Parallel Mechanisms of High Molecular Weight Kininogen Action as a Cofactor in Kallikrein Inactivation and Prekallikrein Activation Reactions†

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ABSTRACT: The mechanism by which high molecular weight kininogen (H-kininogen) potentiates the heparin-accelerated inhibition of plasma kallikrein by antithrombin [Olson, S. T., Sheffer, R., & Francis, A. M. (1993) *Biochemistry* (preceding paper in this issue)] was investigated at $I = 0.15$, pH 7.4, 25 °C. Single-chain, two-chain, and light-chain, but not heavy-chain, forms of H-kininogen were similarly effective in potentiating the heparin-accelerated antithrombin–kallikrein reaction, indicating that the light-chain region of the protein was responsible for promoting kallikrein inactivation and that cleavage of H-kininogen did not significantly affect this promoting activity. H-kininogen potentiation increased in a saturable manner with increasing kininogen concentration, reflecting a K_D (23 ± 8 nM) similar to that previously measured for H-kininogen binding to kallikrein by equilibrium methods. Both H-kininogen-stimulated and unstimulated heparin rate enhancements initially increased with increasing heparin concentration in a manner corresponding to the saturation of antithrombin with heparin (K_D = 10–30 nM). However, at higher heparin concentrations, the stimulated but not the unstimulated heparin rate enhancement decreased in parallel with the saturation of a protein–heparin interaction with a K_D (0.4 ± 0.2 μM) comparable to that directly measured for the H-kininogen–heparin interaction (2.0 ± 0.2 μM). These results implied that H-kininogen stimulation required the formation of a quaternary complex in which antithrombin and H-kininogen–kallikrein complex were bound to the same heparin chain. In keeping with this interpretation, a synthetic heparin pentasaccharide representing the antithrombin binding sequence accelerated the antithrombin–kallikrein reaction to an extent similar to that of full-length heparin chains containing this sequence, but the pentasaccharide acceleration was not stimulated by H-kininogen. The importance of H-kininogen–kallikrein complex binding to heparin for kininogen stimulation was further indicated from the marked salt dependence of the second-order rate constant for the association of H-kininogen–kallikrein complex but not free kallikrein with antithrombin–heparin complex, under conditions where saturation of the two binary complexes was maintained. Kinetic analyses of antithrombin–kallikrein reactions as a function of the inhibitor concentration indicated that the K_D for an initial antithrombin–kallikrein encounter complex was decreased 20-fold by heparin binding to antithrombin and an additional 200-fold by H-kininogen also binding to kallikrein. By contrast, rate constants for the conversion of the encounter complex to a stable complex were comparable for all reactions. Together, these results indicate a heparin accelerating mechanism involving mostly heparin activation of antithrombin in the absence of H-kininogen, with an additional mechanism involving heparin bridging antithrombin and kallikrein contributing in the presence of H-kininogen due to kininogen facilitating the binding of kallikrein to heparin. Zinc ions, at levels which promoted H-kininogen binding to heparin, diminished kininogen potentiation at $I = 0.15$, but increased this potentiation at $I = 0.3$, suggesting that antagonism of inhibitor or kallikrein–kininogen complex binding to heparin by free kininogen can offset the promoting effect of kininogen depending on the kininogen binding affinity for heparin.

High molecular weight kininogen (H-kininogen)[1] is a nonenzymatic plasma glycoprotein that functions as a cofactor in the activation of a number of proenzymes which participate in blood coagulation, fibrinolysis, and complement activation pathways (Griffin & Cochrane, 1976; Kato et al., 1981; Colman, 1984). This cofactor function is localized in two distinct regions of the carboxy-terminal third of the protein (Kerbiriou & Griffin, 1979; Mori & Nagasawa, 1981): one which specifically binds the proenzymes prekallikrein and factor XI and their activated forms (Mandle et al., 1976; Thompson et al., 1977; Kerbiriou et al., 1980; Bouma et al., 1983; Bock & Shore, 1983; Bock et al., 1985; Shimada et al., 1985; Tait & Fujikawa, 1986, 1987), and the other which binds negatively charged surfaces (Han et al., 1975; Sugo et al., 1980; Ikari et al., 1981; Kellerman et al., 1986; Retzios et al., 1987). These two modes of cofactor interaction are thought to allow H-kininogen to mediate proenzyme or enzyme binding to a surface and thereby promote proenzyme activation (Cochrane & Griffin, 1982; Colman, 1984).

In the preceding paper, we showed that in addition to acting as a cofactor in the surface-dependent activation of several proteolytic enzymes, H-kininogen also acts as a cofactor in the heparin-dependent inactivation of one of these enzymes,

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[1] Abbreviations: H-kininogen or Kin, high molecular weight kininogen; L-kininogen, low molecular weight kininogen; Kall, plasma kallikrein; AT, antithrombin; H, heparin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PEG, poly(ethylene glycol).

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plasma kallikrein, by the proteinase inhibitor antithrombin (Olson et al., 1993). Such a mode of action of H-kininogen was predicted on the basis of the observation that the protein binds to heparin (Björk et al., 1989), and by analogy with the established mechanism of action of the protein in promoting proenzyme activation. Thus, we anticipated that H-kininogen might promote the heparin-dependent antithrombin–kallikrein reaction by facilitating kallikrein binding to the heparin surface where a more efficient inactivation of the enzyme by heparin-bound antithrombin could occur [see Scheme I of Olson et al. (1993)]. In the present study, we have investigated this hypothesized mechanism of H-kininogen action by detailed kinetic studies of the potentiating effect of the protein on the heparin-enhanced antithrombin–kallikrein reaction. The results of these studies confirm that H-kininogen functions as a cofactor in kallikrein inactivation by a mechanism which parallels its action as a cofactor in prekallikrein activation.

EXPERIMENTAL PROCEDURES

Proteins. Single-chain H-kininogen was isolated from fresh-frozen plasma as previously described (Kerbiriou & Griffin, 1979; Bock & Shore, 1983). Kinin-free two-chain H-kininogen was purified from outdated plasma as described in the preceding paper (Olson et al., 1993). The H-kininogen light chain was isolated by reduction and alkylation of the two-chain molecule followed by separation from the heavy chain by SP-Sephadex chromatography (Bock & Shore, 1983). Both two-chain and light-chain forms of H-kininogen contained the terminally cleaved light chain lacking the amino-terminal 48 residues of the complete light-chain sequence, as judged from the mobility of the light chains on SDS gel electrophoresis (Nakayasu & Nagasawa, 1979; Mori & Nagasawa, 1981; Bock & Shore, 1983; Tait & Fujikawa, 1986; Retzios et al., 1987). Kinin-free L-kininogen was purified from fresh plasma by papain–Sepharose and DEAE-Sepharose chromatography as described (Björk et al., 1989; Gounaris et al., 1984). All forms of kininogen were treated with D-Phe-L-Phe-L-Arg-chloromethyl ketone to remove any contaminating kallikrein and then dialyzed into 4 mM sodium acetate/0.15 M NaCl, pH 5.3 (storage buffer), as described (Björk et al., 1989). Specific clotting activities of single-chain, two-chain, and light-chain forms of H-kininogen were 27, 23–29, and 42 units/mg, respectively. SDS gel electrophoresis according to Laemmli (1970) showed that single-chain, light-chain, and L-forms of kininogen appeared as single major bands under nonreducing and reducing conditions. The two-chain H-kininogen was a single band under nonreducing conditions but appeared as two major bands under reducing conditions with mobilities indistinguishable from those of the isolated heavy and light chains (Figure 1). Concentrations of kininogens were determined from absorbance measurements at 280 nm utilizing the following absorption coefficients ($L \cdot g^{-1} \cdot cm^{-1}$) and molecular weights, respectively: 0.701 and 108 000 for single- and two-chain H-kininogens (Kerbiriou & Griffin, 1979; Nakayasu & Nagasawa, 1979), 0.59 and 64 000 for L-kininogen (Ryley, 1979; Kellerman et al., 1987), and 0.64 and 30 500 for the H-kininogen light chain (Bock & Shore, 1983; Bock & Halvorson, 1983). Antithrombin and plasma kallikrein were purified and their concentrations determined as described in the preceding paper (Olson et al., 1993).

Heparin. The methyl α-glycoside of the heparin pentasaccharide constituting the antithrombin binding sequence was synthesized as previously described (Petitou et al., 1987). A ~2 mg/mL solution prepared in water gave an average concentration of 1220 ± 40 (SE) μM when analyzed by weight, uronic acid, and antithrombin binding sites (Olson et al., 1992). Full-length heparins of reduced polydispersity and having high affinity for antithrombin were isolated from unbleached porcine intestinal mucosal heparin (Diosynth) by cetylpyridinium chloride precipitation, repeated Sephadex G-100 gel filtration, and affinity chromatography on antithrombin–agarose as described (Olson, 1988). Molecular weights (±~10%) of 7900, 13 100, 17 300, and 21 200 were determined for full-length heparins by sedimentation equilibrium or by analytical gel filtration on a Sephadex G-100 column calibrated with heparin standards whose molecular weights had been measured by sedimentation equilibrium (Olson et al., 1991b). Molar concentrations of heparins were calculated from the dry weight and molecular weight. Concentrations of antithrombin binding sites were determined from the equivalence point of titrations of antithrombin with heparins monitored by the increase in tryptophan fluorescence accompanying heparin binding to the inhibitor (Olson & Shore, 1981). The average number of disaccharide units in heparin chains was calculated on the basis of disaccharide molecular weight of 615 (Lindahl et al., 1984).

All experiments were performed in 0.1 M Hepes buffer containing 0.1 (I = 0.15) or 0.25 (I = 0.3) M NaCl, 1 mM EDTA, and 0.1% PEG 8000, pH 7.40, unless otherwise stated. Proteins and heparins were extensively diluted or dialyzed into experimental buffers prior to use except for kallikrein which was diluted into storage buffer plus 0.1% PEG 8000 in a polyethylene tube coated with PEG 20000 to minimize protein adsorption (Latallo & Hall, 1986).

Kinetic Studies. Reactions of antithrombin and kallikrein in the absence and presence of heparin and/or kininogen were performed under pseudo-first-order conditions as in the preceding paper (Olson et al., 1993). Briefly, reactions were initiated by adding 10 μL of kallikrein to 90 μL of antithrombin plus effectors in pH 7.4 Hepes buffer in a polystyrene or acrylic cuvette coated with PEG 20000, after preincubation of enzyme and inhibitor solutions at 25 °C. After varying intervals of time, reactions were quenched with 0.9–1.0 mL of 200 μM D-Pro-L-Phe-L-Arg-*p*-nitroanilide (S-2302, Kabi) or 50 μM D-Pro-L-Phe-L-Arg-7-amido-4-methylcoumarin (Enzyme Product Systems, Livermore, CA) plus 50–100 μg/mL Polybrene (Aldrich), and the remaining enzyme activity was determined by measuring the initial velocity of substrate hydrolysis at 405 nm for the chromogenic substrate or at excitation and emission wavelengths of 380 and 440 nm, respectively, for the fluorogenic substrate. Substrate concentrations were determined by absorbance measurements at 316 nm for the chromogenic substrate (ϵ = 13 000 M^{-1} cm^{-1}) and at 325 nm for the fluorogenic substrate (ϵ = 20 000 M^{-1} cm^{-1}). In the fluorescence assay, 7-amido-4-methylcoumarin (Sigma; ϵ_{343} = 15 700 M^{-1} cm^{-1}) was used to calibrate the full-scale of the recorder to correspond to 0.1–0.5 μM product. Activities were expressed relative to controls in the absence of inhibitor. Progress curves for antithrombin–kallikrein reactions were analyzed by one- or two-exponential fits as described in the previous paper (Olson et al., 1993). Observed rate constants were computer-fit to appropriate kinetic equations described in the text by nonlinear regression (Duggleby, 1984). Reported errors represent ± 2 SE.

Binding Studies. Binding of H-kininogen to heparin was studied by an affinity partitioning method recently described (Olson et al., 1991a). Briefly, increasing concentrations of H-kininogen (two chain) were added to a series of 0.5-mL microcentrifuge tubes containing 20 μL of a suspension of heparin–Sepharose CL-6B or Sepharose CL-6B

(Pharmacia—LKB Biotechnology, 1 volume of gel to 3 volumes of buffer) or just buffer in a total volume of 200 μL of $I = 0.15$ Hepes buffer. In a second series of tubes, a fixed amount of H-kininogen within the range covered in the first series of tubes together with the same volume of gel suspension or buffer control as before was added along with increasing concentrations of the M_r 7900 full-length heparin, again in a total volume of 200 μL of buffer. Solutions were agitated at room temperature (~22 °C) in a TOMY microtube mixer at a setting of 5 for 1 h, a time shown to result in maximal binding of H-kininogen to the immobilized heparin. Tubes were then centrifuged for 5 min in an Eppendorf microcentrifuge. In the case of the first series of tubes, 100 μL of sample was withdrawn and added to 1.1 mL of buffer in an acrylic cuvette (Sarstedt) and the intrinsic protein fluorescence (λ_{ex} 280 nm, λ_{em} 340 nm) measured in an SLM 8000 spectrofluorometer at 25 °C. The second series of tubes containing heparin was first treated by transferring 145 μL to fresh tubes containing 5 μL of a Polybrene solution (6 mg/mL) to neutralize the heparin. After incubation for 45 min, these tubes were centrifuged, and the protein fluorescence was measured as before. The Polybrene treatment minimally affected the protein fluorescence of H-kininogen in the absence or presence of heparin. Control incubations with buffer or unsubstituted Sepharose gel yielded an indistinguishable linear dependence of measured protein fluorescence on concentration. For each H-kininogen concentration analyzed, the decrease in fluorescence measured in the presence of heparin–Sepharose (F_H) relative to that of the control (F_C) enabled calculation of the concentration of H-kininogen bound to the immobilized heparin from the formula $\{(F_C - F_H)/F_C\} \times [Kin]_0$, where $[Kin]_0$ is the total H-kininogen concentration present in the binding experiment. Analysis of H-kininogen binding to the immobilized heparin in the absence of solution-phase heparin as a function of the H-kininogen concentration was fit empirically by a second-order polynomial expression (Olson et al., 1991a). Binding of the competitor heparin to H-kininogen in the solution phase was analyzed by nonlinear regression fits of the extent of displacement of H-kininogen from the immobilized heparin by the competitor heparin to equations previously described (Olson et al., 1991a).

RESULTS

Potentiating Effects of Single-Chain, Two-Chain, Heavy-Chain, and Light-Chain Forms of H-kininogen on the Heparin-Accelerated Antithrombin–Kallikrein Reaction. The localization of the region of H-kininogen responsible for the stimulating effect of the protein on the heparin-dependent antithrombin–kallikrein reaction demonstrated in the preceding paper (Olson et al., 1993) and the effect of kallikrein cleavage of the single-chain protein to a two-chain species on this stimulation were investigated by comparing the stimulating activities of single-chain and two-chain H-kininogen and the isolated heavy and light chains of the two-chain molecule (Figure 1). In order to study the effect of single-chain H-kininogen on the heparin-dependent antithrombin–kallikrein reaction, it was necessary to employ conditions under which kallikrein cleavage of the cofactor protein to the two-chain form did not occur to any significant extent. This was accomplished by studying the kallikrein inactivation reaction at subnanomolar enzyme concentrations and using a sensitive fluorogenic substrate to monitor residual kallikrein activity. SDS gel electrophoresis under reducing conditions demonstrated that kallikrein cleavage of the single-chain protein was not detectable at the concentrations and reaction times employed in the experiments in Figure 1 either in the absence

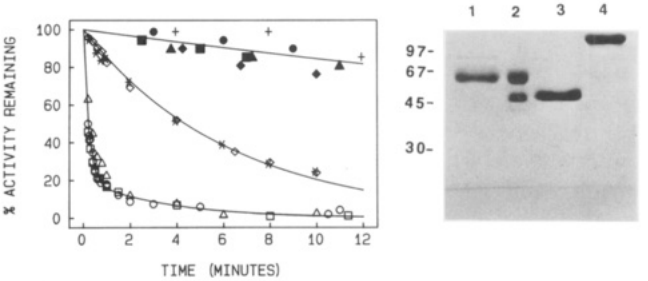


FIGURE 1: Comparison of different forms of H-kininogen as stimulators of the heparin-accelerated antithrombin–kallikrein reaction. (Left) Reactions of 2 μM antithrombin with 0.1 nM kallikrein in the absence [solid symbols and (+)] or presence [open symbols and (*)] of 1 μM heparin containing either no kininogen (+, *), single-chain H-kininogen (▲, △), two-chain H-kininogen (●, ○), L-kininogen (♦, ◇), or the H-kininogen light chain (■, □) at 400 nM in $I = 0.15$ Hepes buffer, pH 7.4, 25 °C. Kallikrein activity was monitored discontinuously by fluorogenic substrate assays as described under Experimental Procedures. Solid lines are either single-exponential fits of combined data sets in the absence or presence of heparin alone or a double-exponential fit of just the two-chain kininogen data when both heparin and kininogen were present. (Right) SDS gel electrophoresis of single-chain (lane 4), two-chain (lane 2), and light-chain (lane 3) forms of H-kininogen and of L-kininogen (lane 1) under reducing conditions. Molecular weights ($\times 10^{-3}$) of standard proteins are indicated on the left.

or in the presence of heparin. Since numerous intrachain disulfide bonds in the H-kininogen heavy chain (Kellerman et al., 1986) are broken in the procedure for separating the two disulfide-linked chains of cleaved kininogen, L-kininogen, consisting of predominantly the heavy-chain portion of H-kininogen (Kerbiriou et al., 1980; Kato et al., 1981), was used to assess the stimulating activity of the heavy chain. The coagulant activity of single-chain H-kininogen was retained in the two-chain and light-chain forms of the protein. SDS gel electrophoresis confirmed the purity of the different forms of H-kininogen and the correspondence of the isolated L-kininogen and H-kininogen light chain to the heavy and light chains, respectively, of the parent two-chain protein (Figure 1).

The effects of the various forms of H-kininogen on the heparin-accelerated inactivation of kallikrein by antithrombin are shown in Figure 1. When present at a concentration sufficient to saturate kallikrein (400 nM), the single-chain, two-chain, and alkylated light-chain forms of H-kininogen all showed comparable stimulating effects on the heparin-enhanced antithrombin–kallikrein reaction, but not on reactions conducted in the absence of heparin. Observed pseudo-first-order rate constants for the predominant fast phase of these cofactor-potentiated reactions, which reflect α-kallikrein inactivation (Olson et al., 1993), were 3.3 ± 0.7, 4.6 ± 0.5, and 5.7 ± 0.5 min^{-1} for single-chain, two-chain, and light-chain potentiated reactions, respectively. In contrast, the heavy chain, i.e., L-kininogen, had no effect on either the heparin-accelerated (0.16 ± 0.02 min^{-1}) or the unaccelerated (0.017 ± 0.01 min^{-1}) reactions. These results indicated that the region of H-kininogen responsible for the potentiating activity was contained in the light chain and that neither cleavage to the two-chain form nor removal of the heavy chain from the protein significantly affected this activity. Subsequent experiments designed to establish the mechanism of H-kininogen potentiation were therefore performed with the two-chain protein.

Dependence of the H-kininogen Potentiating Effect on H-kininogen Concentration. Figure 2 shows the stimulating effect of H-kininogen (Kin) on the pseudo-first-order rate constant (k_{obs}) for the inactivation of kallikrein (Kall) by

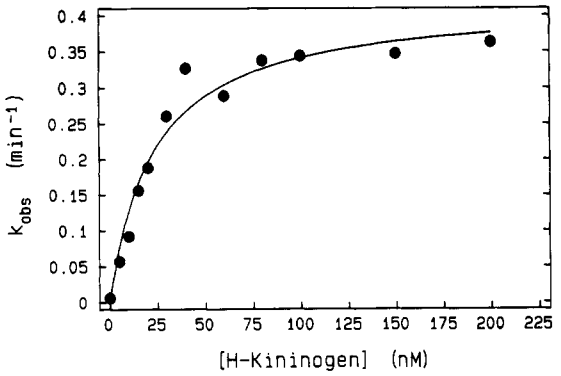


FIGURE 2: H-kininogen concentration dependence of kininogen stimulation of the heparin-dependent antithrombin–kallikrein reaction. k_{obs} for the predominant fast phase of reactions of 1 nM kallikrein, 100 nM antithrombin, 50 nM heparin, and the indicated concentrations of two-chain H-kininogen in I = 0.15 Hepes, pH 7.4 at 25 °C, was determined from a two-exponential fit of kallikrein inactivation curves (see Experimental Procedures). The solid line represents the best fit of the data by eq 1–3 of the text.

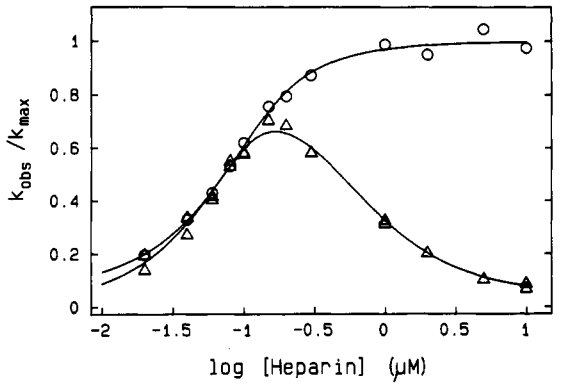


FIGURE 3: Heparin concentration dependence of k_{obs} for H-kininogen-stimulated and unstimulated heparin–antithrombin–kallikrein reactions. k_{obs} for reactions of 100 nM antithrombin with 1 nM kallikrein and the indicated concentrations of heparin was measured in the absence (○) or presence (△) of 200 nM H-kininogen in I = 0.15 Hepes buffer, pH 7.4 at 25 °C, as described under Experimental Procedures. k_{obs} values are expressed relative to extrapolated maximum values of 0.0156 min^{-1} for the reaction in the absence of H-kininogen and of 1.28 min^{-1} for the reaction in the presence of H-kininogen which were determined by fitting of data by eq 4 and 5–7, respectively. Solid lines provide the best fits of data by these equations.

antithrombin (AT) complexed with heparin (H), examined as a function of the H-kininogen concentration. These experiments were analyzed according to Scheme I where $K_{Kin,Kall}$ is the dissociation constant for the H-kininogen–kallikrein interaction and k_H and k'_H are second-order rate constants for the antithrombin–heparin complex inhibition of free or H-kininogen-bound kallikrein, respectively.[2] This scheme assumes that kallikrein or the kallikrein–H-kininogen complex is inhibited by the antithrombin–heparin complex via a second-order kinetic process, a condition assured by working in a range of antithrombin–heparin complex concentration where k_{obs} was linearly dependent on the concentration of this complex (see Figure 6). Complicating effects of free heparin on the reaction (Scheme III) were avoided in these experiments by forming the antithrombin–heparin complex with a molar excess of antithrombin over heparin. The kinetic equations which describe this scheme are (see the Appendix):

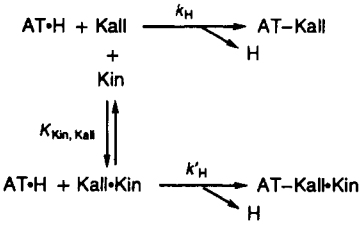
$$k_{obs} = k[AT] + k_H[AT{\cdot}H]\frac{K_{Kin,Kall}}{K_{Kin,Kall} + [Kin]_0} + k'_H[AT{\cdot}H]\frac{[Kin]_0}{K_{Kin,Kall} + [Kin]_0} \quad (1)$$

$$[AT{\cdot}H] = \{[AT]_0 + [H]_0 + K_{AT,H} - \sqrt{([AT]_0 + [H]_0 + K_{AT,H})^2 - 4[AT]_0[H]_0}\}/2 \quad (2)$$

$$[AT] = [AT]_0 - [AT{\cdot}H] \quad (3)$$

where $K_{AT,H}$ is the dissociation constant for the antithrombin–heparin interaction and $[AT]_0$, $[H]_0$, and $[Kin]_0$ represent total concentrations of antithrombin, heparin, and kininogen, respectively. The first term in eq 1 provides the contribution of the free antithrombin reaction with k the second-order rate constant for this reaction with both free and kininogen-bound enzyme (not shown in Scheme I), and the second and third terms provide the contributions of the antithrombin–heparin complex reaction with free and H-kininogen-bound kallikrein, respectively. These equations are valid under conditions where the amount of kininogen complexed with enzyme can be neglected relative to the total kininogen concentration. Such conditions were achieved by maintaining a substantial molar excess of H-kininogen over kallikrein. Figure 2 shows that k_{obs} for the predominant α-kallikrein inactivation phase increased in a saturable manner with increasing H-kininogen concentration, as predicted by eq 1, with no effect of the protein on the amplitude of this reaction phase. A satisfactory fit of the data by this equation was obtained which yielded a value for $K_{Kin,Kall}$ of 23 ± 8 nM, in fair agreement with dissociation constants of 15–17 nM previously determined for the interaction of kallikrein with single-chain and light-chain forms of H-kininogen by equilibrium binding studies under the same experimental conditions (Bock et al., 1985). These results demonstrated that the potentiating effect of H-kininogen was due to the binding of H-kininogen to kallikrein enhancing the reactivity of the enzyme with the antithrombin–heparin complex.

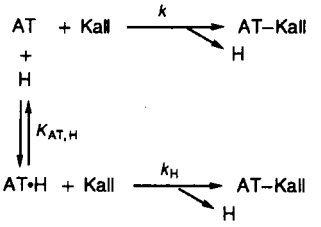
Scheme I



Heparin Concentration Dependence of H-kininogen Stimulation. To investigate the protein–heparin interactions which contribute to H-kininogen's stimulating effect on the heparin-accelerated antithrombin–kallikrein reaction, the heparin concentration dependence of H-kininogen-stimulated and unstimulated heparin-dependent reactions was examined. Figure 3 shows that in the absence of H-kininogen, heparin enhanced the pseudo-first-order rate constant for the antithrombin–kallikrein reaction in a saturable manner, reaching a maximum enhancement at 1 μM heparin which remained constant up to 10 μM polysaccharide. The end point of ~1

[2] Schemes I–III depict heparin dissociating concomitant with the formation of a stable antithrombin–kallikrein complex based on the substantial loss in affinity of heparin for antithrombin demonstrated to accompany the formation of such complexes with other proteinases (Jordan et al., 1979, 1980). It is possible that heparin may remain bound to the inhibitor–enzyme complex in cases where the enzyme is bound to the polysaccharide through H-kininogen. Whether heparin remains bound or is released from the stable inhibitor–enzyme complex during the reaction should not affect kinetic analyses conducted under conditions where heparin does not recycle, i.e., with a large molar excess of antithrombin–heparin complex over enzyme.

Scheme II



mol of heparin/mol of antithrombin characterizing this saturation curve suggested that the heparin rate enhancement was paralleling the saturation of an antithrombin–heparin complex which was a more efficient inhibitor of kallikrein than free antithrombin (Scheme II). In keeping with this scheme, raising the fixed antithrombin concentration by 10-fold shifted the saturation curve to the right and increased the maximum rate constant by similar factors (not shown). This result confirmed that the heparin enhancement of k_{obs} was paralleling the saturation of antithrombin with heparin and was approximately proportional to the concentration of the antithrombin–heparin complex, in accordance with the equation which describes Scheme II (Olson, 1988):

$$k_{obs} = k[AT] + k_H[AT{\cdot}H] \quad (4)$$

where the concentrations of complexed and free antithrombin are given by eq 2 and 3 above. Confirming the above reaction scheme, fitting of the data by the above equations provided a value for $K_{AT,H}$ of 30 ± 7 nM, comparable to the values of 10–14 nM obtained in previous studies by direct equilibrium binding measurements (Olson & Björk, 1991; Olson et al., 1992). Values of 160 ± 90 M^{-1} s^{-1} for k and 2600 ± 70 M^{-1} s^{-1} for k_H were also obtained in these fits, in agreement with the preceding study (Olson et al., 1993).

In the presence of H-kininogen, heparin again enhanced k_{obs} for the predominant α-kallikrein inactivation phase in a saturable manner approximately in parallel with the saturation curve observed in the absence of the kininogen, but a maximum rate enhancement was achieved prior to the saturation point reached without kininogen and was followed by a progressive decline in the heparin enhancement in the range from 0.3 to 10 μM heparin (Figure 3). Such observations suggested that H-kininogen stimulation of the heparin rate enhancement required the formation of a quaternary complex in which antithrombin and H-kininogen–kallikrein complex are bound to the same heparin chain (Jordan et al., 1979, 1980; Griffith, 1982; Nesheim, 1983; Olson, 1988). Thus, the correspondence of the initial ascending phase of the bell-shaped curve to the ascending curve seen in the absence of H-kininogen suggested that this phase reflects the saturation of a complex of antithrombin with heparin whose reaction with kallikrein–H-kininogen complex is stimulated relative to that of free kallikrein. The occurrence of the subsequent descending phase at heparin concentrations which exceeded those required to saturate antithrombin suggested that this phase is due to the binding of kallikrein–H-kininogen complex to free instead of antithrombin-bound heparin chains, resulting in a loss of the kininogen promotion of the reaction. The data were therefore analyzed according to Scheme III. In this scheme, the association of antithrombin–heparin complex with free kallikrein–kininogen complex occurs with the potentiated rate constant k'_H whereas the association of the inhibitor–heparin complex with kallikrein–kininogen complexes bound to separate heparin chains occurs with the unpotentiated rate constant k''_H. Assuming that the fractional saturation of kallikrein–kininogen complex with heparin is equal to the fractional saturation of bulk H-kininogen with the polysaccharide and that the latter is governed by the dissociation constant $K_{Kin,H}$, k_{obs} for Scheme III is given by the equation (see the Appendix):

$$k_{obs} = k[AT] + k'_H[AT{\cdot}H]\frac{[Kin]}{[Kin]_0} + k''_H[AT{\cdot}H]\frac{[Kin{\cdot}H]}{[Kin]_0} \quad (5)$$

where [AT·H[and [AT] are given by eq 2 and 3 above and [Kin·H] and [Kin] are given by the analogous equations:

$$[Kin{\cdot}H] = \{[Kin]_0 + [H]_0 + K_{Kin,H} - \sqrt{([Kin]_0 + [H]_0 + K_{Kin,H})^2 - 4[Kin]_0[H]_0}\}/2 \quad (6)$$

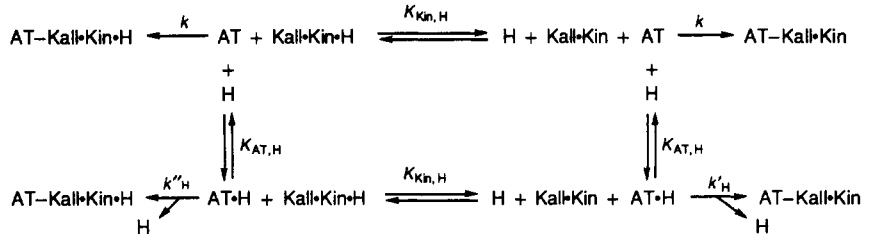
$$[Kin] = [Kin]_0 - [Kin{\cdot}H] \quad (7)$$

Scheme III and eq 5–7 assume that antithrombin and kininogen or kininogen–kallikrein complexes bind independently to heparin and that relatively insignificant levels of complexes in which these proteins are bound to the same heparin chain are formed. This condition was assured by the large difference in affinities of these proteins for heparin which favors their binding to separate heparin chains under the conditions of the experiment. The data in the presence of H-kininogen were satisfactorily fit by eq 5–7 with values for $K_{Kin,H}$ of 0.4 ± 0.2 μM, $K_{AT,H}$ of 10 ± 10 nM, k'_H of 2.1 (±0.1) × 10^5 M^{-1} s^{-1}, and k''_H of 9 (±8) × 10^3 M^{-1} s^{-1}. The error in k''_H indicated that it was indistinguishable from the unstimulated reaction rate constant, k_H, determined in the absence of H-kininogen.

Figure 4 shows the results of direct measurements of the binding of H-kininogen to the heparin employed in the experiment of Figure 3. Binding was measured from the competitive effect of this heparin on the observable binding of H-kininogen to heparin immobilized on Sepharose (Olson et al., 1991a). The binding of H-kininogen to the immobilized heparin in the absence of the competitor heparin is shown in the left-hand panel of Figure 4 while the displacement of bound H-kininogen from the matrix-linked heparin by the competitor heparin is shown in the right-hand panel. At each competitor heparin concentration used in the displacement experiment, the concentration of H-kininogen bound to the solution-phase heparin is rigorously given by the difference between the total H-kininogen concentrations yielding an equivalent extent of saturation of the immobilized heparin in the presence and absence of competitor (Olson et al., 1991a). The fit of the displacement data by the equations defining this relationship (Figure 4) provided an apparent dissociation constant, $K_{Kin,H}$, of 2.0 ± 0.2 μM for the H-kininogen–heparin solution-phase interaction. Due to the low binding densities of ≤0.3 kininogen molecules bound per heparin chain that were achieved in these experiments, as calculated from the ratio of bound kininogen to total heparin, this dissociation constant corresponds to the binding of a single H-kininogen molecule per heparin chain. The directly determined dissociation constant was somewhat greater than the kinetic value determined above under similar low binding density conditions, suggesting that kallikrein binding to H-kininogen modestly enhances the affinity of kininogen for heparin. This differential affinity indicates the kinetic dissociation constant should be regarded as an approximation of the kallikrein–kininogen affinity for heparin.

Dependence of H-kininogen Potentiating Activity on Heparin Chain Length. The heparin chain-length requirements for H-kininogen potentiation of the heparin-accelerated antithrombin–kallikrein reaction were investigated to deter-

Scheme III



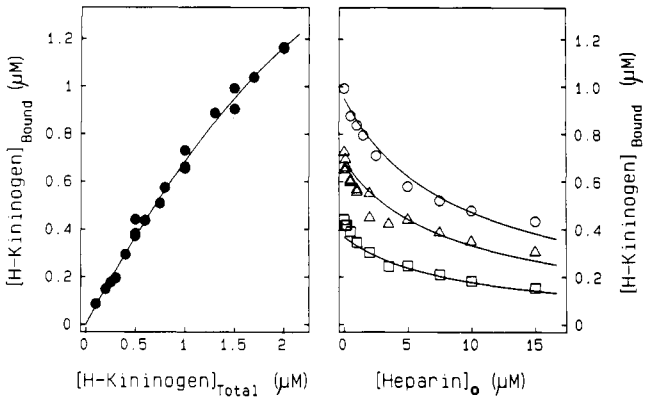


FIGURE 4: Equilibrium binding of H-kininogen to heparin. (Left) H-kininogen binding to immobilized heparin in the absence of solution-phase heparin in $I = 0.15$ Hepes buffer, pH 7.4 at room temperature, was quantified as a function of the total H-kininogen concentration ($[Kin]_0$) as described under Experimental Procedures. The solid line is an empirical fit of the data by the second-degree polynomial equation: $[Kin]_{bound} = -0.102[Kin]_0^2 + 0.785[Kin]_0$. (Right) Displacement of H-kininogen from heparin–agarose by a competitor 26-saccharide heparin was measured as a function of heparin concentration as detailed under Experimental Procedures. Displacement curves were obtained at fixed total H-kininogen concentrations of 0.5 (□), 1.0 (△), and 1.5 μM (○), with solid lines representing the best fit of all data by an apparent K_D of 2.0 μM for the H-kininogen–competitor heparin interaction using equations described previously (Olson et al., 1991a).

mine whether the sequence-specific pentasaccharide region of heparin involved in binding antithrombin (Choay et al., 1981, 1983; Thunberg et al., 1982; Atha et al., 1985; Olson et al., 1992) was sufficient to produce the kininogen-potentiating effect or whether larger heparin chains containing the antithrombin binding sequence were necessary to express potentiating activity. Table I compares the stimulating effects of H-kininogen on second-order rate constants for heparin-accelerated reactions with pentasaccharide and full-length heparin chains of 26, 44, 56, and 70 saccharides in length, all containing the pentasaccharide binding sequence. In the absence of H-kininogen, full-length heparin chains enhanced the second-order inhibition rate constant to an extent comparable to that of the pentasaccharide, with rate enhancements increasing by ~3-fold from the pentasaccharide to the largest full-length heparin chain. In the presence of H-kininogen, however, full-length and pentasaccharide heparins produced markedly different enhancement of the second-order inhibition rate constant due to the substantial stimulations of full-length heparin rate enhancements by the protein but the lack of stimulation of the pentasaccharide rate enhancement. Second-order rate constants for the kininogen-stimulated heparin-accelerated reactions again increased about 3-fold from the smallest to the largest full-length heparin chains, similar to the chain-length dependence of the unstimulated rate constants.

Salt Dependence of H-kininogen-Stimulated and Unstimulated Heparin Rate Enhancements. The salt dependence of heparin rate enhancements was used to further probe the role

Table I: Chain-Length Dependence of H-kininogen-Stimulated and Unstimulated Heparin Accelerations of Antithrombin–Kallikrein Reactions[a]

heparin chain length (saccharides)	k_H (M^{-1} s^{-1})	k'_H (M^{-1} s^{-1})
5	$(1.8 \pm 0.3) \times 10^3$	$(1.8 \pm 0.4) \times 10^3$
26	$(2.2 \pm 0.4) \times 10^3$	$(0.9 \pm 0.2) \times 10^5$ [b]
44	$(2.8 \pm 0.5) \times 10^3$	$(1.9 \pm 0.1) \times 10^5$
56	$(5.2 \pm 1.7) \times 10^3$	$(2.8 \pm 0.3) \times 10^5$
70	$(5.3 \pm 0.4) \times 10^3$	$(2.8 \pm 0.1) \times 10^5$

[a] Second-order rate constants for unstimulated (k_H) and H-kininogen-stimulated (k'_H) reactions of antithrombin–heparin complex with kallikrein (Scheme I) in $I = 0.15$ Hepes buffer, pH 7.4, 25 °C, were determined by dividing observed pseudo-first-order rate constants (k_{obs}) by the antithrombin–heparin complex concentration after correction for the free antithrombin reaction as described under Experimental Procedures. Concentrations of antithrombin–heparin complexes were calculated from measured dissociation constants of 15 nM for full-length heparin interactions and of 40 nM for the pentasaccharide interaction (Olson & Björk, 1991; Olson et al., 1992). Reaction mixtures contained 100 nM antithrombin and 25 or 50 nM heparin with 1 nM kallikrein in the absence or presence of 200 nM H-kininogen. Pentasaccharide reactions were done with 2 μM antithrombin and 0.5 or 1 μM pentasaccharide with and without 200 nM H-kininogen. [b] Values of $(1.7 \pm 0.2) \times 10^5$, $(2.1 \pm 0.5) \times 10^5$, and $(2 \pm 1) \times 10^5$ M^{-1} s^{-1} were obtained in the experiments of Figures 2, 3, and 6 on the basis of larger data sets.

of kallikrein–H-kininogen complex binding to heparin in kininogen stimulation of the heparin-dependent antithrombin–kallikrein reaction. If H-kininogen stimulation is due to heparin bridging antithrombin and kallikrein–H-kininogen complex bound to the same polysaccharide chain, then the association of kallikrein–H-kininogen complex with heparin-bound antithrombin should be strongly dependent on salt concentration due to the salt dependence of kallikrein–H-kininogen complex binding to heparin (Olson & Björk, 1991; Olson et al., 1992). Table II shows the effect of doubling the ionic strength on second-order rate constants for pentasaccharide and full-length (26-saccharide) heparin-accelerated antithrombin–kallikrein reactions in the absence and presence of H-kininogen. These rate constants were measured under conditions where heparin chains were saturated with antithrombin, and where kallikrein was saturated with H-kininogen at the two salt concentrations, based on measured dissociation constants for the binary complex interactions (see above and Experimental Procedures). Such conditions ensured that any observed salt dependence of the rate constants reflected a salt-dependent association between the two binary complexes rather than any salt-dependent dissociation of the binary complexes. In the absence of H-kininogen, second-order rate constants for the association of antithrombin or antithrombin complexes with pentasaccharide and 26-saccharide heparins with kallikrein were independent of doubling the ionic strength from $I = 0.15$ to $I = 0.3$. Likewise, the reactions of antithrombin and antithrombin–pentasaccharide complex with kallikrein complexed with H-kininogen were independent of doubling the ionic strength. Contrasting these results, the reaction between antithrombin–full-length heparin and kallikrein–H-kininogen binary complexes was markedly

Table II: Salt Dependence of Association Rate Constants (M^{-1} s^{-1}) for the Reactions of Antithrombin or Antithrombin–Heparin Complex with Free or H-kininogen-Complexed Kallikrein[a]

	kallikrein		kallikrein–H-kininogen complex	
	$I = 0.15$	$I = 0.30$	$I = 0.15$	$I = 0.30$
AT	$(1.6 \pm 0.5) \times 10^2$	$(1.7 \pm 0.4) \times 10^2$	$(1.6 \pm 0.6) \times 10^2$	$(1.5 \pm 0.1) \times 10^2$
AT–H5	$(1.8 \pm 0.3) \times 10^3$	$(2.2 \pm 0.1) \times 10^3$	$(1.8 \pm 0.4) \times 10^3$	$(1.9 \bullet 0.2) \times 10^3$
AT–H26	$(2.3 \pm 0.5) \times 10^3$	$(1.9 \pm 0.7) \times 10^3$	$(1.9 \pm 0.4) \times 10^5$	$(4.2 \pm 0.4) \times 10^3$

[a] Second-order association rate constants (±2 SE $N = 2$) for antithrombin–kallikrein reactions were determined by dividing observed pseudo-first-order rate constants (k_{obs}) by the antithrombin or antithrombin–heparin complex concentration after correction, in the latter case, for the free antithrombin reaction. k_{obs} was measured for reactions of 2 μM antithrombin with 10–12 nM kallikrein in the absence or presence of either or both 1 μM pentasaccharide (H5) or 26-saccharide (H26) heparins and 0.2 ($I = 0.15$) or 0.5 ($I = 0.3$) μM H-kininogen in pH 7.4 Hepes buffer, 25 °C, at the indicated ionic strengths as described under Experimental Procedures. For the reaction between AT–H26 and kallikrein–H-kininogen binary complexes at $I = 0.15$, the ratio $k_{obs}/[AT–H26]$ was further corrected for saturation of an intermediate enzyme–inhibitor encounter complex by multiplying by the factor $1 + [AT–H26]/K_{E,I}$, where $K_{E,I}$ is the dissociation constant for encounter complex formation given in Table III. Antithrombin–heparin complex concentrations were calculated on the basis of dissociation constants previously determined (Olson et al., 1992).

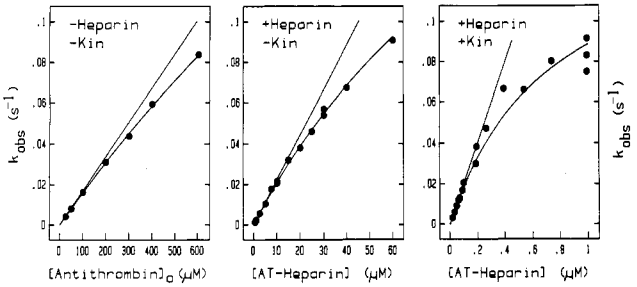


FIGURE 5: Inhibitor concentration dependence of k_{obs} for H-kininogen-stimulated and unstimulated reactions of antithrombin or antithrombin–heparin complex with kallikrein. k_{obs} for reactions of 10 nM kallikrein with varying concentrations of antithrombin or antithrombin–heparin complex was determined in the absence or presence of 200 nM H-kininogen in $I = 0.15$ Hepes buffer, pH 7.4 at 25 °C, from single- or double-exponential fits of kallikrein inactivation curves, as detailed under Experimental Procedures. Antithrombin–heparin complex was formed with a molar excess of antithrombin and the complex concentration calculated on the basis of a measured K_D of 15 nM. Correction of k_{obs} for the excess free antithrombin reaction was made in such cases. Curved lines are fits by eq 8 of the text, and straight lines represent the initial slopes of these fits.

affected by salt, the rate enhancement decreasing from 1200-fold at $I = 0.15$ to 24-fold at $I = 0.3$. This decreased heparin enhancement was not due to the failure to saturate kallikrein with kininogen, since doubling the kininogen concentration had no effect on the lower rate enhancement.[3]

Inhibitor Concentration Dependence of H-kininogen-Stimulated and Unstimulated Reaction Rates. To determine whether antithrombin inhibited kallikrein in a two-step process involving an initial encounter between inhibitor and proteinase followed by the transformation to a stable inhibitor–proteinase complex (Olson & Shore, 1982) and to identify the steps affected by heparin and H-kininogen, the inhibitor concentration dependence of pseudo-first-order rate constants for kallikrein inactivation reactions in the absence and presence of the two effectors was investigated. To minimize any competitive effects of excess free heparin or H-kininogen on the association of antithrombin–heparin complex with free kallikrein or kallikrein–kininogen complex, these experiments were performed under conditions where heparin chains were fully complexed with antithrombin and where the free kininogen concentration was well below the K_D for kininogen binding to heparin. Figure 5 shows that reactions of

[3] This result is consistent with the interpolated K_D of 60 nM for the H-kininogen–prekallikrein interaction at $I = 0.3$ based on the previously reported ionic strength dependence of the interaction together with the observation that proenzyme and enzyme forms of kallikrein bind H-kininogen indistinguishably (Bock et al., 1985).

Table III: Kinetic Parameters Characterizing the Two-Step Reaction between Antithrombin or Antithrombin–Heparin Complex and Free or H-kininogen-Complexed Kallikrein[a]

inhibitor	enzyme	$K_{E,I}$ (μM)	k_I (s^{-1})
AT	Kall	3400 ± 1000	0.6 ± 0.2
AT–H26	Kall	140 ± 30	0.3 ± 0.1
AT–H26	Kall–Kin	0.7 ± 0.2	0.16 ± 0.03

[a] Kinetic parameters ± 2 SE for the two-step reaction of Scheme IV were determined from fits of the data in Figure 5 by eq 8 for reactions where E represents either kallikrein (Kall) or kallikrein–H-kininogen complex (Kall–Kin) and I represents either antithrombin (AT) or antithrombin–26-saccharide heparin complex (AT–H26) as indicated.

antithrombin or antithrombin–heparin complex with kallikrein, in the absence or presence of H-kininogen levels sufficient to fully complex the enzyme, all showed a saturable dependence of k_{obs} values on inhibitor or inhibitor–heparin complex concentrations, as judged from the progressive decline of k_{obs} values from an initial linear inhibitor concentration dependence. Such behavior indicated that the reversible formation of an antithrombin–kallikrein encounter complex precedes the essentially irreversible formation of a more stable complex according to Scheme IV (Olson & Shore, 1982) where E is kallikrein or kallikrein–H-kininogen complex, I is antithrombin or antithrombin–heparin complex, $K_{E,I}$ is the dissociation constant for the encounter complex interaction, and k_I is the first-order rate constant for conversion of the encounter complex to a stable complex. Similar limiting rate constants appeared to be approached for all reactions, suggesting comparable first-order rate constants for conversion of the encounter complex to a stable complex. However, the inhibitor or inhibitor–heparin complex concentration range over which saturation of the encounter complex occurred was very different for each of these reactions. In the absence of heparin and H-kininogen, saturation was observed to occur over inhibitor concentrations from 100 to 600 μM, whereas in the presence of heparin alone, the corresponding range of antithrombin–heparin complex concentrations over which saturation occurred was reduced 10-fold, i.e., from 10 to 60 μM. With both heparin and H-kininogen present, saturation was observable over a concentration range that was reduced a further 100-fold, i.e., from 0.1 to 1 μM. The dependence of k_{obs} on inhibitor concentration for Scheme IV is given by the equation (Olson & Shore, 1982):

$$k_{obs} = \frac{k_I[I]_0}{K_{E,I} + [I]_0} \quad (8)$$

where $[I]_0$ is the total concentration of inhibitor, i.e., antithrombin or antithrombin–heparin complex. Fitting of the data of Figure 5 by the above equation (solid lines) yielded

Scheme IV

$$E + I \underset{}{\overset{K_{E,I}}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_I} E\text{–}I$$

values for the kinetic parameters, $K_{E,I}$ and k_I, given in Table III.[4] The values obtained for these parameters confirmed that the primary effect of both heparin and H-kininogen was to promote the initial encounter between antithrombin and kallikrein, with heparin alone decreasing the $K_{E,I}$ for the initial encounter complex by 20-fold and H-kininogen and heparin together producing a further 200-fold reduction. In contrast, heparin and H-kininogen had relatively small effects on k_I, heparin alone reducing this rate constant by 2-fold and H-kininogen and heparin producing a further 2-fold decrease.

Effect of Zinc Ions on the Potentiating Activity of H-kininogen. Zinc ions at concentrations found in plasma (~10 μM) were previously shown to greatly enhance the binding of H-kininogen to heparin (Björk et al., 1989). Figure 6 shows the effect of 10 μM zinc ions on second-order rate constants for the reaction of antithrombin with kallikrein in the absence and presence of heparin and H-kininogen measured under conditions where antithrombin or antithrombin–heparin complex concentrations were subsaturating with respect to antithrombin–kallikrein encounter complex formation (Table III). Zinc ions decreased 6-fold H-kininogen's stimulating effect on the heparin-accelerated antithrombin–kallikrein reaction at physiological ionic strength without significantly affecting reactions in the absence of kininogen. In contrast zinc ions produced a 4-fold increase in the smaller stimulating effect of H-kininogen on the heparin-dependent reaction at double the ionic strength. Again, zinc ions had no significant effect on the reaction at this ionic strength except when both heparin and H-kininogen were present. In no case was there any evidence of a rate-enhancing effect of zinc ions at $I = 0.15$ when a range of metal ion concentrations was examined (0.1–10 μM) at subsaturating (10 nM) and saturating (100 nM) concentrations of kininogen. The dependence of the zinc-stimulating effect at $I = 0.3$ examined over the same range of metal ion concentrations indicated that the effect was maximal at 1–2 μM zinc ions.

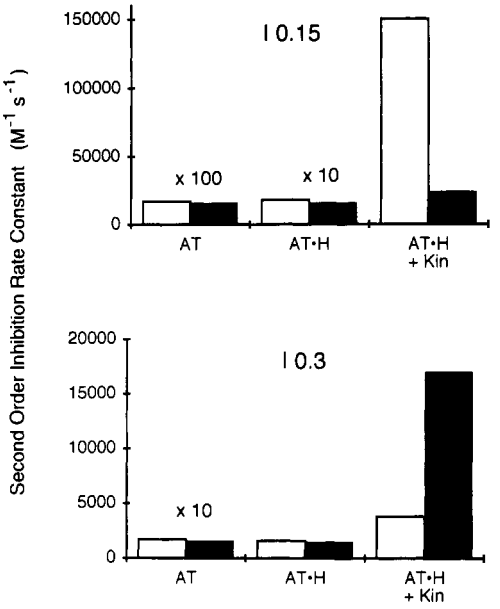


FIGURE 6: Effect of zinc ions on H-kininogen-stimulated and unstimulated heparin–antithrombin–kallikrein reactions. Second-order rate constants were determined as in Table II from pseudo-first-order rate constants for reactions of 200 nM antithrombin with 10 nM kallikrein in the presence of 100 nM heparin and 200 nM H-kininogen as indicated ($I = 0.15$), or of 2 μM antithrombin with 10 nM kallikrein in the presence of 1 μM heparin and 1 μM H-kininogen as indicated ($I = 0.3$). Reactions were conducted either in the absence (open bars) or in the presence (closed bars) of 10 μM zinc chloride in pH 7.4 Hepes buffer at 25 °C.

DISCUSSION

The goal of this study was to elucidate the mechanism by which H-kininogen potentiates the heparin-accelerated inhibition of plasma kallikrein by antithrombin and whether this mechanism was analogous to the previously established mechanism by which H-kininogen promotes the surface-dependent activation of prekallikrein and other proenzymes. H-kininogen promotes prekallikrein activation by facilitating the binding of the proenzyme to a surface through separate proenzyme and surface binding regions on the protein (Cochrane & Griffin, 1982; Colman, 1984). The binding of the proenzyme–kininogen complex to the surface enhances the rate of proenzyme activation by surface-bound factor XIIa due to a bridging or surface approximation effect [see Scheme I of Olson et al. (1993)].

Several observations made in the present study strongly support a parallel mechanism for H-kininogen promotion of the heparin-dependent inactivation of plasma kallikrein. First of all, the light-chain region of H-kininogen responsible for the proenzyme-activating activity of the protein (Kerbiriou & Griffin, 1979; Mori & Nagasawa, 1981) as well as for the binding of heparin (Björk et al., 1989) and kallikrein (Schapira et al., 1982; Bock et al., 1985) is also responsible for the protein promoting the heparin-dependent antithrombin–kallikrein reaction. This finding is consistent with the surface and proenzyme/enzyme binding regions of the light chain mediating both proenzyme-activating and enzyme-inactivating actions of H-kininogen. Second, the potentiating effect of H-kininogen parallels the specific binding of H-kininogen to kallikrein characterized in previous studies (Bock et al., 1985), indicating that the potentiation is due to an enhanced reactivity of the H-kininogen–kallikrein complex as compared to free kallikrein with heparin-bound antithrombin. Third, H-kininogen stimulation exhibits a bell-shaped dependence on heparin concentration, suggesting that the stimulation results from the bridging of antithrombin and kallikrein–H-kininogen complex bound to the same heparin chain in a quaternary complex (Jordan et al., 1979, 1980; Griffith, 1982; Nesheim, 1983; Olson, 1988). Consistent with this conclusion, direct binding measurements confirmed that H-kininogen stimulation paralleled the formation of antithrombin–heparin complexes in the initial ascending phase of the heparin concentration dependence whereas the loss in stimulation in the subsequent descending phase was correlated with the binding of kallikrein–H-kininogen complexes to free rather than antithrombin-bound heparin chains. Fourth, H-kininogen stimulation requires full-length heparin chains which are larger than the antithrombin

[4] Our kinetic analysis does not account for free kininogen competing with kininogen–kallikrein complexes for binding to the antithrombin–heparin complex. Such competition will be minimal when the free kininogen concentration is well below the K_D for kininogen binding to heparin or when the antithrombin–heparin complex concentration is well below the K_D for its association with kallikrein–kininogen complexes, as in the experiments of Figures 2 and 3. Competition will be evident as nonproductive binding when antihrombin–heparin complex levels approach saturation of kallikrein–kininogen complexes as in the experiment of Figure 5. The effect of this competition can be estimated from the ratio of dissociation constants for kininogen–kallikrein complex and free kininogen binding to heparin determined in this study of 0.4 μM/2 μM or 0.2. Such a ratio would indicate that measured k_I and $K_{E,I}$ values are 20% lower than their true values (Olson & Björk, 1991).

binding pentasaccharide region since no kininogen stimulation is observable with the heparin pentasaccharide. This observation is in keeping with the idea that heparin chains have to be large enough to accommodate both antithrombin and the kallikrein–H-kininogen complex in order for heparin to act as a bridge between antithrombin and kallikrein (Laurent et al., 1978; Oosta et al., 1981; Lane et al., 1984; Danielsson et al., 1986; Olson et al., 1992). Fifth, H-kininogen stimulating activity increases as the chain length of full-length heparins increases, as is characteristic of a heparin bridging mechanism (Laurent et al., 1978; Hoylaerts et al., 1984). This observation can be explained by an increased number of nonspecific binding sites on larger heparin chains facilitating the capture of the kallikrein–kininogen complex and the diffusion of this complex on the polysaccharide surface to its target site adjacent to specifically bound antithrombin (Richter & Eigen, 1974; Winter et al., 1981). Sixth, the stimulated association of antithrombin–heparin and kallikrein–H-kininogen binary complexes is strongly dependent on salt, whereas the unstimulated association between antithrombin–heparin complex and free kallikrein is salt-independent. The salt dependence of the association of proteinases with antithrombin–heparin complex measured under conditions where heparin is saturated with the inhibitor was previously shown to provide a diagnostic of the contribution of an ionic proteinase–heparin interaction to heparin rate enhancement (Olson & Björk, 1991; Olson et al., 1992). In the case of the heparin-accelerated antithrombin–thrombin reaction, the strong salt dependence of the heparin rate enhancement was correlated with a parallel salt dependence of thrombin binding to heparin, and the rate enhancement was completely accounted for by the binding energy of the proteinase–heparin interaction (Olson & Björk, 1991). The marked salt dependence of the kininogen-potentiated but not the unpotentiated reaction is thus compatible with this potentiation resulting from the binding of H-kininogen–kallikrein complex to heparin, promoting the interaction of kallikrein with heparin-bound antithrombin. Finally, H-kininogen potentiation results from the protein promoting the initial encounter between antithrombin and kallikrein in an intermediate quaternary complex rather than affecting the rate of conversion of the intermediate encounter complex to a stable complex. The rate constant for the latter step was in fact reduced 2–4-fold from values measured in the absence of kininogen, although the large extrapolations required to determine this rate constant suggest these differences may be due to experimental error. This finding supports a bridging mechanism for kininogen potentiation in which the binding of H-kininogen–kallikrein complex to heparin promotes the encounter of the enzyme with heparin-bound antithrombin (Olson & Shore, 1982; Olson & Björk, 1991). Comparison of K_D values for the binding of free and enzyme-complexed forms of H-kininogen to heparin (0.4–2.0 μM) with the K_D for forming the encounter complex (0.7 μM) indicates that the binding energy of the kininogen–heparin interaction is sufficient to account for kininogen promotion of encounter complex formation. Together, the above observations provide compelling evidence for parallel mechanisms of action of H-kininogen as a cofactor in surface-dependent kallikrein inactivation and prekallikrein activation reactions.

While our findings support a bridging mechanism for the kininogen promotion of the heparin-dependent antithrombin–kallikrein reaction, they argue against a bridging mechanism for the smaller heparin rate enhancement observed in the absence of kininogen. Thus, the comparable rate-enhancing effects of pentasaccharide and full-length heparins on the antithrombin–kallikrein reaction imply that heparin binding to just antithrombin is sufficient to produce the bulk of these rate enhancements (Choay et al., 1981, 1983; Oosta et al., 1981; Lane et al., 1984; Danielsson et al., 1986; Olson et al., 1992), in agreement with a previous study (Holmer et al., 1981). The observation that the unstimulated heparin rate enhancement parallels the formation of antithrombin–heparin binary complexes and is not diminished at higher heparin concentrations further supports the idea that this enhancement is mostly due to antithrombin binding to heparin with no significant contribution of kallikrein binding to the polysaccharide (Jordan et al., 1980). Finally, the salt independence of the rate-enhancing effects of the heparin pentasaccharide and a full-length heparin containing 26 saccharides is in keeping with kallikrein binding to heparin not being involved in these rate enhancements (Olson et al., 1992). Heparin binding to just antithrombin may promote the reaction with kallikrein as a result of the conformational change induced in the inhibitor by the polysaccharide (Olson & Shore, 1981; Olson et al., 1981; Kress & Catanese, 1981; Chang, 1989; Olson et al., 1992). According to our results, this conformational change would act by promoting the initial encounter between antithrombin and kallikrein rather than the conversion to a stable complex. The promotion may occur either through an increased accessibility of the inhibitor reactive bond to the proteinase or through an enhanced complementarity of the reactive bond region of the inhibitor to the proteinase active site (Rosenberg & Damus, 1973; Carrell et al., 1991).

The antithrombin conformational change has similarly been shown to be the predominant mechanism of heparin acceleration of the reaction of antithrombin with factor Xa (Jordan et al., 1980; Choay et al., 1983; Lane et al., 1984; Owen & Owen, 1990; Olson et al., 1992) and possibly with factor XIIa as well (Holmer et al., 1981). In contrast, a bridging mechanism appears to be primarily responsible for heparin acceleration of antithrombin inhibition of thrombin (Laurent et al., 1978; Jordan et al., 1979, 1980; Griffth, 1982; Nesheim, 1983; Lane et al., 1984; Hoylaerts et al., 1984; Olson, 1988; Olson & Björk, 1991; Olson et al., 1992), factor IXa (Jordan et al., 1980; Holmer et al., 1981), and possibly also factor XIa (Holmer et al., 1981). The antithrombin–kallikrein reaction thus provides an interesting example of an antithrombin–proteinase reaction which displays both types of heparin-accelerating mechanisms. A factor Xa-type antithrombin conformational change mechanism thus appears to predominate in the absence of H-kininogen whereas a thrombin-type heparin bridging mechanism becomes an additional more prominent contributor in the presence of kininogen. The present findings thus support our previous conclusion that antithrombin conformational change and bridging mechanisms make additive contributions to heparin's rate-enhancing effect on antithrombin–proteinase reactions (Olson et al., 1992). They further demonstrate that the expression of a bridging mechanism component may require other plasma proteins to mediate the binding of the proteinase to heparin. An intriguing possibility suggested by these findings is that unidentified plasma proteins may exist to promote the binding to heparin of proteinases such as factor Xa, whose inhibition by antithrombin–heparin complex is accelerated mostly by the antithrombin conformational change. Such proteins could result in much greater heparin rate enhancements for antithrombin inhibition of certain proteinases than have been reported in *in vitro* studies.

It is of interest that the ability of H-kininogen to stimulate the heparin-dependent antithrombin–kallikrein reaction is not

significantly affected by kallikrein cleavage of the protein to the two-chain form. Such a result is consistent with our previous observation that both single- and two-chain forms of H-kininogen bind heparin with indistinguishable affinities in the presence of zinc ions (Björk et al., 1989) and implies that these two kininogen forms also have similar affinities for heparin in the absence of metal ions. However, this result contrasts with the reported enhancement of the procoagulant activity of H-kininogen upon kallikrein cleavage to the two-chain species (Scott et al., 1984), a behavior similar to that of other cofactor proteins (Mann et al., 1988). This enhanced cofactor activity was shown to be due to the tighter binding of two-chain H-kininogen to a kaolin surface. Whether kininogen cleavage induces stronger binding to procoagulant surfaces but not to anticoagulant surfaces such as heparin warrants further investigation.

Zinc ions were found to decrease the stimulating effect of H-kininogen on the heparin-accelerated antithrombin–kallikrein reaction at physiological ionic strength, but enhance this stimulating effect at higher ionic strength. The different effects of the metal ion can be explained by our previous observation that zinc ions greatly enhance the binding of H-kininogen to heparin (Björk et al., 1989). This enhanced binding may result in free kininogen competing with antithrombin or the kininogen–kallikrein complex for binding to heparin at the H-kininogen concentrations required to saturate kallikrein, similar to the action of other heparin binding proteins (Lane, 1989). Under such conditions, a balance between antagonizing and stimulating effects of kininogen on the heparin-dependent antithrombin–kallikrein reaction may result. By contrast, the kininogen concentrations required to saturate kallikrein in the absence of zinc ions are much less than the K_D for kininogen binding to heparin, consistent with no significant antagonizing effect of free kininogen under such conditions. That H-kininogen effectively competes with antithrombin for binding to heparin in the presence of zinc ions and at physiological ionic strength is suggested by the observations that (1) H-kininogen and antithrombin are eluted from heparin–agarose in the presence of zinc ions at comparable salt concentrations (~0.7 vs 0.8 M; Björk et al., 1989) and (2) the K_D for antithrombin binding to heparin (10–20 nM; Olson & Björk, 1991; Olson et al., 1992) is similar to the apparent K_I for H-kininogen neutralization of the heparin acceleration of the antithrombin–thrombin reaction (~20 nM; Björk et al., 1989). At higher ionic strength, however, a significant separation in antithrombin and H-kininogen binding affinites for heparin may exist, comparable to that observed in the absence of zinc, due to the substantial nonelectrostatic contribution to the binding energy of the specific antithrombin–heparin interaction but probable electrostatic nature of the H-kininogen–heparin interaction (Olson & Björk, 1991; Olson et al., 1992). Since the promoting effect of H-kininogen dominates under conditions where the affinity of antithrombin for heparin greatly exceeds that of H-kininogen for heparin, kininogen promotion could be expected at higher ionic strength.

Zinc ions did not affect the kininogen-stimulated enhancement by heparin of the rate of kallikrein inactivation in plasma (Olson et al., 1993), most likely because of zinc binding proteins which greatly reduce the free metal ion concentration (Cunningham et al., 1990). However, the release of high levels of zinc ions from activated platelets or neutrophils could result in localized effects of the metal ion at sites of vessel injury (Bernardo et al., 1993). Whether these effects are antagonizing or promoting could depend on competing surfaces and metal ion binding proteins which may mimic the effect of ionic strength.

The dual function of H-kininogen as a cofactor in both prekallikrein activation and kallikrein inactivation is a novel finding with little precedent among other cofactor proteins which function in blood coagulation. Thus, the specific association of cofactor proteins such as factors V and VIII with coagulation proteinases activates a procoagulant action of these proteinases (Mann et al., 1988), but at the same time blocks rather than enhances their inactivation by antithrombin in the absence or presence of heparin (Miletich et al., 1978; Barrowcliffe et al., 1987). However, a recent report indicates that the cofactor protein, tissue factor, similarly can act both to enhance factor VII activation by factor Xa as well as to stimulate factor VIIa inactivation by antithrombin–heparin complex on a membrane surface (Lawson et al., 1993). The ability of H-kininogen to promote both kallikrein formation and kallikrein inactivation suggests that the regulation of these opposing actions cannot be controlled by the cofactor protein itself. One possible means of controlling such alternative actions is through the specificity of the surface component. Thus, surfaces such as activated platelets or endothelial cells have been shown to expose specific cell receptors for H-kininogen that direct a proenzyme activating mode of action (Greengard & Griffin, 1984; van Iwaarden et al., 1988; Schmaier et al., 1988). Similarly, the surfaces, heparin or heparan sulfate, contain a specific pentasaccharide "receptor" for antithrombin that results in an antiproteinase mode of action of the protein (Choay et al., 1981, 1983; Thunberg et al., 1982; Marcum et al., 1986). The appropriate action of H-kininogen may therefore critically depend on the surfaces available for the protein to bind and their temporal presentation during vascular injury. Such control of these opposing kininogen actions may be vital for effective regulation of kallikrein activity.

APPENDIX

Equation 1 of the text is derived from the differential equation for the disappearance of active kallikrein according to Scheme I:

$$\frac{-d[Kall]_0}{dt} = k[AT][Kall] + k[AT][Kall\cdot Kin] + k_H[AT\cdot H][Kall] + k_H'[AT\cdot H][Kall\cdot Kin] \quad (1A)$$

where $[Kall]_0$ represents the sum of free and H-kininogen-complexed forms of active kallikrein

$$[Kall]_0 = [Kall] + [Kall\cdot Kin] \quad (2A)$$

and where rate constants and abbreviations are those defined for Scheme I, except for the addition of the free antithrombin reaction with kallikrein which is governed by the rate constant k for both free and H-kininogen-complexed enzyme. Under the pseudo-first-order conditions, $[AT]_0$, $[H_0] \gg [Kall]_0$, antithrombin and antithrombin–heparin complex concentrations can be considered constant and given by eq 2 and 3 of the text. From the expression for the dissociation constant for the kallikrein–H-kininogen interaction:

$$K_{Kin,Kall} = \frac{[Kall][Kin]}{[Kall\cdot Kin]} \quad (3A)$$

eq 2A can be written

$$[Kall]_0 = [Kall]\left(1 + \frac{[Kin]}{K_{Kin,Kall}}\right) \quad (4A)$$

Under the experimental conditions employed, $[Kin]_0 \gg$

$[Kall]_0$, the free H-kininogen concentration, [Kin], can be equated with the total, i.e., $[Kin]_0$. From eq 2A and 4A, the differential eq 1A can then be written:

$$\frac{-d[Kall]_0}{dt} = k[AT][Kall]_0 + k_H[AT\cdot H]$$

$$[Kall]_0\frac{K_{Kin,Kall}}{K_{Kin,Kall} + [Kin]_0} + k_H'[AT\cdot H]$$

$$[Kall]_0\frac{[Kin]_0}{K_{Kin,Kall} + [Kin]_0} = k_{obs}[Kall]_0 \quad (5A)$$

where k_{obs} is given by eq 1 of the text. Equation 5A indicates that free and H-kininogen-complexed α-kallikrein will be inhibited by a pseudo-first-order decay process with an observed rate constant given by eq 1 of the text.

Equation 5 of the text is derived from the differential equation for the disappearance of kallikrein–H-kininogen complex according to Scheme III:

$$\frac{-d[Kall\cdot Kin]_0}{dt} = k[AT][Kall\cdot Kin] + k[AT][Kall\cdot Kin\cdot H] +$$
$$k_H'[AT\cdot H][Kall\cdot Kin] + k_H''[AT\cdot H][Kall\cdot Kin\cdot H] \quad (6A)$$

where $[Kall\cdot Kin]_0$ represents the sum of the concentrations of kallikrein–H-kininogen complex bound and not bound to heparin

$$[Kall\cdot Kin]_0 = [Kall\cdot Kin] + [Kall\cdot Kin\cdot H] \quad (7A)$$

and where rate constants and reacting species are as defined in Scheme III. If it is assumed that kallikrein binding to H-kininogen has no effect on H-kininogen binding to heparin, then it follows that

$$[Kall\cdot Kin] = \frac{[Kin]}{[Kin]_0}[Kall\cdot Kin]_0 \quad (8A)$$

$$[Kall\cdot Kin\cdot H] = \frac{[Kin\cdot H]}{[Kin]_0}[Kall\cdot Kin]_0 \quad (9A)$$

Under the conditions of the experiments, $[AT]_0$, $[H]_0$, $[Kin]_0 \gg [Kall\cdot Kin]_0$, the concentrations of free and heparin-bound antithrombin and free and heparin-bound H-kininogen are given by eq 2, 3, 6, and 7 of the text and can be considered constant during the reaction. These equations assume that the binding of antithrombin to heparin does not affect H-kininogen binding to the polysaccharide and vice versa. This assumption is reasonable given (1) the large separation in K_Ds for antithrombin and kininogen binding to heparin which favors the binding of these proteins to separate heparin chains under the conditions employed, (2) the specific binding of antithrombin and presumably nonspecific binding of kininogen to different sites on heparin, and (3) conditions resulting in low kininogen binding densities on heparin. From eq 7A, 8A, and 9A, differential eq 6A can be written:

$$\frac{-d[Kall\cdot Kin]_0}{dt} = k[AT][Kall\cdot Kin]_0 + k_H'[AT\cdot H]\frac{[Kin]}{[Kin]_0}$$

$$[Kall\cdot Kin]_0 + k_H''[AT\cdot H]\frac{[Kin\cdot H]}{[Kin]_0}[Kall\cdot Kin]_0 = k_{obs}$$

$$[Kall\cdot Kin]_0 \quad (10A)$$

where k_{obs} is given by eq 5. Equation 10A indicates that kallikrein activity will decay in a first-order kinetic process with an observed pseudo-first-order rate constant given by eq 5.

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