Fractionation of heparin by chromatography on a tissue plasminogen activator-Sepharose column

(affinity chromatography)

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ABSTRACT Heparin stimulates the activity of tissue plasminogen activator (t-PA) and binds to t-PA. To study this interaction, a complex between t-PA and N-acetylated heparin was formed and then linked to Sepharose. This procedure selectively links the t-PA to the column because the acetylated heparin has no free amino groups. The procedure also protects the heparin-binding site(s) on the enzyme during coupling to the matrix. The t-PA column separates heparin into two fractions, one with low affinity for t-PA and one with high affinity. Both fractions of heparin effectively accelerate inactivation of thrombin by antithrombin III. However, the fractions differ in their ability to stimulate t-PA: the low-affinity heparin has no effect on the activity of t-PA, whereas the high-affinity heparin enhances this activity. These heparin fractions will be useful in characterizing the biochemical basis and physiological consequences of the heparin–t-PA interaction.

Heparin can bind many proteins of biological interest, including antithrombin III (AT-III), clotting factors (1), growth factors (2, 3), apolipoproteins (4), fibronectin (5), and plasminogen activators (6–8). The interaction between heparin and AT-III has been extensively studied. The anticoagulant potency of heparin, due primarily to its binding to AT-III (9, 10), depends on the molecular size of the preparation (11–14) and a particular polysaccharide sequence (15–17). This work was aided by fractionation of heparin on columns of immobilized AT-III and chemically analyzing the fractions that bound most tightly (15, 16, 18–22).

Heparin has been reported to enhance fibrinolysis *in vitro* and *in vivo* (23). By using purified components of the fibrinolytic system, heparin has been shown to enhance the activity of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (6, 7, 24). Whereas commercial heparin binds tightly to both t-PA and Lys-plasminogen, low-molecular-weight heparin does not bind t-PA and binds Lys-plasminogen only weakly (25). These low-molecular-weight heparins do not stimulate plasmin formation by t-PA but still retain anticoagulant properties, such as binding to AT-III and acceleration of the inactivation of thrombin. Thus, size of the mucopolysaccharide appears important for its interaction with the fibrinolytic system.

To study heparin–t-PA interaction, we sought a way to fractionate heparin on columns of immobilized t-PA. This approach was successful only when t-PA was protected by N-acetylated heparin during the coupling of t-PA to the matrix, similar to the protocol used for purification of AT-III-binding heparins (18). The chromatography has separated heparin into two fractions, one with high affinity for t-PA and the other with low affinity for t-PA. The anticoagulant activities of both fractions are similar. These two fractions

should be useful in exploring the biochemical basis and clinical consequences of the heparin–t-PA interaction.

EXPERIMENTAL PROCEDURES

Materials

Heparin (calcium salt) was derived from porcine intestinal mucosa (148 units/mg; Sigma). CNBr-activated Sepharose 4B and Sephadex G-75 (superfine) were purchased from Pharmacia. The chromogenic plasmin substrate D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (S-2251) was purchased from Kabi (Stockholm). Thrombin substrate D-hexahydrotyrosyl-L-alanyl-L-arginine-*p*-nitroanilide diacetate salt was from American Diagnostica (New York).

The proteins used were obtained as follows: plasminogen-free bovine fibrinogen (26, 27); soluble fibrinogen fragments (28); human Glu-plasminogen (29); human Lys-plasminogen, two-chain t-PA purified from human melanoma (Bowes) cells (fibrinolytic activity 650,000–750,000 international units/mg), urokinase-type plasminogen activator high-molecular-weight form, 63,000 units/mg (American Diagnostica); human recombinant t-PA (Genentech); human thrombin 3000 NIH units/mg (Sigma); AT-III 5 international units/mg (Helen Laboratories).

Methods

Preparation of Active t-PA-Sepharose. *Acetylation of heparin-agarose.* Heparin-agarose (15 ml, 780 μ g of heparin/ml of bed volume) was washed with 10% methanol, centrifuged, and resuspended with 5 ml of 100% methanol. After cooling to 5°C, 25 mg of NaCO₃ was added with gentle stirring. Acetic anhydride (100 μ l) was added slowly, and the pH was maintained at 7.0–7.5 with Na₂CO₃. Samples of beads were tested for free amino groups with 2,4,6-trinitrobenzene-sulfonic acid (TNBS) in a saturated borate solution. Additional acetic anhydride was added when results of the TNBS reaction were positive (orange-yellow color). The final gel was collected on a sintered glass filter and washed with 100 ml of H₂O, 100 ml of 0.5 M NaCl, and stored at 5°C in 0.5 M NaCl.

Binding of t-PA to acetylated heparin-agarose. Acetylated and nonacetylated heparin-agarose (20 μ l of packed gel, 780 μ g of heparin/ml of gel) and 10 ng of t-PA in 80 μ l of phosphate-buffered saline were incubated for 45 min at 25°C. The resin was centrifuged, and the supernatant was removed. After a quick wash of the resin with phosphate-buffered saline, the bound protein was eluted with 80 μ l of 2%

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Abbreviations: AT-III, antithrombin III; t-PA, tissue-type plasminogen activator; LA-heparin and HA-heparin, fraction of heparin with low and high affinity for t-PA, respectively.

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SDS/5% glycerol/phosphate-buffered saline. Samples were electrophoresed and analyzed by casein-agar zymography (30, 31).

Acetylation of heparin. Heparin was N-acetylated following the procedure for acetylation of heparin-agarose described above. Heparin (50 mg) was dissolved in 5 ml of aqueous 10% methanol. After addition of 25 mg of NaCO₃ and cooling to 4°C, 150 μ l of acetic anhydride was added in 25- μ l fractions over a period of 30 min. The final reaction mixture was dialyzed against H₂O and lyophilized.

Preparation of active t-PA-Sepharose. Before immobilization of t-PA to CNBr-activated Sepharose 4B, the t-PA was complexed with N-acetylated heparin: 20 mg of t-PA was dissolved in 10 ml of 0.2 M Hepes/5% glycerol, pH 7.4, and dialyzed against the same buffer. N-acetylated heparin (2.0 ml of solution at 20 mg/ml) in the Hepes/glycerol buffer was added dropwise to the t-PA solution with gentle mixing. N-acetylated heparin was used to protect the heparin-binding sites of t-PA and prevent their destruction during the coupling reaction. The CNBr-activated Sepharose 4B (4 g of dry weight) was coupled to t-PA in the presence of N-acetylated heparin by mixing overnight at 4°C and then for 1 hr at room temperature. Excess ligand (t-PA) and acetylated heparin were removed by washing with Hepes buffer, and the remaining active groups on the Sepharose were blocked with 0.1 M Tris-HCl, pH 8.0, for 4 hr. The protein content of the gel was $\approx$ 0.6 mg of t-PA per ml of Sepharose suspension. The activity of the gel was analyzed by following the generation of amidolytic plasmin activity of a mixture of Lys-plasminogen and t-PA-Sepharose beads. This assay was also performed in the presence of either heparin or fibrinogen fragments (6). Beads were centrifuged at different times, and A₄₀₅ of the supernatant was measured. The data are expressed as the slopes of plots of A vs. time squared; these slopes measure the rate (V₀) of plasmin generation by t-PA.

Fractionation of Heparin on a t-PA Column. Heparin (200 μ g) was chromatographed on a column of t-PA-Sepharose at 4°C. The column (2.5 $\times$ 8 cm) was equilibrated with 0.1 M Tris-HCl/75 mM NaCl, pH 8.0. Fractions (2 ml) were collected until the eluate was completely devoid of heparin ($\approx$ 3 column volumes), as measured by the Azure A method (see below). Bound heparin was then eluted from the column with 0.1 M Tris-HCl/2.5 M NaCl, pH 8.0. Both the fractions obtained with the loading buffer (low-affinity fractions, LA-heparin) and those obtained with the high-ionic-strength buffer (high-affinity fractions, HA-heparin) were pooled separately, dialyzed against H₂O, and lyophilized. The dried LA-heparin fraction was dissolved in 100 μ l of loading buffer, rechromatographed, and processed as above.

Assay Procedures. **Assay for heparin.** Heparin concentrations were determined by a modification of the Azure-A procedure of Jacques and Wollin (32). Aliquots (20 μ l) of the fractions were added to 200 μ l of Azure-A solution (20 μ g/ml in 0.02 M Tris-HCl, pH 8.0), and the A₄₉₀ was determined in a Bio-Tek model EL308 microplate reader. A standard curve was generated with known heparin concentrations.

Assay for t-PA activity. Lys-plasminogen (final concentration, 0.42 μ M) was incubated at 37°C with t-PA and the chromogenic substrate, S2251 (0.3 mM), and different concentrations of heparins (unfractionated heparin, LA-heparin, HA-heparin) in 0.1 M Tris-HCl, pH 8.1/0.01% Tween 80. Generation of plasmin and initial rates of hydrolysis of the substrate were determined as described (6).

Assay for thrombin inhibition. Inhibition of amidolytic thrombin activity by AT-III and the LA- and HA-heparin fractions was analyzed at 25°C as follows: Heparin fractions (0–200 ng/ml) were incubated with AT-III (50 μ l, 0.3 units/ml) in 0.05 M Tris-HCl, pH 8.4/7.5 mM EDTA/0.17 M NaCl. After 3 min, 50 μ l of thrombin (10 NIH units/ml in 0.15 M NaCl) was added. Two minutes later, 50 μ l of substrate

solution (0.3 mM chromogenic substrate D-hexahydrotyrosyl-L-alanyl-L-arginine-p-nitroanilide diacetate salt in polybrene at 0.4 mg/ml) was added, and the A₄₀₅ changes were measured.

Gel Filtration of Heparins. Gel filtration of heparins (unfractionated, LA-, and HA-heparin fractions) was performed on a Sephadex G-75 column (1 $\times$ 60 cm, void volume 11.2 ml) equilibrated with 0.1 M sodium phosphate buffer/1 mM EDTA/0.001% Triton X-100, pH 7.0. The column was calibrated with polystyrene sulfonates of known molecular weights. They were detected in the eluate by absorbance at 240 nm. The heparin content of the fractions ($\approx$ 1 ml per fraction) was measured by Azure-A.

RESULTS

t-PA-Sepharose. Initially, heparin was fractionated on a column in which free t-PA had been linked to Sepharose 4B by using CNBr in a conventional manner. As shown in Fig. 1A, no heparin was retained on the column. One explanation for this result was the destruction of the heparin-binding region of t-PA during enzyme immobilization. To fix t-PA to Sepharose and still maintain heparin-binding capacity, we coupled a t-PA-heparin complex to the matrix. This strategy presented an additional problem: heparin, a glycosaminoglycan, contains primary amino groups that can react with activated CNBr-Sepharose. To prevent heparin coupling from occurring, the free amino groups of heparin were acetylated with acetic anhydride before complex formation with t-PA.

Before using this strategy, it was necessary to determine whether N-acetylated heparin would bind to t-PA, as described for unmodified heparin (6). The affinity was measured by incubating the protein with the acetylated or unmodified heparin-agarose and analyzing the supernatant-associated

FIG. 1. Fractionation of heparin on t-PA-Sepharose prepared without (A) and with (B) protection of the heparin-binding site of t-PA during coupling. Two-milliliter-fractions were collected until the eluate was completely devoid of heparin. The column was then washed with high-salt buffer. Fractions obtained with the equilibration buffer were pooled together (LA-heparin), and the bound heparin eluted with high salt buffer (B) was also pooled (HA-heparin).

FIG. 2. Binding of t-PA to acetylated heparin-agarose. Packed acetylated (+Ac) or nonacetylated (-Ac) heparin-agarose beads were incubated with t-PA. The bound protein found in the pellet (P) and the supernatant or unbound protein (S) were analyzed by gel electrophoresis and casein-agar zymography as described. rt-PA, recombinant t-PA.

protein and the bound material. Fig. 2 shows that N-acetylated heparin binds t-PA effectively enough to be used as a protective agent during the immobilization procedure.

Because acetylated heparin bound t-PA, a coupling procedure was performed with a t-PA-acetylated heparin complex. In this case, only the t-PA will contain free amino groups and be coupled by the CNBr procedure, but the heparin-binding site of t-PA should be protected. After such coupling, the activity of the t-PA covalently bound to Sepharose beads was measured by incubation of t-PA-Sepharose beads with Lys-plasminogen with or without either fibrinogen fragments or heparin. Fig. 3 shows that t-PA-Sepharose can activate plasminogen to plasmin and is stimulated by heparin. The stimulation by fibrinogen fragments is not as great as when t-PA is free in solution. One explanation is that there are fewer fibrinogen binding sites on the t-PA-Sepharose because they were not protected during the coupling procedure. The t-PA bound to Sepharose is quite stable: the enzyme activity did not decrease after a 14-hr incubation at room temperature or after 4 mo at 4°C.

Fractionation of Heparin. When heparin was fractionated on immobilized t-PA that had its heparin-binding site protected during the coupling reaction, $\approx 30\%$ bound to the column (Fig. 1, compare B with A, column made with unprotected t-PA). The bound material could be recovered by elution with high-ionic-strength buffer. After dialysis, when this HA-heparin fraction was reapplied to the column, it was quantitatively bound. To ensure that the flow-through fraction was devoid of components showing high affinity for the column, the unbound material was passed through the column twice more. By the third application, no high-affinity material was seen. The final flow-through fraction was designated as LA-heparin.

FIG. 3. Plasminogen-activating activity of t-PA-Sepharose. The activity was determined as described. rt-PA, recombinant t-PA.

FIG. 4. Effect of heparin fractions obtained after t-PA-Sepharose chromatography on the activity of t-PA. t-PA (0.09 nM) was incubated at 25°C in 200 μ l of 0.1 M Tris-HCl, pH 8.1/0.01% (vol/vol) Tween 80-containing heparin fractions (unfractionated heparin, LA-heparin, or HA-heparin)/0.42 μ M Lys-plasminogen/0.3 mM D-valyl-L-leucyl-L-lysine-*p*-nitroanilide. The initial rate of plasmin generation is plotted vs. the heparin concentrations.

Fig. 1 illustrates two points. (i) Acetylated heparin has to be used during immobilization of t-PA to Sepharose in order to be able to fractionate heparin. (ii) At least two types of heparin occur, with respect to affinity toward t-PA.

Characterization of the Fractions. *Effects of LA- and HA-heparin fractions on plasminogen activation.* The ability of the HA-heparin and LA-heparin to stimulate the activation of plasminogen by t-PA was determined. Fig. 4 shows that HA-heparin at 15 μ g/ml enhanced t-PA activity 3.7-fold. However, when the LA-heparin fraction was assayed in the same manner, no significant enhancement of t-PA activity was observed over the same range of concentrations. Unfractionated heparin, under the same assay conditions, increases t-PA-mediated plasminogen activation 2-fold (6).

Effect of LA- and HA-heparin fractions on inhibition of thrombin by AT-III. The heparin fractions obtained after t-PA affinity chromatography were compared with unfractionated heparin for their ability to stimulate the inactivation of thrombin by AT-III. Fig. 5 shows that all preparations of heparin (prefractionation, LA-, and HA-heparin) were effective at inactivating thrombin via AT-III.

Molecular Size Fractionation. To define further the different properties of each heparin fraction eluted from t-PA-Sepharose, the heparin preparations were subjected to molecular sieve chromatography on Sephadex G-75 (Fig. 6).

FIG. 5. Thrombin inhibition by AT-III and heparin fractions obtained after t-PA-Sepharose chromatography. Heparin fractions were assayed for ability to potentiate the inhibition of thrombin by AT-III. The initial rate of remaining thrombin activity is plotted vs. increased heparin concentrations.

FIG. 6. Sephadex G-75 elution profile of heparin fractions from a t-PA-Sepharose column. Fraction size: 1 ml; flow rate: 3.4 ml/hr.

The gel filtration chromatography showed, judging from the elution positions of appropriate markers, that the HA-heparin had an average molecular weight of 16,500, whereas the unfractionated and LA-heparins had average molecular weights of 15,500 and 13,100, respectively.

DISCUSSION

We have used t-PA affinity chromatography to partition heparin into two fractions: a low-affinity and a high-affinity fraction. Both have similar anticoagulant activity as assayed by the acceleration of thrombin inactivation by AT-III. Plasminogen activation showed that although the low-affinity fraction did not influence t-PA activity, the high-affinity fraction enhanced t-PA activity. These results suggest that the fibrinolytic enhancing activity of heparin depends upon a heparin-t-PA complex. However, the promotion by heparin of plasminogen activation could depend not only on its binding to t-PA but also on the simultaneous binding of both t-PA and plasminogen. The molecular size of the high-affinity fraction (average M_r of 16,500) might be able to accommodate both proteins to bring substrate and enzyme in proximity and thereby augment plasminogen activation. The low-affinity fraction had a slightly smaller M_r of 13,100; however, studies on partially depolymerized heparin show that the inability of the LA-heparin to stimulate t-PA activity or bind t-PA is not simply a consequence of this size difference (25).

There are two potentially useful extensions of the work described here: (i) Characterization of the high-affinity fraction may reveal the exact nature of the heparin fragment (i.e., size, structure) responsible for enhancement of fibrinolytic activity. (ii) Our results show that a heparin preparation can be isolated that does not bind t-PA or stimulate its activity but does retain its anticoagulant activity. Removal of the fibrinolytic stimulating activity in commercial preparations of heparin may reduce some of the undesirable consequences associated with heparin treatment.

It has been demonstrated that heparin seems to form a complex with t-PA at a molar ratio of 1:1 (33). This interaction of t-PA with heparin has been used to purify the protease, based on its strong affinity for immobilized heparin (8). Localization of the heparin-binding sites on t-PA by analyzing deletion mutants has suggested that the finger domain and the second kringle are involved in heparin binding (34). However, Soeda *et al.* (35), using porcine t-PA and separating the functionally active A and B chains after mild reduction and alkylation, provided evidence that heparin forms complexes with both t-PA and plasminogen through their catalytic B chains. The difference in these results might be due to the methods by which regions of the t-PA molecule were obtained.

The interaction between heparin and t-PA may be important with regard to the therapeutic uses of heparin and t-PA (see ref. 6) and also in circumstances where endogenous t-PA and "heparin-like" species might coexist. Some examples include the following: the surface of endothelial cells (36); the nervous system, where heparin sulfates are present as part of the extracellular matrix and plasminogen activators have been associated with neuronal migration and development (37); the preovulatory follicle, where the interaction of follicular fluid proteoglycans and plasminogen activator might be involved in ovulation and in the degradation of basement membrane glycoproteins and proteoglycans (38, 39); in the secondary mouse oocyte (40); and in the pituitary (41). Clearly, a better understanding of the binding of t-PA to heparin is needed to understand the physiological consequences of such interactions and also to elucidate the role of t-PA in environments where fibrinolysis does not appear to be the primary function. In such cases, heparin-like species might modulate t-PA activity.

The t-PA affinity chromatography also may be a method for identifying and purifying other moieties that interact with t-PA and influence its activity. Given the interest in thrombolytic therapy for coagulation disorders, this aspect could eventually aid in the design of new therapies.

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