Binding of Heparin to Human High Molecular Weight Kininogen†

Ingemar Björk,‡ Steven T. Olson,§ Roberta G. Sheffer,§ and Joseph D. Shore*,§

Division of Biochemical Research, Henry Ford Hospital, Detroit, Michigan 48202, and Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Box 575, S-751 23 Uppsala, Sweden

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ABSTRACT: The binding of heparin to high molecular weight kininogen (H-kininogen) was analyzed by the effect of kininogen in decreasing the heparin-induced enhancement of the rate of inactivation of thrombin by antithrombin. The conditions were arranged so that the heparin-catalyzed antithrombin–thrombin reaction, monitored in the presence of the reversible thrombin inhibitor *p*-aminobenzamidine, followed pseudo-first-order kinetics and the observed rate constant (k_{obsd}) varied linearly with the heparin concentration. In the absence of metal ions, H-kininogen minimally affected k_{obsd}, measured at a constant concentration of heparin with high affinity for antithrombin (30 nM), at $I = 0.15$, pH 7.4 and 25 °C. However, at a saturating concentration of Zn^{2+} (10 μM), k_{obsd} was reduced to 50% at ~20 nM H-kininogen and to that of the uncatalyzed reaction at $\geq$~0.2 μM H-kininogen. Conversely, at a saturating concentration of H-kininogen (0.5 μM), k_{obsd} was decreased to 50% at ~0.6 μM Zn^{2+} and to the k_{obsd} of the uncatalyzed reaction at $\geq$10 μM Zn^{2+}. Other metal ions were effective in the order $Zn^{2+} \sim Ni^{2+} > Cu^{2+} \sim Co^{2+} \sim Cd^{2+}$. The single-chain and two-chain forms of H-kininogen and the H-kininogen light chain reduced the heparin enhancement in the presence of Zn^{2+} to the same extent, whereas low molecular weight kininogen had no influence. Heparin with low affinity for antithrombin reversed the effect of H-kininogen, together with Zn^{2+}, in decreasing the rate enhancement caused by high-affinity heparin at concentrations consistent with the two heparin species binding similarly to H-kininogen. In the absence of metal ions, the effect of H-kininogen on the rate of the heparin-catalyzed antithrombin–thrombin reaction increased with decreasing pH below 7.4 in a manner indicating involvement of protonated histidine residues. A lower metal-dependent heparin-neutralizing ability was observed in H-kininogen-deficient than in normal plasma. These findings suggest that heparin with both high and low affinity for antithrombin can bind with appreciable affinity to the histidine-rich region of the light-chain portion of H-kininogen. At physiological pH, such binding must be mediated by divalent metal ion binding to unprotonated histidine residues, while at lower pH the polysaccharide binds directly to protonated histidines. Like histidine-rich glycoprotein, H-kininogen may compete with antithrombin for heparin during heparin therapy.

Heparin acts as an anticoagulant by binding to the plasma proteinase inhibitor antithrombin. This binding leads to a dramatic increase of the rate by which antithrombin inactivates serine proteinases of the intrinsic coagulation system, primarily thrombin and factor X_a [for reviews, see Björk and Lindahl (1982) and Björk and Danielsson (1986)]. In plasma, heparin binds also to other proteins besides antithrombin, e.g., albumin and fibrinogen (Longas et al., 1980), fibronectin (Hayashi & Yamada, 1982), vitronectin (Preissner & Müller-Berghaus, 1986), and histidine-rich glycoprotein (Lijnen et al., 1983a; Lane et al., 1986; Peterson et al., 1987). The binding of heparin to the latter protein has been studied in some detail. The two heparin forms with high and low affinity for antithrombin (Lam et al., 1976; Höök et al., 1976) bind indistinguishably to histidine-rich glycoprotein. The apparent dissociation constant for this binding has been estimated to be (1–5) $\times 10^{-8}$ M at near-neutral pH for heparin chains with M_r 11 500–25 000 (Lijnen et al., 1983a; Peterson et al., 1987). The interaction presumably involves histidine residues in a region of the protein abundant in such residues (Lijnen et al., 1983a; Koide et al., 1986; Peterson et al., 1987). Metal ions have been implicated to participate in the binding of heparin to human histidine-rich glycoprotein (Lijnen et al., 1983a), although this was not found for the rabbit protein (Peterson et al., 1987). The anticoagulant activity of heparin chains longer than about 18 monosaccharide units (M_r ~5400) is readily neutralized by histidine-rich glycoprotein, whereas the activity of smaller chains becomes increasingly resistant to such neutralization with decreasing chain length (Lane et al., 1986). The binding of heparin to histidine-rich glycoprotein may thus play a role in modulation of the activity of heparin administered in vivo (Lijnen et al., 1983a; Lane et al., 1986).

Kininogens are multifunctional plasma proteins which occur in two partially identical forms, high molecular weight and low molecular weight kininogen (H-kininogen and L-kininogen, respectively)[1] (Habal et al., 1974; Komiya et al., 1974; Kato et al., 1981; Lottspeich et al., 1985; Takagaki et al., 1985; Kellerman et al., 1986). Both forms are precursor molecules for vasoactive peptides, kinins (Kato et al., 1981), and act as inhibitors of cysteine proteinases (Ohkubo et al., 1984; Sueyoshi et al., 1985; Müller-Esterl et al., 1985; Higashiyama et al., 1986). H-Kininogen also participates as a cofactor in the contact activation phase of blood clotting (Griffin &

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* Address correspondence to this author.

‡ Swedish University of Agricultural Sciences.

§ Henry Ford Hospital.

[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H-kininogen, high molecular weight kininogen; L-kininogen, low molecular weight kininogen; Mes, 4-morpholineethanesulfonic acid; PEG, poly(ethylene glycol); D-Phe-Phe-Arg-CH_2Cl, D-phenylalanyl-L-phenylalanyl-L-arginyl chloromethyl ketone.

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Cochrane, 1976; Kato et al., 1981). The latter effect is mediated by a histidine-rich surface binding region (Han et al., 1975; Sugo et al., 1980; Ikari et al., 1981; Kellerman et al., 1986; Retzios et al., 1987) and binding sites for factor XI and prekallikrein (Kerbiriou et al., 1980; Bouma et al., 1983; Bock & Shore 1983; Bock et al., 1985; Shimada et al., 1985; Tait & Fujikawa, 1986, 1987) in the light-chain portion of the protein. In this work, we show that heparin binds tightly also to H-kininogen, apparently to the histidine-rich region of the light chain, and that this binding is dependent on divalent metal ions at physiological pH. We also provide evidence that H-kininogen, like histidine-rich glycoprotein, is effective at neutralizing heparin in plasma.

Materials and Methods

Materials. The isolation and properties of the single-chain and two-chain forms of human H-kininogen have been described previously (Kerbiriou & Griffin, 1979; Bock & Shore, 1983). Before use, solutions of the proteins were made 10 μM in the kallikrein inhibitor D-Phe-Phe-Arg-CH_2Cl (Calbiochem, San Diego, CA) and were dialyzed for 18 h at 4 °C against 0.1 M Hepes/NaOH, 0.1 M NaCl, and 0.1% (w/v) PEG 6000, pH 7.4, containing 10 μM D-Phe-Phe-Arg-CH_2Cl to inactivate any contaminating kallikrein. They were then dialyzed extensively against the Hepes buffer without chloromethyl ketone. The H-kininogen light chain was prepared by reduction and alkylation of the two-chain form of the protein, followed by chromatography on sulfopropyl-Sephadex (Pharmacia, Uppsala, Sweden), as described earlier (Bock & Shore, 1983). The preparations of both the two-chain form of H-kininogen and its light chain contained predominantly the terminally cleaved light chain, lacking the first 48 residues of the complete light-chain sequence (Nakayasu & Nagasawa, 1979; Mori & Nagasawa, 1981; Bock & Shore, 1983; Tait & Fujikawa, 1986; Retzios et al., 1987).

L-Kininogen was isolated from human plasma by affinity chromatography on matrix-linked, inactivated papain. The procedure developed by Gounaris et al. (1984) was modified in that purified, ~95% active papain (Burke et al., 1974), rather than commercial papain, was used and was inactivated with iodoacetamide instead of iodoacetic acid. This modification allowed elution of the bound kininogen with 3 M sodium thiocyanate in 0.1 M Tris-HCl, 0.1 M NaCl, and 100 μM EDTA, pH 8.0, and thus obviated the use of high pH. Further separation was done by ion-exchange chromatography on DEAE-Sepharose CL6B (Pharmacia) under essentially the same conditions as those used previously for separation of H- and L-kininogens by fast protein liquid chromatography (Gounaris et al., 1984). The L-kininogen preparation appeared >90% homogeneous in sodium dodecyl sulfate/polyacrylamide gel electrophoresis in 10% slab gels under reducing conditions (Laemmli, 1970). It reacted in immunodiffusion with antibodies against the human H-kininogen heavy chain (Miles Scientific, Naperville, IL) but did not react with antibodies against the light chain from this protein. Radioimmunoassay (Carretero et al., 1976) showed that essentially no kinin could be released by trypsin, indicating that the kinin segment must have been excised during the preparation procedure. Before use, the protein was treated with D-Phe-Phe-Arg-CH_2Cl as described for H-kininogen.

Antithrombin and heparin species with low and high affinity for antithrombin (low-affinity and high-affinity heparin; M_r ~7900) were prepared by methods detailed in previous publications (Höök et al., 1976; Olson & Shore, 1982). Human α-thrombin (specific activity 2800 NIH units/mg) was a generous gift from Dr. John Fenton (New York State Department of Health, Albany, NY).

Protein concentrations were determined by absorbance measurements at 280 nm. The following specific absorption coefficients (in liters per gram per centimeter) and molecular weights were used in the calculations: 0.701 and 108 000 for H-kininogen (Kerbiriou & Griffin, 1979; Nakayasu & Nagasawa, 1979); 0.59 and 64 000 for L-kininogen (Ryley, 1979; Kellerman et al., 1987); 0.64 and 30 500 for the light chain of H-kininogen (Bock & Shore, 1983; Bock & Halvorson, 1983); 0.65 and 58 000 for antithrombin (Nordenman et al., 1977). The concentration of thrombin was determined by fluorometric active-site titration (Jameson et al., 1973). Concentrations of high-affinity heparin were determined by titrations, monitored by measurements of tryptophan fluorescence, into antithrombin at low ionic strength (Olson & Shore, 1982), whereas low-affinity heparin concentrations were determined by dry-weight analyses.

All buffers were treated with Chelex 100 before addition of metal ions or EDTA.

Kinetic Analysis of Heparin Binding to Kininogen. The binding of high-affinity heparin to H- or L-kininogen was analyzed by the apparent decrease caused by kininogen of the accelerating effect of the polysaccharide on the antithrombin–thrombin reaction. Most analyses were done in 0.1 M Hepes/NaOH, 0.1 M NaCl, and 0.1% (w/v) PEG 6000, pH 7.4 (I = 0.15), although studies in the presence of Cu^{2+} and of the pH dependence of the binding were done in other buffer systems (see legends to Table I and Figure 5). In most experiments, the rate of the antithrombin–thrombin reaction was monitored by the decrease of the fluorescence of the thrombin inhibitor *p*-aminobenzamidine, caused by displacement of this probe from the enzyme by antithrombin (Evans et al., 1982; Olson & Shore, 1982). Kininogen (0–0.5 μM), high-affinity heparin (0–30 nM), thrombin (0.25 μM), *p*-aminobenzamidine (1 mM), and divalent metal ions (0–100 μM) or EDTA (1 mM) were mixed in a fluorescence cuvette, and the solution was allowed to equilibrate to 25.0 °C for about 5 min. The reaction was then started by the addition of 5 μM antithrombin. All concentrations given are those in the final reaction volume of 1 mL. The decrease in fluorescence intensity was monitored as a function of time in a Perkin-Elmer 650-10 S spectrofluorometer (Perkin-Elmer, Norwalk, CN) with excitation and emission wavelengths of 340 and 370 nm and bandwidths of 5 and 10 nm, respectively.

In control experiments and in studies of the pH dependence of the binding of high-affinity heparin to H-kininogen, the rate of the heparin-catalyzed antithrombin–thrombin reaction in the presence of *p*-aminobenzamidine was analyzed by the discontinuous assay of residual thrombin activity, instead of by the decrease in probe fluorescence. The conditions were the same as those described above, except that the thrombin concentrations were appreciably lower, minimally 20 nM. At different times, portions of the reaction mixture were added to a polystyrene cuvette with 1 mL of 0.02 M sodium phosphate, 0.25 M NaCl, 100 μM EDTA, 0.1% (w/v), and PEG 6000, pH 7.4, containing 100 μM thrombin substrate D-phenylalanyl-L-pipecolyl-L-arginyl *p*-nitroanilide (S-2238; KabiVitrum, Stockholm, Sweden) and 100 μg/mL polybrene (Aldrich, Milwaukee, WI) to neutralize the heparin. The final thrombin concentration in the cuvette was 2–6 nM. The rate of release of *p*-nitroaniline from the substrate was monitored at 25 °C by continuous recording of the absorbance at 405 nm.

The binding of low-affinity heparin to H-kininogen was studied by competition with high-affinity heparin. The con-

ditions of these experiments were the same as in the analyses of the binding of high-affinity heparin to kininogen by the fluorescence method, except that the buffer was 0.1 M Hepes/NaOH, 0.25 M NaCl, and 0.1% (w/v) PEG 6000, pH 7.4 (I = 0.3), and the concentration of high-affinity heparin was increased to 0.1 μM. The concentration of H-kininogen was 0.4 μM, and the low-affinity heparin concentration was varied between 0 and 0.4 μM. The displacement of high-affinity heparin from H-kininogen by low-affinity heparin was evaluated by the effect of the displaced high-affinity heparin on the rate of the antithrombin–thrombin reaction, monitored by the decrease in *p*-aminobenzamidine fluorescence in the manner described above.

Affinity Chromatography Analysis of Heparin Binding to Kininogen. Affinity chromatography of H-kininogen on heparin–Sepharose (Pharmacia) was done with a 0.7 × 17 cm column, equilibrated with 0.1 M Hepes/NaOH and 0.1 M NaCl, pH 7.4, containing 2 mM EDTA or 10 μM $ZnSO_4$. An amount of 1 mg of protein was applied to the column, which was eluted at a flow rate of 20 mL/h with a linear gradient (total volume 100 mL) to the Hepes buffer containing 1 M NaCl. At the end of the gradient, the column was eluted with buffer containing 2 M NaCl. Fractions of 2-mL volume were collected. The protein concentration was analyzed by tryptophan fluorescence with excitation and emission wavelengths of 280 and 340 nm and bandwidths of 5 and 10 nm, respectively. The fluorescence intensities of the fractions were related to that of a H-kininogen standard with known concentration. Chromatography of H-kininogen on dextran sulfate–agarose (Pierce, Rockford, IL) was done in a similar manner.

Heparin Binding to Kininogen in Plasma. The ability of H-kininogen in plasma to neutralize high-affinity heparin in a Zn^{2+}-dependent manner was assessed by comparisons of the heparin-accelerated inactivation of thrombin added to normal plasma and plasma deficient in both L- and H-kininogen (Donaldson et al., 1976). Thrombin inactivation was measured either with a chromogenic substrate or by clotting times. Portions of 1 mL of both types of plasma (obtained from George King Biomedical, Overland Park, KS) were first dialyzed for ~20 h against 150 mL of 0.1 M Hepes/NaOH, 0.1 M NaCl, and 0.1% (w/v) PEG 6000, pH 7.4 (I = 0.15), containing 9 g of Chelex 100 (Bio-Rad, Richmond, CA), to remove endogenous metal ions and added citrate. In some experiments, the kininogen-deficient plasma was then reconstituted by addition of the two-chain form of H-kininogen to a concentration of 1 μM. In measurements of thrombin inactivation with a chromogenic substrate, the reaction mixture consisted of plasma (5 μL), high-affinity heparin (0 or 6 nM), Zn^{2+} (0 or 10 μM), and thrombin (5 nM) in a final volume of 0.5 mL in a polystyrene cuvette; the buffer was the Hepes buffer described above. The reaction was started by addition of thrombin and was continued for 3 min at 25 °C. The inactivation of thrombin was then stopped by addition of 0.5 mL of Hepes buffer containing 200 μM thrombin substrate S-2238 and 100 μg/mL polybrene. Remaining thrombin activity was immediately determined from the rate of substrate hydrolysis at 25 °C, monitored by the absorbance increase at 405 nm. Thrombin activity at zero time was measured by addition of the substrate solution to the reaction mixture before thrombin. In clotting time measurements of thrombin inactivation, the reaction mixture consisted of plasma (100 μL), high-affinity heparin (0 or 7 nM), Zn^{2+} (0 or 10 μM), and thrombin (5 nM, i.e., 0.5 NIH unit/mL), all in the Hepes buffer in a final volume of 1 mL in a polystyrene cuvette at 37 °C. The clotting time was determined spectrophotometrically by measurements of turbidity at 350 nm. The reaction was started by addition of thrombin after the base-line absorbance (A_{350} ~ 0.25) had been offset and recorded. Formation of a clot was apparent from the sharp rise and eventual plateauing of the turbidity (ΔA_{350} ~ 0.15). The clotting time was defined as the intersection of the line drawn through the sigmoidal turbidity curve in the linear region corresponding to the maximum rate of absorbance change with the initial base-line absorbance. A linear relationship between log clotting time and log thrombin concentration was obtained over the thrombin concentration range of 1–10 nM, with clotting times at these concentrations from 230 to 23 s.

Results

Use of a Kinetic Probe To Measure Heparin Binding to Kininogen. The binding of heparin with high affinity for antithrombin (Lam et al., 1976; Höök et al., 1976) to H- or L-kininogen was assessed by the effect of the kininogens in reducing the amount of the polysaccharide available to increase the rate of the antithrombin–thrombin reaction. Recent studies by a number of investigators have led to the conclusion that high-affinity heparin accelerates the reaction between antithrombin and thrombin in a catalytic fashion by binding both inhibitor and proteinase, thus being formally equivalent to a two-substrate enzyme [for reviews, see Björk and Lindahl (1982) and Björk and Danielsson (1986)]. To obtain a convenient quantitative measure of the concentration of high-affinity heparin not bound to kininogen, we therefore carried out the analyses at catalytic heparin concentrations, a thrombin concentration substantially below the apparent K_M of the "substrate" thrombin for the "enzyme" high-affinity heparin [achieved partly by the use of a high concentration of the thrombin inhibitor *p*-aminobenzamidine, which increases this apparent K_M; see Olson and Shore (1986)], and a large excess of antithrombin over thrombin. Since the antithrombin concentration remains essentially constant under these conditions, the heparin-catalyzed inhibition of thrombin by antithrombin can be treated as a one-substrate enzyme reaction with thrombin as the substrate. Moreover, if the thrombin concentration is sufficiently low, so that it can be neglected in comparison with the apparent K_M, the disappearance of thrombin is given by

$$-\frac{d[T]_t}{dt} = \left(\frac{k_{cat}}{K_M}\right)_{app}[H]_0[T]_t + k_{0,app}[T]_t$$

In this equation, $[T]_t$ is the thrombin concentration at time t, $(k_{cat}/K_M)_{app}$ is the apparent specificity constant for heparin catalysis of the antithrombin–thrombin reaction (and is a function of the antithrombin and *p*-aminobenzamidine concentrations used), $[H]_0$ is the concentration of high-affinity heparin, and $k_{0,app}$ is the apparent pseudo-first-order rate constant for the uncatalyzed antithrombin–thrombin reaction. Integration of this equation gives

$$[T]_t = [T]_0 e^{-k_{obsd}t}$$

where $[T]_0$ is the thrombin concentration at t = 0 and $k_{obsd} = (k_{cat}/K_M)_{app}[H]_0 + k_{0,app}$. Thus, under these conditions, the disappearance of thrombin follows first-order kinetics with an observed pseudo-first-order rate constant, k_{obsd}. Moreover, k_{obsd} increases linearly with the concentration of high-affinity heparin. A decrease of k_{obsd} measured at a constant high-affinity heparin concentration caused by kininogen therefore must be proportional to the amount of heparin bound to this protein, provided that a direct effect of kininogen on the rate of the

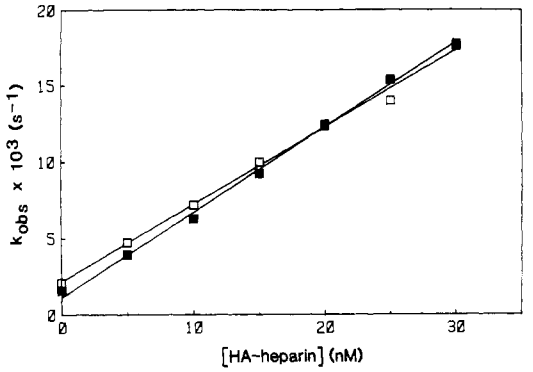

FIGURE 1: Observed pseudo-first-order rate constants (k_{obsd}) for the antithrombin–thrombin reaction, monitored by displacement of *p*-aminobenzamidine from the enzyme as a function of the concentration of high-affinity (HA) heparin without kininogen and with or without Zn^{2+} at $I = 0.15$, pH 7.4. (□) 1 mM EDTA; (■) 10 μM Zn^{2+}. Other conditions were as described under Materials and Methods.

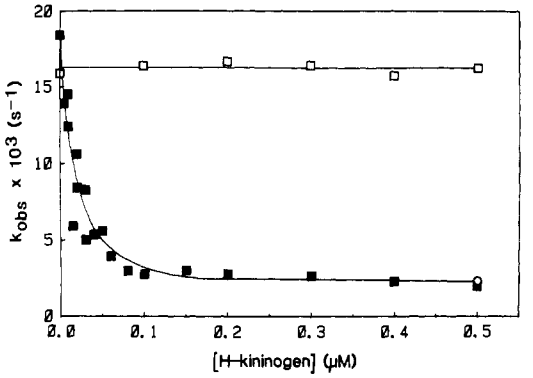

FIGURE 2: Observed pseudo-first-order rate constants (k_{obsd}) for the high-affinity heparin-catalyzed antithrombin-thrombin reaction, monitored by displacement of *p*-aminobenzamidine from the enzyme, as a function of the concentration of the two-chain form of H-kininogen with or without Zn^{2+} at $I = 0.15$, pH 7.4. (□) 1 mM EDTA and 30 nM high-affinity heparin; (■) 10 μM Zn^{2+} and 30 nM high-affinity heparin; (○) 10 μM Zn^{2+} and no heparin. Other conditions were as described under Materials and Methods.

antithrombin–thrombin reaction can be excluded.

In most experiments, the rate of the antithrombin–thrombin reaction was monitored by continuous assay of the decrease of the fluorescence intensity of *p*-aminobenzamidine caused by antithrombin displacing this inhibitor from the active site of thrombin (Evans et al., 1982; Olson & Shore, 1982). Suitable conditions for this assay were first developed by studies in the absence of kininogen at pH 7.4 and ionic strength 0.15, conditions under which most subsequent analyses of heparin binding to kininogen were carried out. The lowest thrombin concentration and highest *p*-aminobenzamidine concentration compatible with acceptable experimental precision were found to be 0.25 μM and 1 mM, respectively. For reactions in the presence of high-affinity heparin, these conditions were such that the thrombin concentration was somewhat too high to be completely negligible in comparison with the apparent K_M of thrombin for the heparin used. Thus, the first-order plots showed a slight initial curvature, indicating that pseudo-first-order conditions were not attained until after a certain lag period. In these cases, the observed rate constant, k_{obsd}, was evaluated from the limiting slope of the plots, i.e., from the linear region from about 40% to about 90% of the reaction. For reactions in the absence of heparin, strictly linear first-order plots were obtained.

Several experiments were done to verify that the assumptions implicit in the theoretical analysis were satisfied and that k_{obsd} is a valid measure of the concentration of high-affinity heparin under the conditions developed. Although plagued by considerable noise, experiments monitored by *p*-aminobenzamidine fluorescence at half the optimal thrombin concentration, 0.125 μM, gave values for k_{obsd} at several concentrations of high-affinity heparin that were within 10% of those measured at the higher thrombin concentration. Discontinuous assays of residual thrombin activity were also used to monitor experiments performed under the same conditions as those monitored by fluorescence (i.e., in the presence of *p*-aminobenzamidine), except that the thrombin concentration was reduced from 0.125 μM to as low as 20 nM. These analyses similarly gave k_{obsd} values that were independent of the thrombin concentration and indistinguishable within experimental error (±15%) from those measured by the fluorescence method. Experiments at different concentrations of high-affinity heparin further showed that k_{obsd}, measured by the fluorescence assay, increased linearly with the heparin concentration (Figure 1). In these experiments, rate constants that were identical within experimental error were obtained in the absence and presence of 10 μM Zn^{2+}, both without and with heparin (Figure 1). This comparison was made because Zn^{2+} (or certain other metal ions) was found to be essential for binding of heparin to kininogen at physiological pH and therefore was included at concentrations up to 10 μM in most analyses (see below). Zn^{2+} thus does not affect the rate of either the uncatalyzed or the heparin-catalyzed antithrombin–thrombin reaction. Together, the independence of k_{obsd} on thrombin concentration and the linearity of k_{obsd} with the concentration of high-affinity heparin convincingly demonstrate that the assumptions discussed above are fulfilled under the conditions used.

Metal Ion Requirement for Heparin Binding to H-Kininogen at Physiological pH. All studies of the binding of high-affinity heparin to kininogen were done at 25 °C and an ionic strength of 0.15 with the highest concentration of high-affinity heparin used in the control experiments described above (Figure 1), i.e., 30 nM. Initial studies, monitored by the fluorescence method, were done with the two-chain form of H-kininogen at physiological pH, 7.4. At this pH, two-chain H-kininogen negligibly affected the heparin-accelerated rate of the antithrombin–thrombin reaction in the absence of metal ions, whereas a marked decrease of the rate was observed at a saturating concentration (10 μM; see below) of Zn^{2+} (Figure 2). The rate constant, k_{obsd}, was thus reduced to 50% at a kininogen concentration of ~20 nM and to that of the uncatalyzed reaction at kininogen concentrations ≥0.2 μM. Additional studies at saturating concentrations (0.25 or 0.5 μM) of kininogen but varying concentrations of Zn^{2+} showed an analogous decrease of k_{obsd} to 50% at a Zn^{2+} concentration of ~0.6 μM and to k_{obsd} of the uncatalyzed reaction at Zn^{2+} concentrations ≥10 μM (Figure 3). Control experiments (Figure 3) demonstrated that two-chain H-kininogen did not affect the rate of the uncatalyzed antithrombin–thrombin reaction at any Zn^{2+} concentration investigated and furthermore that Zn^{2+} in these concentrations negligibly affected both the uncatalyzed and catalyzed reactions, as was also concluded previously. Taken together, these experiments thus strongly indicate that two-chain H-kininogen reduces the rate of the heparin-accelerated antithrombin–thrombin reaction by binding tightly to high-affinity heparin in the presence of Zn^{2+}, thereby decreasing the amount of polysaccharide that can catalyze the inactivation of the enzyme by the inhibitor.

We also investigated the effect of different divalent metal ions, together with two-chain H-kininogen, in decreasing the rate of the heparin-accelerated antithrombin–thrombin reaction at pH 7.4. All metal ions investigated had no measurable

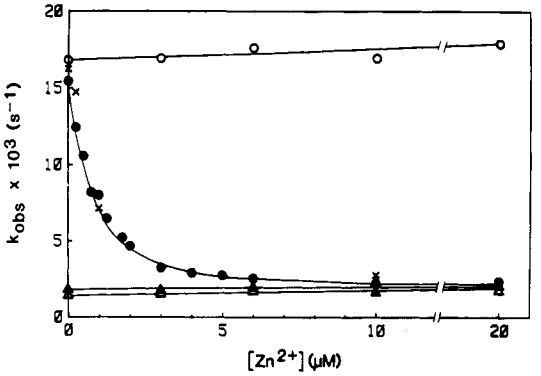

FIGURE 3: Observed pseudo-first-order rate constants (k_{obsd}) for the uncatalyzed and high-affinity heparin-catalyzed antithrombin–thrombin reaction, monitored by displacement of *p*-aminobenzamidine from the enzyme, as a function of Zn^{2+} concentration with or without the two-chain form of H-kininogen at I = 0.15, pH 7.4. (○) No kininogen and 30 nM high-affinity heparin; (●) 0.5 μM kininogen and 30 nM high-affinity heparin; (×) 0.25 μM kininogen and 30 nM high-affinity heparin; (△) no kininogen and no heparin; (▲) 0.5 μM kininogen and no heparin. Experiments without Zn^{2+} were done in the presence of 1 mM EDTA. Other conditions were as described under Materials and Methods.

Table I: Effect of Different Metal Ions, Together with the Two-Chain Form of H-Kininogen, in Decreasing the Enhancement by High-Affinity Heparin of the Rate of the Antithrombin–Thrombin Reaction, Monitored by Displacement of *p*-Aminobenzamidine from the Enzyme at I = 0.15, pH 7.4[a]

metal ion	decrease of heparin rate enhancement (%) for metal ion concn (μM) of 1	10	100
Cd^{2+}	10	62	95
Co^{2+}	11	62	93
Cu^{2+}	18	67	ND[b]
Ni^{2+}	65	95	98
Zn^{2+}	62	97	97

[a] The analyses were done with 0.25 μM kininogen and 30 nM high-affinity heparin. Other conditions were as described under Materials and Methods. The buffer was 0.1 M Hepes/NaOH, 0.1 M NaCl, and 0.1% (w/v) PEG 6000, pH 7.4, in all analyses except those with Cu^{2+}. In these experiments, the Hepes concentration was decreased to 0.023 M (with an increase in NaCl concentration to maintain an ionic strength of ~0.15), because difficulties were encountered in dissolving Cu^{2+} salts in the 0.1 M Hepes buffer. In spite of the lower Hepes concentration, solutions containing ≥100 μM Cu^{2+} could not be prepared. All other metal ions used were easily dissolved in the more concentrated Hepes buffer at concentrations up to 100 μM and showed the same effect at both Hepes concentrations, indicating negligible binding to this buffer component. The decrease of the heparin rate enhancement was expressed as the decrease of k_{obsd} in percent of the maximal decrease. The latter was taken as the decrease from k_{obsd} of the heparin-catalyzed reaction, measured in the presence of kininogen but in the absence of metal ions (i.e., with 1 mM EDTA), to k_{obsd} of the uncatalyzed reaction, measured in the presence of kininogen at the respective metal ion concentrations. [b] ND, not determined.

effect on the uncatalyzed antithrombin–thrombin reaction or on the heparin-catalyzed reaction in the absence of kininogen (data not shown) but markedly reduced the rate of the heparin-catalyzed reaction in the presence of 0.25 μM kininogen (Table I). However, Ni^{2+} and Zn^{2+} were considerably more effective than Cd^{2+}, Co^{2+}, and Cu^{2+}, giving the same decrease of k_{obsd} as the latter ions at about 10-fold lower concentrations. These findings indicate that the binding of high-affinity heparin to kininogen at pH 7.4 can occur in the presence of several divalent metal ions but is tightest with Ni^{2+} and Zn^{2+} of those metal ions studied.

Binding of Heparin to Different Forms of Kininogen. The effect of different forms of kininogen on the heparin-catalyzed reaction between antithrombin and thrombin was next studied. These experiments, which also were monitored by the fluorescence method, were made at pH 7.4 in the presence of 10 μM Zn^{2+}, a concentration shown to be saturating for two-chain H-kininogen, and at two kininogen concentrations, corresponding to about half-saturating and saturating concentrations with two-chain H-kininogen. L-kininogen was found to be completely without effect, indicating negligible binding of heparin to this form. However, the one-chain and two-chain forms of H-kininogen and the free H-kininogen light chain were equally effective in reducing k_{obsd} of the heparin-accelerated antithrombin–thrombin reaction in the presence of Zn^{2+} (Table II). Thus, all these forms of H-kininogen bind high-affinity heparin with about the same affinity.

Table II: Effect of Different Forms of Kininogen, Together with Zn^{2+}, in Decreasing the Enhancement by High-Affinity Heparin of the Rate of the Antithrombin–Thrombin Reaction, Monitored by Displacement of *p*-Aminobenzamidine from the Enzyme at I = 0.15, pH 7.4[a]

kininogen form	decrease of heparin rate enhancement (%) for kininogen concn (nM) of 25	250
L, two-chain	0	0
H, two-chain	52	100
H, one-chain	50	94
H, light chain	51	98

[a] The analyses were done with 10 μM Zn^{2+} and 30 nM high-affinity heparin. Other conditions were as described under Materials and Methods. The decrease of the heparin rate enhancement was expressed as the decrease of k_{obsd} in percent of the maximal decrease. The latter was taken as the decrease from k_{obsd} of the heparin-catalyzed reaction, measured in the presence of Zn^{2+} but in the absence of kininogen, to k_{obsd} of the uncatalyzed reaction, measured in the presence of Zn^{2+} at the respective kininogen concentrations.

Binding of Heparin with Low and High Affinity for Antithrombin to H-Kininogen. The binding of low-affinity heparin to H-kininogen in the presence of Zn^{2+} was evaluated by experiments in which this heparin species was allowed to compete with high-affinity heparin for binding to the protein. The resulting displacement of high-affinity heparin from H-kininogen was monitored by the fluorescence method. In these studies, the ionic strength was increased to 0.3, because low-affinity heparin alone was found to appreciably accelerate the antithrombin–thrombin reaction at I = 0.15, thereby making it difficult to accurately assess the contribution of high-affinity heparin to the observed rate constant. Several lines of evidence (not presented) suggested that this effect represented an inherent activity of low-affinity heparin and was not due to contamination by high-affinity heparin.

At I = 0.3, however, low-affinity heparin had only a small effect on the rate of the antithrombin–thrombin reaction, while high-affinity heparin retained considerable accelerating ability, although lower than at I = 0.15 (Figure 4). Due to weaker binding of high-affinity heparin to the proteins involved in the measurements at I = 0.3 than at I = 0.15, the concentration of the polysaccharide was increased to 0.1 μM; control experiments showed that k_{obsd} was linear up to this concentration in the absence of kininogen and low-affinity heparin and was unaffected by Zn^{2+}. Other control experiments showed that the effect of low-affinity heparin on the rate of the antithrombin–thrombin reaction was additive to that of high-affinity heparin in the absence of kininogen (Figure 4). A concentration of the two-chain form of H-kininogen of 0.4 μM

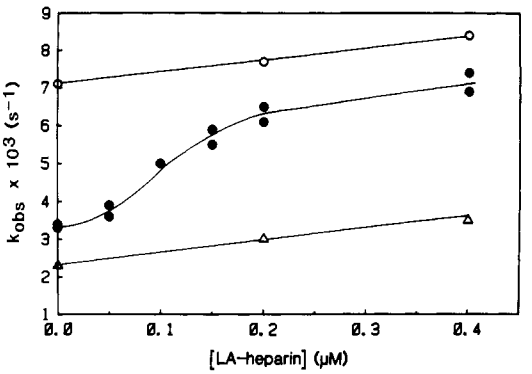

FIGURE 4: Observed pseudo-first-order rate constants (k_{obsd}) for the high-affinity heparin-catalyzed antithrombin–thrombin reaction, monitored by displacement of *p*-aminobenzamidine from the enzyme, as a function of the concentration of low-affinity (LA) heparin in the presence of H-kininogen and Zn^{2+} at I = 0.3, pH 7.4. (○) No kininogen and 0.1 μM high-affinity heparin; (●) 0.4 μM kininogen and 0.1 μM high-affinity heparin; (△) no kininogen and no high-affinity heparin. The concentration of Zn^{2+} was 10 μM in all measurements. Other conditions were as described under Materials and Methods.

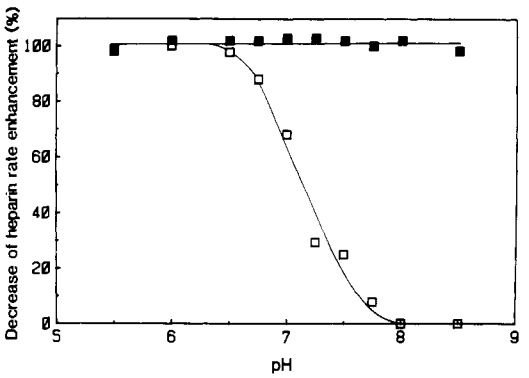

FIGURE 5: Dependence on pH of the effect of the two-chain form of H-kininogen, with or without Zn^{2+}, in decreasing the enhancement by high-affinity heparin of the rate of the antithrombin–thrombin reaction in the presence of *p*-aminobenzamidine at I = 0.15, monitored by discontinuous assay of residual thrombin activity. (□) 1 mM EDTA; (■) 10 μM Zn^{2+}. The high-affinity heparin, kininogen, and thrombin concentrations were 30 nM, 0.25 μM, and 0.1 μM, respectively. The buffer was 0.05 M Mes/0.05 M Hepes and 0.1% (w/v) PEG 6000, adjusted to the desired pH with HCl or NaOH; NaCl was added to an ionic strength of 0.15. Other conditions were as described under Materials and Methods. Kininogen and antithrombin solutions in 0.01 M Hepes/NaOH, 0.1 M NaCl, and 0.1% (w/v) PEG 6000, pH 7.0, were diluted extensively into the desired buffer immediately before use without any measurable effect on the pH of the reaction mixture. The decrease of the heparin rate enhancement was expressed as the decrease of k_{obsd} in percent of the maximal decrease. The latter was taken as the decrease from k_{obsd} of the heparin-catalyzed reaction, measured in the absence or presence of Zn^{2+} and the absence of kininogen, to k_{obsd} of the uncatalyzed reaction, measured in the absence or presence of Zn^{2+} and the presence of kininogen.

was used in the competition experiments; this concentration caused about an 80% reduction of k_{obsd} in the presence of high-affinity heparin and 10 μM Zn^{2+} but in the absence of low-affinity heparin (Figure 4). Increasing the concentration of low-affinity heparin resulted in an increase of k_{obsd} (Figure 4), compatible with competition between low-affinity and high-affinity heparin for kininogen decreasing the binding of the high-affinity species to the protein. A 50% increase of k_{obsd} was apparent at 0.1–0.2 μM low-affinity heparin, a concentration comparable to that of high-affinity heparin present, while k_{obsd} was close to the value observed in the absence of kininogen at 0.4 μM low-affinity heparin. These data thus are consistent with low-affinity heparin binding to H-kininogen with an affinity at least as high as that of the high-affinity heparin species.

Dependence of the Interaction between Heparin and H-Kininogen on pH. The pH dependence at I = 0.15 of the ability of the two-chain form of H-kininogen, with or without Zn^{2+}, to decrease the rate of the high-affinity heparin-catalyzed antithrombin–thrombin reaction was studied by discontinuous assay of residual thrombin activity, but still in the presence of *p*-aminobenzamidine to increase the apparent K_M of thrombin for high-affinity heparin. The discontinuous assay method, although more cumbersome than the continuous fluorescence method, was chosen to exclude complications due to pH-dependent changes of the fluorescent enhancement of *p*-aminobenzamidine on binding to thrombin. It also allowed the use of a lower thrombin concentration, sufficiently low to be well below the apparent K_M of thrombin throughout the pH range covered, as experimentally confirmed from the independence of k_{obsd} on thrombin concentration at the two extreme pH values of this range.

Several control experiments (not shown) were first performed. The rate constant, k_{obsd}, of the uncatalyzed antithrombin–thrombin reaction was found to decrease ~15-fold from pH 8.5 to 5.5; this decrease was not detectably affected by either 10 μM Zn^{2+} or 0.25 μM H-kininogen, or both. Similarly, k_{obsd} for the antithrombin–thrombin reaction in the presence of high-affinity heparin decreased ~10-fold over the same pH interval, and this decrease was not affected by Zn^{2+} alone. However, H-kininogen together with Zn^{2+} reduced k_{obsd} of the heparin-catalyzed reaction to that of the uncatayzed reaction at all pH values studied (Figure 5), in agreement with previous results at pH 7.4. H-Kininogen without Zn^{2+} had no effect on the heparin-accelerated rate of the antithrombin–thrombin reaction at pH ≥8 but progressively decreased this rate at lower pH values (Figure 5). Thus, at pH ≤~6, H-kininogen alone was able to reduce k_{obsd} to that of the uncatalyzed reaction, indicating strong binding of heparin to H-kininogen without Zn^{2+} at these more acidic pH values. The midpoint of the transition was at pH ~7. Analysis of the data by a Hill-type plot [log $[Y/(I - Y)]$ vs pH, where Y represents the fractional decrease in heparin rate enhancement] gave a slope greater than unity (2.0 ± 0.2), suggesting that protonation of amino acid residues involved in heparin binding occurs cooperatively. A small effect of H-kininogen in the absence of Zn^{2+} was observed at pH 7.4, in apparent contrast to previous experiments with the fluorescence method at this pH. This difference may be due partly to experimental error, somewhat higher in the discontinuous assay method, and partly to the use of different buffer systems in the two analyses.

Demonstration of an Interaction between Heparin and H-Kininogen by Affinity Chromatography. The tight binding of H-kininogen to heparin in the presence of Zn^{2+} at pH 7.4 and the negligible binding of L-kininogen were verified by affinity chromatography on heparin–agarose. In the absence of Zn^{2+}, the two-chain form of H-kininogen eluted from the column at ~0.25 M NaCl, while a considerably higher NaCl concentration, ~0.7 M, was required to elute the protein in the presence of 10 μM Zn^{2+} (Figure 6). In contrast, L-kininogen did not bind to the column either in the absence or in the presence of Zn^{2+} (not shown). This behavior suggested that chromatography on heparin–agarose with or without the metal ion can be used to separate the two kininogen forms, a conclusion that was confirmed experimentally. A similar Zn^{2+}-dependent tighter binding of H-kininogen to immobilized dextran sulfate was also observed.

H-Kininogen Neutralization of Heparin in Plasma. The ability of H-kininogen, together with Zn^{2+}, in plasma to

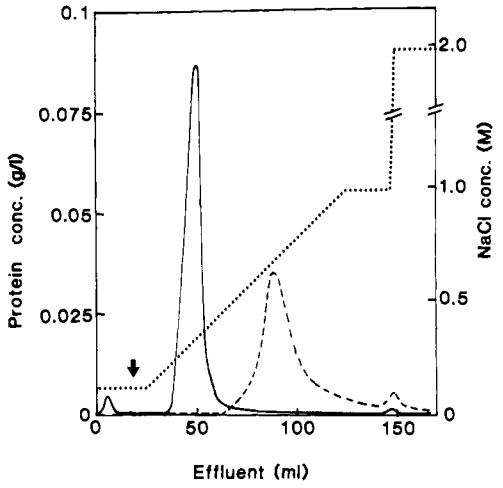

FIGURE 6: Affinity chromatography of the two-chain form of H-kininogen on matrix-linked heparin in the absence and presence of Zn^{2+} at pH 7.4. (—) Protein concentration in 2 mM EDTA; (---) protein concentration in 10 μM Zn^{2+}; (···) NaCl concentration, measured by conductance. The conditions and analyses of the experiments are described under Materials and Methods. The arrow marks the start of the eluting salt gradient.

Table III: Inactivation of Thrombin, Measured with a Chromogenic Substrate, in Normal, H-Kininogen-Deficient, and Reconstituted, H-Kininogen-Deficient Plasma in the Absence and Presence of High-Affinity Heparin and Zn^{2+} at I = 0.15, pH 7.4[a]

addition	thrombin activity remaining (%)		
	normal plasma	H-kininogen-deficient plasma	reconstituted, H-kininogen-deficient plasma
no high-affinity heparin, no Zn^{2+}	102	100	100
no high-affinity heparin, 10 μM Zn^{2+}	102	92	97
6 nM high-affinity heparin, no Zn^{2+}	0	0	0
6 nM high-affinity heparin, 10 μM Zn^{2+}	52 ± 0[b]	40 ± 3[b]	73 ± 2[b]

[a] Experimental details are given under Materials and Methods. Remaining thrombin activity was calculated as percent of the thrombin activity at zero time, measured as described under Materials and Methods. [b] Average of two analyses, with range.

neutralize heparin was studied by addition of thrombin to normal or H-kininogen-deficient plasma in the absence and presence of high-affinity heparin and Zn^{2+}. In one set of experiments, heparin-neutralizing activity in 100-fold dilutions of both types of plasma was assessed by measurements with a chromogenic substrate of the amount of thrombin inactivated in a fixed time interval (Table III). Control experiments showed that no thrombin was inhibited in the absence of high-affinity heparin under the conditions used, either with or without Zn^{2+}. The concentration of high-affinity heparin was chosen such that in the absence of Zn^{2+} the added thrombin was just inactivated, both by normal and by H-kininogen-deficient plasma. Addition of Zn^{2+} to 10 μM, approximately the plasma concentration (Whitehouse et al., 1983; Woo & Cannon, 1984), appreciably decreased the amount of thrombin inactivated in the presence of high-affinity heparin in normal plasma, reflecting Zn^{2+}-dependent heparin-neutralizing ability. This neutralizing ability was lower in H-kininogen-deficient plasma, and, moreover, addition of H-kininogen to the deficient plasma to 1 μM, approximately the normal plasma concentration of the protein (Adam et al., 1985), appreciably increased the heparin-neutralizing ability in the presence of Zn^{2+}.

Table IV: Inactivation of Thrombin, Measured by Clotting Times, in Normal, H-Kininogen-Deficient, and Reconstituted, H-Kininogen-Deficient Plasma in the Absence and Presence of High-Affinity Heparin and Zn^{2+} at I = 0.15, pH 7.4[a]

addition	thrombin clotting time (s)		
	normal plasma	H-kininogen-deficient plasma	reconstituted, H-kininogen-deficient plasma
no high-affinity heparin, no Zn^{2+}	46 ± 1 (2)	44 ± 0 (2)	46 ± 1 (2)
no high-affinity heparin, 10 μM Zn^{2+}	47 ± 1 (4)	41 ± 1 (4)	44 ± 1 (4)
7 nM high-affinity heparin, no Zn^{2+}	>400[b] (2)	>400[b] (2)	>400[b] (2)
7 nM high-affinity heparin, 10 μM Zn^{2+}	83 ± 1 (4)	217 ± 24 (6)	150 ± 9 (7)

[a] Experimental details are given under Materials and Methods. Average thrombin clotting times and their standard errors are presented with the number of determinations in parentheses. [b] Clotting not detectable in 400 s.

In a second set of experiments, thrombin clotting times in 10-fold dilutions of plasma were used to assess heparin-neutralizing activity (Table IV). Control experiments in the absence of high-affinity heparin, either with or without Zn^{2+}, gave similar clotting times of 41–47 s in normal, H-kininogen-deficient, and H-kininogen-reconstituted, deficient plasma. High-affinity heparin concentrations were chosen to produce a doubling of the clotting time in normal plasma in the presence of 10 μM Zn^{2+}. In the absence of Zn^{2+}, this level of heparin resulted in the failure of normal plasma to clot in 400 s, indicating a marked heparin-neutralizing activity that was dependent on the presence of Zn^{2+}. When heparin and Zn^{2+} were added to H-kininogen-deficient plasma in the same concentrations as to normal plasma, a substantially greater prolongation of the clotting time compared with normal plasma was observed. Moreover, this prolongation was reduced by the addition of two-chain kininogen to the deficient plasma. Again, no clotting was observed in 400 s in these plasmas when heparin was present without Zn^{2+}, demonstrating the metal ion dependence of the heparin-neutralizing ability. These two sets of experiments thus show that H-kininogen in plasma is effective at neutralizing heparin at plasma Zn^{2+} concentrations.

Discussion

The data presented here show that H-kininogen, together with Zn^{2+} or certain other metal ions, can completely inhibit the acceleration caused by high-affinity heparin of the antithrombin–thrombin reaction at physiological pH. All evidence indicates that this effect is due to H-kininogen binding to heparin and competing for the polysaccharide with either antithrombin or thrombin, or with both proteins, as suggested for similar competition by histidine-rich glycoprotein (Lane et al., 1986; Peterson et al., 1987). However, the mechanism of this competition may not be simple. For instance, the binding of H-kininogen to heparin is likely to be nonspecific, and several molecules of the protein may thus have to bind to each polysaccharide chain to completely exclude antithrombin or thrombin binding. Partial inhibition of the accelerating effect of heparin when fewer than this number of molecules of H-kininogen are bound to each heparin chain is

also possible. Moreover, the metal ions may interact with either heparin or H-kininogen, or with both molecules, to mediate the binding, and the number of such ions involved in these interactions is unknown. Therefore, analysis of the data with a simple, two-state model for the competitive effect does not appear meaningful at present. Nevertheless, the concentrations at which competition occurs in the presence of Zn^{2+} at physiological pH suggest a tight binding between H-kininogen and heparin under these conditions, with an affinity comparable to that between heparin and histidine-rich glycoprotein (Lijnen et al., 1983a; Peterson et al., 1987). Tight binding is further indicated by the affinity chromatography experiments, in which H-kininogen eluted from matrix-linked heparin in the presence of Zn^{2+} at pH 7.4 at an ionic strength similar to that required to elute antithrombin (Höök et al., 1976; Danielsson & Björk, 1981). As was also found for histidine-rich glycoprotein (Lijnen et al., 1983a; Lane et al., 1986), heparin species with low and high affinity for antithrombin bound with comparable affinity to H-kininogen, demonstrating that the specific antithrombin binding pentasaccharide sequence in heparin [see reviews by Björk and Lindahl (1982) and Björk and Danielsson (1986)] is not required for binding of the polysaccharide to either of these two other proteins.

Although divalent metal ions are required for the binding of heparin to H-kininogen at physiological pH, this requirement varies with pH. The binding in the absence of such ions thus increases with decreasing pH with an apparent pK_a consistent with involvement of protonated histidine residues in the binding. The requirement for protonated histidine residues apparently can be alleviated at physiological and higher pH by divalent metal ions, most efficiently Zn^{2+} and Ni^{2+}, binding to the unprotonated form of histidine (Porath et al., 1975) and mediating a tight binding of the polysaccharide due to their positive charge. Binding of metal ions to unprotonated histidines would also be expected to shift the pK_a of these histidines to a lower value. The participation of histidine residues in Zn^{2+} binding to H-kininogen is supported by the finding that chemical modification of histidines abolishes this binding (Retzios et al., 1987). Evidence for involvement of protonated histidine residues has also been presented for the binding of heparin to histidine-rich glycoprotein (Peterson et al., 1987). However, the participation of divalent metal ions in the binding of heparin to this protein at physiological pH may depend on the species. Thus, binding of heparin to human histidine-rich glycoprotein at this pH has been shown to be dependent on divalent metal ions (Lijnen et al., 1983a), whereas the polysaccharide binds to the rabbit protein with similar affinity both in the absence and in the presence of Zn^{2+} (Peterson et al., 1987).

The analyses of the binding of heparin to different forms of kininogen show that the polysaccharide binds to the light-chain portion of H-kininogen and also that excision of the kinin part and the adjacent 48-residue segment of the light chain does not affect this binding. Moreover, the involvement of histidine residues inferred above suggests that the binding occurs to the histidine-rich region of the H-kininogen light chain. This region is responsible for binding of H-kininogen to negatively charged surfaces, thereby mediating the binding of prekallikrein and factor XI to these surfaces (Han et al., 1975; Sugo et al., 1980; Kerbiriou et al., 1980; Bock & Shore, 1983; Bock et al., 1985; Tait & Fujikawa, 1986, 1987). It is likely that the binding of H-kininogen to such surfaces shows characteristics similar to those of its binding to the negatively charged polysaccharide heparin and therefore also is affected by divalent metal ions and pH. This possibility is supported by the observation that the binding of H-kininogen to dextran sulfate, a molecule acting as a soluble surface in contact activation reactions, is considerably stronger in the presence than in the absence of Zn^{2+}. The similar metal ion dependent binding of the single- and two-chain forms of H-kininogen to a negatively charged surface observed in this work contrasts with a previous report that the two-chain form of H-kininogen bound substantially tighter to a kaolin surface than the single-chain form, although in the absence of metal ions (Scott et al., 1984). Whether the different behavior observed in the two studies is due to the presence of metal ions or to the different properties of the surfaces used remains to be determined. The effects of metal ions and pH may be relevant for the physiological regulation of the surface binding of H-kininogen, since Zn^{2+} influences the binding at concentrations much below that in plasma [10–25 μM, most of which, however, is protein bound; see Whitehouse et al. (1983), Woo and Cannon (1984), and Magneson et al. (1987)] and the major change of the binding with pH occurs around neutral pH. Such a regulatory effect of Zn^{2+} is supported by the observations that the binding of H-kininogen to stimulated platelets and to endothelial cells requires Zn^{2+} ions (Greengard & Griffin, 1984; van Iwaarden et al., 1988). Interestingly, recent studies have shown that also other reactions involved in the contact activation phase of blood clotting are markedly affected by Zn^{2+} or other metal ions (Bock et al., 1981; Shimada et al., 1984; Shore et al., 1987a,b).

Previous studies have indicated that heparin binding to histidine-rich glycoprotein in plasma may decrease the effective concentration of heparin administered during antithrombotic prophylaxis and therapy (Lijnen et al., 1983a; Lane et al., 1986). H-Kininogen would be expected to have a similar effect, since the two proteins are present in human plasma in similar concentrations (1–1.5 μM; Lijnen et al., 1981, 1983b; Adam et al., 1985) and have comparable, metal-dependent affinities for heparin. Indeed, this work shows that H-kininogen in plasma has an appreciable, metal-dependent heparin-neutralizing ability. It may appear from the data presented by Lijnen et al. (1983a) that histidine-rich glycoprotein has a significantly larger effect in this respect. However, these data were obtained with plasma made deficient in histidine-rich glycoprotein by treatment with (carboxymethyl)cellulose, which is likely also to have eliminated H-kininogen, based on the tight binding of this protein to a cation exchanger (Kerbiriou & Griffin, 1979). A definite conclusion regarding the relative effectiveness of the two proteins in neutralizing heparin in plasma thus cannot be drawn at present.

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Registry No. Heparin, 9005-49-6; antithrombin, 9000-94-6.

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