin were achieved. Hence, there is no experimental basis for considering heparin as merely a catalyst; rather, the published in vitro data (3,13) upon which the catalytic mechanism is based, suggest the possibility that heparin was involved in a stoichiometric fashion. Moreover, Rosenberg and Damus noted that their results could be explained by the formation of a heparin-thrombin complex, but rejected the possibility because they could show no inhibi-

tion of thrombin (esterase or clotting activities) by heparin alone (3). We have since shown that heparin alone inhibits both thrombin clotting and amidase activities, and from the latter data, forms a strong 1:1 complex with thrombin (17).

Human plasma contains the following protease inhibitors, all claimed to inhibit thrombin (14-16): α_2-antithrombin = 2.1×10^{-6} M; α_2-macroglobulin = 0.9×10^{-6} M; α_1-antitrypsin = 18.5×10^{-6} M. When thrombin levels as low as 1×10^{-10} M are added to human plasma, there is no inhibition by this formidable excess of protease inhibitors (17). This same conclusion could be deduced from the control mixture of Table I above, in which 2×10^{-8} M thrombin caused a 30-second clotting time in the plasma assay system in spite of the excess plasma inhibitors. Heparin converts this inert inhibitory system to a powerful and instantaneous antithrombin system (Tables I and II). That heparin participates in this antithrombin system stoichiometrically allows us to rationalize the clinical effectiveness of heparin. We postulate that in the presence of excess plasma protease inhibitors, a molar thrombin challenge experienced by a patient will require an equivalent molar blood level of heparin in order for complete anticoatulation to be realized.

The maximum thrombin challenge in human plasma is simply equal to the prothrombin concentration, normally about 1×10^{-6} M (19,20). This is also near the molarity of the heparin concentration achieved during complete anticoagulation clinically, namely, about 1-2 USP units per ml or $0.6\text{-}1.2 \times 10^{-6}$ M heparin (18). Postoperative deep vein thrombosis is prevented by heparin levels of 0.05-0.15 units/ml (21) or about $0.03\text{-}0.11 \times 10^{-6}$ M heparin; therefore, these patients must have experienced thrombin challenges of $0.03\text{-}0.11 \times 10^{-6}$ M thrombin (or less).

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