High Molecular Weight Kininogen Potentiates the Heparin-Accelerated Inhibition of Plasma Kallikrein by Antithrombin: Role for Antithrombin in the Regulation of Kallikrein†

Steven T. Olson,* Roberta Sheffer, and Ann Marie Francis

Division of Biochemical Research, Henry Ford Hospital, Detroit, Michigan 48202

Received March 29, 1993; Revised Manuscript Received June 25, 1993®

ABSTRACT: The effects of previously characterized interactions of high molecular weight kininogen (H-kininogen) with plasma kallikrein and with heparin on the regulation of kallikrein by the heparin-activated inhibitor, antithrombin, were investigated. H-kininogen, at levels sufficient to fully complex kallikrein, greatly potentiated the acceleration of antithrombin inhibition of kallikrein produced by heparin with high affinity for antithrombin. At $I = 0.15$, pH 7.4, 25 °C, kininogen thus maximally increased the heparin enhancement of the second-order rate constant for the antithrombin–kallikrein reaction from 13-fold (1.6×10^2 M^{-1} s^{-1} to 2.1×10^3 M^{-1} s^{-1}) to 1200-fold (1.9×10^5 M^{-1} s^{-1}). In contrast, H-kininogen had no effect on the antithrombin–kallikrein reaction in the absence of heparin, nor did the protein enhance the rate constants of 1.7×10^4 and 3.4×10^4 M^{-1} s^{-1} for kallikrein reactions with its primary plasma inhibitors C1-inhibitor and α_2-macroglobulin, respectively, in the absence or presence of heparin. Consistent with these results, SDS gel electrophoresis of the ^{125}I-labeled kallikrein–inhibitor complexes formed in a mixture of these kallikrein inhibitors at their relative plasma concentrations indicated that antithrombin effectively competed with C1-inhibitor and α_2-macroglobulin for kallikrein, accounting for 54% of the total kallikrein complexes, only when both heparin and H-kininogen were present. Similarly, the presence of therapeutic levels of heparin (~1 unit/mL) in normal, factor XII-deficient, and prekallikrein-deficient plasmas enhanced the rate of inactivation of added kallikrein by 2.3-fold and significantly altered the partitioning of radiolabeled kallikrein from predominantly C1-inhibitor and α_2-macroglobulin complexes (86–92%) to mostly antithrombin complexes (50–53%). Experiments in antithrombin-deficient and H-kininogen-deficient plasmas confirmed that the enhanced kallikrein inactivation rate and predominant formation of antithrombin–kallikrein complexes in heparinized plasma were dependent on antithrombin and H-kininogen. The contribution of antithrombin to kallikrein inhibition in plasma remained significant (~40–70%) at optimal concentrations of unfractionated or size- and antithrombin affinity-fractionated heparin, in the presence of plasma levels of calcium and zinc ions, at 37 °C, and with minimal plasma dilution. These results suggest that antithrombin and H-kininogen may play important roles in the regulation of kallikrein activity in the presence of heparin or heparin-like glycosaminoglycans.

High molecular weight kininogen (H-kininogen)[1] is a nonenzymatic plasma glycoprotein with multiple functions. In addition to being a precursor of the vasoactive nonapeptide bradykinin (Kato et al., 1981), the protein acts as an inhibitor of cysteine proteinases (Ohkubo et al., 1984; Müller-Esterl et al., 1985; Sueyoshi et al., 1985), as an anti-cell-adhesion agent (Asakura et al., 1992), as an antagonist of thrombin-induced platelet aggregation (Meloni & Schmaier, 1991), and as a cofactor in the surface-dependent activation of the proenzymes prekallikrein, factor XI, and factor XII, which participate in blood coagulation, fibrinolysis, and complement activation pathways (Griffin & Cochrane, 1976a; Kato et al., 1981). The anti-cell-adhesion and proenzyme activation functions of the protein are contained in its carboxy-terminal light chain produced by kallikrein excision of bradykinin from the single-chain protein (Kerbiriou & Griffin, 1979; Mori & Nagasawa, 1981; Asakura et al., 1992). Two regions of this light chain appear to mediate the action as a cofactor in proenzyme activation: (1) a histidine-rich region which is involved in the binding to a negatively charged activating surface (Han et al., 1975; Sugo et al., 1980; Ikari et al., 1981; Kellerman et al., 1986; Retzios et al., 1987); and (2) a region which specifically interacts with prekallikrein, factor XI, and the activated forms of these proenzymes (Kerbiriou et al., 1980; Schapira et al., 1982b; Scott et al., 1982; Bouma et al., 1983; Bock and Shore, 1983; van der Graaf et al., 1984; Bock et al., 1985; Shimada et al., 1985; Tait & Fujikawa, 1986, 1987). The specificity and high affinity of the latter interactions result in the proenzymes circulating in plasma as tight complexes with H-kininogen (Mandle et al., 1976; Thompson et al., 1977). On the basis of these two types of interactions, H-kininogen is thought to function as a cofactor by promoting the binding of the tightly-associated proenzymes to a negatively charged surface via the surface binding region of the cofactor, thereby allowing rapid activation of the proenzymes by surface-bound factor XIIa to occur (Cochrane & Griffin, 1982; Colman, 1984). In a similar fashion, kininogen is believed to promote kallikrein activation of factor XII on a surface by facilitating

† Supported by an Established Investigatorship of the American Heart Association and NIH Grants HL-25670 and HL-39888.

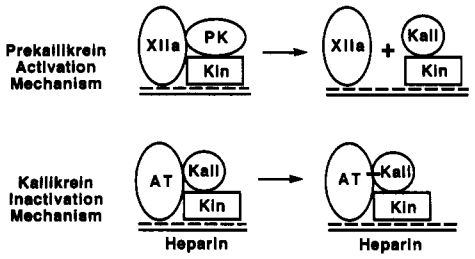
* Correspondence should be addressed to this author at the Division of Biochemical Research, Education and Research Building, Room 3126, Henry Ford Hospital, 2799 W. Grand Blvd., Detroit, MI 48202. Telephone: 313-876-3196. FAX: 313-876-2380.

® Abstract published in *Advance ACS Abstracts,* November 1, 1993.

[1] Abbreviations: SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, poly(ethylene glycol); BSA, bovine serum albumin; FFRCK, D-Phe-L-Phe-L-Arg-chloromethyl ketone; Hep, heparin; H-kininogen or Kin, high molecular weight kininogen; C1, C1-inhibitor; AT, antithrombin; α_2M, α_2-macroglobulin; Kall or K, kallikrein; PK, prekallikrein.

0006-2960/93/0432-12136$04.00/0

Scheme I



kallikrein binding to the surface.

In addition to the functions of H-kininogen enumerated above, we and our colleagues recently showed that H-kininogen binds with high affinity to the glycosaminoglycan heparin through the histidine-rich surface binding region involved in the binding to proenzyme-activating surfaces (Björk et al., 1989). Such binding was predicted on the basis of the homology between H-kininogen and a well-established heparin binding protein in plasma, histidine-rich glycoprotein, in a common histidine-rich region of these proteins (Lijnen et al., 1983; Koide et al., 1986). The binding of either protein to heparin is promoted by divalent metal ions such as zinc and copper (Lijnen et al., 1983; Björk et al., 1989). This binding antagonizes the anticoagulant action of heparin by interfering with its ability to accelerate the inactivation of certain blood coagulation proteinases by their primary protein inhibitor antithrombin (Lijnen et al., 1983; Björk et al., 1989). The interference results from the binding of such proteins to heparin, blocking the binding of the inhibitor, the proteinase, or both to the surface of the polysaccharide chain (Peterson et al., 1987).

The binding of H-kininogen to the proteinases kallikrein and factor XIa as well as to heparin suggested that H-kininogen might have a promoting rather than antagonizing effect on the heparin-accelerated inactivation of kallikrein and factor XIa by antithrombin. Thus, by analogy to the action of the protein as a cofactor in proenzyme activation, we hypothesized that H-kininogen might similarly act as a cofactor to promote proteinase inactivation (Scheme I). In this antiproteinase mode of action, we envisioned that specific complexes of H-kininogen with kallikrein or factor XIa could promote the binding of the proteinases to the negatively charged heparin surface and thereby enhance their inactivation by surface-bound antithrombin. Such a hypothesis would explain why heparin only modestly accelerates the reactions of antithrombin with kallikrein and factor XIa in the absence of the cofactor protein (Colman et al., 1989).

In the present study, we confirm this hypothesis by demonstrating that H-kininogen greatly potentiates heparin's accelerating effect on the inactivation of plasma kallikrein by antithrombin. This effect is shown to be specific for the reaction of antithrombin with kallikrein, as no comparable effects are seen on the reactions of kallikrein with its primary plasma protein inhibitors, C1-inhibitor and α_2-macroglobulin. Consistent with the predictions of these findings, antithrombin is shown to be a primary inhibitor of kallikrein in plasma containing therapeutic levels of heparin, and this effect is demonstrated to be dependent on the presence of H-kininogen.

EXPERIMENTAL PROCEDURES

Proteins. Kinin-free two-chain H-kininogen was purified from outdated plasma as a byproduct of the purification of antithrombin (see below). H-kininogen was eluted in the heparin–Sepharose chromatography step at ~0.25 M NaCl (Björk et al., 1989) and further processed as described for the purification of H-kininogen from plasma (Kerbiriou & Griffin, 1979; Bock & Shore, 1983). SDS gel electrophoresis of the resulting purified protein confirmed that it was the kinin-free 2-chain form of the protein containing predominantly the terminally cleaved light chain missing the first 48 residues of the amino terminus (Nakayasu & Nagasawa, 1979; Mori & Nagasawa, 1981; Bock & Shore, 1983; Tait & Fujikawa, 1986; Retzios et al., 1987). The protein was treated with D-phenylalanyl-L-phenylalanyl-L-arginine-chloromethyl ketone (FFRCK) to remove any contaminating kallikrein and then dialyzed against 4 mM sodium acetate/0.15 M NaCl, pH 5.3 (storage buffer), as detailed previously (Björk et al., 1989).

Prekallikrein was purified according to Bock and Shore (1983) and activated with 1% α-factor XIIa prepared from purified factor XII by autoactivation (Bock et al., 1985; Shore et al., 1987). Following activation of the zymogen, monitored from the appearance of amidolytic activity toward the substrate D-prolyl-L-phenylalanyl-L-arginyl-*p*-nitroanilide (S-2302, Kabi), the reaction was quenched with an equal volume of 0.2 M sodium acetate, 1 mM benzamidine, and 1 mM EDTA, pH 5.3 at 4 °C. α-Kallikrein (≥80% α-form) was then isolated by soybean trypsin inhibitor–agarose affinity chromatography, essentially as described for the isolation of factor Xa (Bock et al., 1989), and dialyzed against storage buffer. Control experiments demonstrated that factor XIIa as well as prekallikrein eluted from the affinity column under conditions where kallikrein was bound. The concentration of active kallikrein was determined by active-site titration in kinetics buffer (see below) with fluorescein mono-*p*-guanidinobenzoate (Melhado et al., 1982; Bock et al., 1989). The observation of indistinguishable fluorescein burst amplitudes over the range of titrant concentrations from 5 to 15 μM and a proportional dependence of such amplitudes on the enzyme concentration indicated that the burst amplitude could be equated with the active enzyme concentration (Bock et al., 1989). On the basis of the concentration measured from the absorbance at 280 nm together with the absorption coefficient of 1.17 L g^{-1} cm^{-1} (Bock & Shore, 1983) and molecular weight of 82 000 (Heimark & Davie, 1981), the enzyme from two preparations ranged from 75% to 85% active.

Antithrombin, C1-inhibitor, and α_2-macroglobulin were simultaneously purified from outdated plasma. The plasma was first fractionated with PEG 4000 to obtain fractions precipitating between 4 and 14% which contained α_2-macroglobulin (Barrett et al., 1979), and between 14 and 35% which contained antithrombin and C1-inhibitor (Thaler & Schmer, 1975; Salvesen et al., 1985). α_2-Macroglobulin was subsequently purified from the 4–14% PEG fraction by chromatography on zinc-chelating Sepharose 6B (Kurecki et al., 1979), Blue Sepharose, and Sephacryl S-300 (Virca et al., 1978). Antithrombin and C1-inhibitor were purified from the 14–35% PEG fraction. The antithrombin purification followed the method of Thaler and Schmer (1975) with modifications (Olson, 1988) and involved chromatography on heparin–Sepharose, DEAE-Sepharose, and Sephacryl S-200. The unbound protein fraction from the initial heparin–Sepharose chromatography step contained C1-inhibitor activity (assayed as anti-kallikrein activity with the use of the substrate S-2302). C1-inhibitor was further purified from this fraction by DEAE-Sepharose, Blue Sepharose, ConA–Sepharose and finally Sephacryl S-200 essentially as published (Reboul et al., 1977; Salvesen et al., 1985).

SDS–polyacrylamide gel electrophoresis according to Laemmli (1970) indicated that all purified proteins appeared as a single major band under nonreducing conditions [except for kallikrein which appeared as a doublet due to variant forms of the light chain (Bock et al., 1985)]. Under reducing conditions, kallikrein and H-kininogen each yielded two major bands corresponding to the disulfide-linked heavy and light chains of these proteins, whereas the three inhibitors still appeared as single bands, although, in the case of α_2-macroglobulin, with a reduced molecular weight characteristic of the monomeric subunit (Barrett et al., 1979). The isolated α_2-macroglobulin inhibited 1.7–1.9 mol of active-site-titrated β-trypsin/mol of inhibitor when analyzed by the method of Ganrot (1966). Concentrations of inhibitors and H-kininogen were determined from the 280-nm absorbance with the use of absorption coefficients (L g^{-1} cm^{-1}) and molecular weights of 0.65 and 58 000 for antithrombin (Nordenman et al., 1977), 3.6 and 100 000 for C1-inhibitor (Harrison, 1983), 0.90 and 725 000 for α_2-macroglobulin (Dunn & Spiro, 1967; Hall & Roberts, 1978), and 0.701 and 108 000 for two-chain H-kininogen (Kerbiriou & Griffin, 1979; Nakayasu & Nagasawa, 1979), respectively.

Glycosaminoglycans. Heparin (sodium salt) with a narrow molecular weight distribution (M_r ~7900) and containing a single high-affinity binding site for antithrombin was prepared from commercial heparin (Diosynth) by gel filtration and antithrombin–agarose affinity chromatography (Olson, 1988). Heparin concentrations were determined by titrations of antithrombin with heparin monitored by the enhancement of tryptophan fluorescence which accompanies the binding of the polysaccharide to the inhibitor (Olson & Shore, 1981). Unfractionated heparin (sodium salt, grade II from porcine intestinal mucosa) with an anticoagulant activity of 159–169 USP units/mg was obtained from Sigma. Molar concentrations were estimated from the dry weight assuming an average molecular weight of 15 000 (Laurent et al., 1979). The unfractionated heparin was used as a standard to determine the specific activity of the fractionated heparin based on the increase in clotting time produced by the polysaccharide in an activated partial-thromboplastin time coagulation assay (Barrowcliffe et al., 1989). Dermatan sulfate (sodium salt, Miles Scientific) was treated with nitrous acid to remove any contaminating heparin, as described (Tollefsen et al., 1983). The resulting glycosaminoglycan failed to accelerate the inactivation of 20 nM thrombin by 200 nM antithrombin when tested up to 4 μg/mL, confirming the absence of heparin activity in the preparation.

Plasmas. Normal plasma and plasmas deficient in factor XII, prekallikrein, or both L- and H-forms of kininogen were obtained from George-King. Plasma specifically depleted of antithrombin was prepared by adsorption with heparin–Sepharose (LKB—Pharmacia Biotechnology) following the procedure of Hoogendoorn et al. (1980), with modifications. Briefly, normal plasma (2 mL) was dialyzed against 100 volumes of 0.1 M Hepes/0.4 M NaCl, pH 7.4, buffer for 4 h at room temperature, applied to a 1-mL heparin–Sepharose column equilibrated with dialysis buffer, and then eluted with the same buffer. Fractions (0.2 mL) containing plasma were identified from the 280-nm absorbance of a 200-fold-diluted sample. The central portion of the plasma peak (~1.2 mL) was pooled (<10% dilution) and dialyzed against 100 volumes of 0.1 M Hepes/0.1 M NaCl, pH 7.4, buffer (I = 0.15) overnight at room temperature. Removal of antithrombin (>99%) from the plasma was confirmed from the absence of detectable factor Xa inhibition (≤5%) when this plasma (up to 50 μL) was incubated with 5 nM factor Xa and 50 nM fractionated heparin in I = 0.15 Hepes buffer in a total volume of 100 μL for 1 min at 25 °C. Control incubations with 0.5–2.5 μL of normal plasma showed a heparin-dependent inhibition of up to 80% factor Xa activity. Assays for the heparin binding plasma proteins H-kininogen [by the ability of the plasma to correct the clotting time of H-kininogen-deficient plasma (Griffin & Cochrane, 1976b)] and heparin cofactor II [using a dermatan sulfate-dependent antithrombin assay (Tollefsen et al., 1983)] indicated >80% normal H-kininogen and heparin cofactor II activity. Such assays were consistent with the selective removal of antithrombin from the plasma as more rigorously shown in previous studies (Hoogendoorn et al., 1980). Although not tested, protein C inhibitor is also unlikely to have been substantially removed from the deficient plasma by the high salt conditions employed for removing antithrombin, based on its lower affinity for heparin–agarose (Pratt et al., 1992).

Kinetic Studies. The rate of inactivation of kallikrein by purified protein inhibitors or in plasma was monitored under pseudo-first-order conditions (i.e., at least a 10-fold molar excess of inhibitor over enzyme) by assaying residual enzymatic activity after varying reaction times in the absence or presence of effectors. Briefly, the inhibitor along with heparin and/or H-kininogen, when present, was added in 0.1 M Hepes, 0.1 M NaCl, 1 mM EDTA, and 0.1% PEG 8000, pH 7.4 (kinetics buffer), in a total volume of 90 μL to a series of polystyrene semi-microcuvettes coated with PEG 20 000 to minimize protein adsorption (Latallo et al., 1986). After preincubation at 25 °C, 10 μL of a prewarmed kallikrein solution in storage buffer made up in a PEG-coated polypropylene tube was added to initiate the reaction. Reactions were then quenched after varying incubation times with 0.9 mL of 200 μM S-2302 containing 100 μg/mL Polybrene to neutralize heparin when present. The residual enzyme activity was determined from the initial linear rate of substrate hydrolysis at 405 nm (<5% substrate consumption). Activities were expressed relative to control samples incubated without inhibitor. Quenching of reactions was confirmed from the absence of inactivation when substrate was added to the inhibitor-containing sample before the enzyme. Inactivation experiments conducted in plasma were similarly performed except that PEG-coating of the cuvettes was unnecessary and no EDTA was present in the reaction buffer.

Kinetic data obtained for kallikrein reactions with antithrombin and C1-inhibitor were well described by first-order single-exponential reactions for at least 3 half-lives (>90% inactivation) with the exception of the antithrombin reaction in the presence of both heparin and H-kininogen. Single-exponential reactions were analyzed graphically by a semi-logarithmic plot or by nonlinear least-squares fitting. Reactions of kallikrein with antithrombin in the presence of both heparin and H-kininogen or with α_2-macroglobulin exhibited biexponential decays which were computer-fit by the equation:

$$A_t = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + A_3$$

where A_t is the kallikrein activity, i.e., the initial rate of substrate hydrolysis, at time t, A_1 is the activity associated with the first exponential process having a rate constant k_1, A_2 is the activity associated with the second exponential process having a rate constant k_2, and A_3 is the limiting activity after the reaction is complete. The latter parameter is zero for the antithrombin reaction, but is nonzero for the α_2-macroglobulin reaction due to the ability of enzyme bound to this inhibitor

to still hydrolyze the small chromogenic substrate, albeit at a reduced rate (Schapira et al., 1982b; van der Graaf et al., 1984).

Kallikrein inactivation in normal and deficient plasmas was described in most cases by a single-exponential process in the absence or presence of heparin over the extent of the reaction measured (~90%) (see Results). To account for the α_2-macroglobulin contribution to the inhibition, these reactions were fit by a single-exponential function with a finite end point, i.e., with A_2 and k_2 in the above equation set to zero. Typically, the rate of kallikrein inactivation was monitored for 3–4 half-lives, and an end point was then measured after 8–10 half-lives. In those cases where a slower phase was detectable, fitting by a two-exponential function indicated that the rate constant for the predominant kinetic phase was underestimated typically by less than 10% and at most 20% by the fitting of data by a single-exponential function.

Stoichiometry of the Antithrombin–Kallikrein Reaction. Kallikrein was reacted with 0–1.4 molar equiv of antithrombin in kinetics buffer (40-μL total volume) in PEG-coated cuvettes at 25 °C. Reactions were conducted with 240 nM enzyme for 30 h in the absence of effectors or with 50 nM enzyme for 24 h in the presence of 100 nM heparin alone or 100 nM heparin plus 200 nM H-kininogen. The residual kallikrein activity was then measured by the chromogenic substrate assay described above. Reactions were judged to be ≥90% complete on the basis of measured second-order rate constants.

Radiolabeling of Kallikrein. Kallikrein was labeled with Na^{125}I (Amersham) using the Bolton–Hunter reagent and Iodobeads (Pierce) according to the manufacturer's directions. After separation of the labeled protein from excess reagents by Sephadex G-25 chromatography in storage buffer plus 1 mg/mL BSA, it was applied to a 6-mL soybean trypsin inhibitor–agarose column equilibrated in the same buffer containing 1 M NaCl, 1 mM EDTA, and 1 mM benzamidine. The column was washed with 10 column volumes of this high-salt buffer followed by 5 column volumes of low-salt buffer (0.15 M NaCl) which eluted a nonbinding radioactive peak containing labeled BSA and presumably inactive enzyme. Active kallikrein was then eluted with the low-salt buffer containing 0.5 M benzamidine. Radioactive fractions were pooled and concentrated followed by dialysis against storage buffer and freezing at −70 °C. On the basis of the rate of chromogenic substrate hydrolysis produced by the labeled enzyme together with the K_M and k_{cat} determined for kallikrein acting on the substrate, a specific radioactivity of 2.6 μCi/μg was calculated for the preparation (equivalent to ~0.1 mol of ^{125}I/mol of kallikrein), and 30% total recovery of the starting enzyme activity was obtained.

SDS Gel Electrophoresis and Autoradiography. ^{125}I-Labeled kallikrein (~2 nM) was incubated with purified inhibitors or plasma in the absence or presence of effectors in kinetics buffer at 25 °C for 15 min, a time shown to produce complete inactivation of enzyme activity. Samples were made 1% in SDS in the absence of reducing agent and boiled for 2 min. Electrophoresis on 5% polyacrylamide gels was then performed according to Laemmli (1970). Gels were fixed overnight in 10% 2-propanol/10% acetic acid, dried onto filter paper, and exposed to X-ray film (Kodak) with an intensifying screen for periods of up to 24 h. Band intensities were quantified in underexposed autoradiograms by integration with an LKB laser densitometer. Band intensities were confirmed to lie within a range where the intensity was linearly dependent on concentration by calibration with enzyme–inhibitor complexes made from purified proteins. A small fraction of the labeled kallikrein (12–15%) did not appear to complex with inhbitors, possibly due to dissociation of enzyme–inhibitor complexes during electrophoresis or to residual inactive labeled enzyme.

Conversion of α-Kallikrein to β-Kallikrein. Kallikrein in storage buffer was added to an equal volume of 0.2 M Hepes, 0.1 M NaCl, and 0.2% PEG 8000, pH 7.4, to jump the pH. A sample of the enzyme was withdrawn and quick-frozen in dry ice/ethanol as a control. The remainder was incubated at 25 °C, and samples were withdrawn after 4-, 8-, and 24-h incubation and similarly quick-frozen and stored at −70 °C. These samples were then examined by SDS gel electrophoresis on a 10% gel under reducing and nonreducing conditions. Reducing gels showed a nearly complete (94%) loss of the M_r 51 000 heavy chain over the 24-h reaction time with the concomitant appearance of two new bands with lower apparent molecular weights of ~19 000 and 32 000, characteristic of the β-kallikrein heavy-chain cleavage products (Colman et al., 1985). Nonreduced samples showed no change in mobility, consistent with the expected heavy-chain cleavage within a disulfide bond. The reaction was quantitated by integrating the intensities of the heavy- and light-chain bands in the reduced samples. The summed intensities of the heavy-chain cleavage products of β-kallikrein relative to those of all heavy-chain bands was assumed to reflect the fractional conversion to β-kallikrein, based on the invariance of the latter sum over the reaction (<12% decrease). Minimal losses in kallikrein enzymatic activity (5–15%) were detected over the 24-h period required to complete the reaction.

Effect of Kallikrein on H-kininogen Procoagulant Activity. Kallikrein (20 nM) was incubated with 1 μM two-chain H-kininogen in kinetics buffer at 25 °C. After varying incubation times up to 1 h, the enzyme was inactivated by adding 50 nM FFRCK and the sample incubated for a further 15 min. The clotting time of a 50-fold dilution of the sample was then determined. Control samples in which FFRCK was added to the kallikrein before adding the H-kininogen or which contained H-kininogen alone gave indistinguishable baseline activities, indicating that FFRCK had no effect on the clotting activity at the levels employed.

RESULTS

Effects of H-kininogen and Heparin on the Antithrombin–Kallikrein Reaction. Figure 1 shows the effects of kinin-free two-chain H-kininogen and an M_r 7900 heparin with high affinity for antithrombin on the rate of inactivation of α-kallikrein by antithrombin under pseudo-first-order conditions, i.e., with a large molar excess of inhibitor over enzyme. Heparin alone accelerated the kallikrein inactivation rate, but only to a relatively modest extent, in agreement with previous reports (Colman et al., 1989). H-kininogen greatly potentiated the acceleration of kallikrein inactivation in the presence of heparin, whereas the protein had no effect on the reaction rate when the polysaccharide was absent from the reaction. The potentiating effect of H-kininogen was maximal at concentrations between 200 and 400 nM, which were below the plasma concentration of ~700 nM. Such concentrations were in a range expected to saturate the specific interaction of kininogen with kallikrein, given the K_D of ~15 nM previously measured for this interaction under the conditions of these experiments (Bock et al., 1985).

In contrast to the single-exponential decay of kallikrein activity observed when just heparin or H-kininogen alone or neither effector was present, the potentiated reaction in the presence of both effectors was biexponential with ~80% of

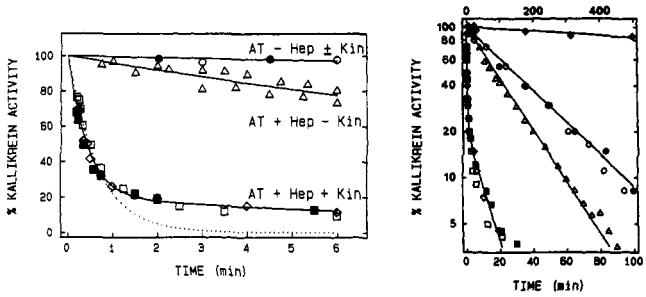


FIGURE 1: Effect of heparin and H-kininogen on the antithrombin–kallikrein reaction. Reactions of 0.55 μM antithrombin with 4 nM (◊) or 20 nM (all other symbols) α-kallikrein in the absence of added components (○) or in the presence of 0.4 μM H-kininogen alone (●), 0.28 μM heparin alone (△), or both 0.28 μM heparin and H-kininogen at 0.2 (□, ◊) or 0.4 μM (■). Reactions were in I = 0.15, pH 7.4 Hepes buffer at 25 °C and monitored from the loss in kallikrein enzymatic activity as detailed under Experimental Procedures. (♦) denotes a control incubation of enzyme in the absence of inhibitor. Solid lines represent fits by a single-exponential function except for reactions containing both heparin and H-kininogen which were fit by a double-exponential. The dotted line shows a single-exponential fit of the latter reaction for comparison. The left panel highlights the progress curve for the kininogen-potentiated reaction while the right panel shows full progress curves for all reactions as semi-log plots with the upper time scale referring to reactions in the absence of heparin and the lower time scale to reactions in the presence of heparin.

the activity decaying much faster than the remaining ~20% (right panel of Figure 1). The relative amplitudes of these two phases appeared to be independent of the antithrombin and heparin concentrations when H-kininogen was saturating, although the rates of the two phases were dependent on the concentrations of these species. This biphasic behavior did not result from proteolytic inactivation of kininogen by kallikrein during the reaction since the procoagulant activity of H-kininogen, measured in a clotting assay, was not decreased, nor was any degradation of the protein detected by SDS gel electrophoresis, when 1 μM H-kininogen was incubated with kallikrein (20 nM) for up to 1 h under the conditions employed in the experiment of Figure 1. Moreover, lowering the enzyme concentration by 5-fold, which should have decreased the rate of any such proteolytic inactivation, had no effect on the biphasic kallikrein inactivation curves (Figure 1). The possibility that the kininogen potentiation was diminished by the product antithrombin–kallikrein complex generated during the reaction was also ruled out by the observation of indistinguishable biphasic inactivation curves when reactions were conducted in the absence or presence of reaction product at levels equivalent to that of the enzyme. Finally, the possibility that H-kininogen and heparin were enhancing a substrate reaction of antithrombin with kallikrein that was depleting the levels of inhibitor (Björk & Fish, 1982; Olson, 1985) was also eliminated from the observation that the measured stoichiometry of kallikrein inactivation by antithrombin of 1.3 ± 0.1 mol of antithrombin/mol of kallikrein was unaffected by heparin or H-kininogen added alone or together at levels sufficient to saturate the inhibitor and enzyme, respectively.

Examination of the kallikrein preparation by SDS gel electrophoresis showed evidence of sufficient β-kallikrein in the preparation (~13%) to account for the amplitude of the observed slow phase in the kininogen-potentiated reaction. β-Kallikrein is produced from α-kallikrein by autolytic cleavage in the heavy chain which contains the kininogen binding region, without affecting the light chain which contains the enzyme active site (Colman et al., 1985; Bock et al., 1985). This cleavage significantly reduces the kininogen binding affinity of the enzyme (Colman et al., 1985). β-Kallikrein produced by autolytic cleavage of the predominantly α-kallikrein preparation was inactivated by antithrombin at the same rate as the parent preparation in the absence or presence of heparin alone, indicating equivalent rates of reaction of the two forms of kallikrein with the free or heparin-bound inhibitor. However, H-kininogen only weakly potentiated the heparin-enhanced reaction of the inhibitor with β-kallikrein to an extent similar to that of the slow phase of the parent kallikrein reaction. These results thus suggested that the biexponential inactivation of kallikrein by antithrombin in the presence of heparin and H-kininogen was due to two forms of the enzyme which bind kininogen with differing affinities and/or which differentially promote the interaction of the enzyme with antithrombin bound to heparin.

Pseudo-first-order rate constants for the reactions of antithrombin or antithrombin–heparin complex with α-kallikrein in the absence of H-kininogen were proportional to the inhibitor or inhibitor–polysaccharide complex concentrations, respectively, over a range encompassing the concentrations employed in Figure 1, consistent with these being simple biomolecular reactions characterized by the second-order rate constants shown in Table I. In contrast, pseudo-first-order rate constants for the predominant fast phase of the kininogen-potentiated reaction showed evidence of saturation at the levels of antithrombin–heparin complex utilized in the experiment of Figure 1, but exhibited a similar proportional dependence at lower concentrations from which a second-order rate constant could be determined (Olson et al., 1993). On the basis of these second-order rate constants, H-kininogen was found to augment the heparin rate enhancement of the antithrombin–kallikrein reaction from a modest 13-fold to a substantial 1200-fold.

Effect of H-kininogen and Heparin on the Reaction of Kallikrein with Its Primary Plasma Inhibitors. To determine whether the potentiating effect of H-kininogen on the heparin-accelerated antithrombin–kallikrein reaction might be of physiologic significance, it was necessary to examine the effects of H-kininogen and heparin on the inactivation of kallikrein by the two main inhibitors of this enzyme in plasma, C1-inhibitor and α_2-macroglobulin (Schapira et al., 1982a; van der Graaf et al., 1983a). Figure 2 shows that heparin and H-kininogen alone or in combination and at plasma concentrations of kininogen (0.2–1 μM) had no detectable effect on the rate of kallikrein inactivation by C1-inhibitor measured under pseudo-first-order conditions. Observed pseudo-first-order inactivation rate constants for this reaction were proportional to the inhibitor concentration and yielded a second-order rate constant comparable to that reported by others, given the differences in temperature in these studies (Table I; Schapira et al., 1982b; van der Graaf et al., 1983b; Silverberg et al., 1986). As with the antithrombin reaction, α- and β-forms of kallikrein reacted with C1-inhibitor at indistinguishable rates, in agreement with past studies (Colman et al., 1985).

Figure 3 shows the effects of heparin and H-kininogen on the inactivation of kallikrein by α_2-macroglobulin, also studied under pseudo-first-order conditions. The reaction of kallikrein with α_2-macroglobulin was accompanied by an incomplete loss of chromogenic substrate activity (Figure 3), due to the retention of kallikrein activity toward small substrates in the α_2-macroglobulin–kallikrein complex (Schapira et al., 1982b; van der Graaf et al., 1984). As with the C1-inhibitor reaction, heparin had no effect on the rate of complex formation with this inhibitor either in the absence or in the presence of

Table I: Second-Order Rate Constants for Kallikrein–Inhibitor Reactions[a]

inhibitor	kallikrein	second-order rate constant (M^{-1} s^{-1})			
		–Hep–Kin	+Hep – Kin	–Hep + Kin	+Hep + Kin
C1-inhibitor	α	$(1.7 \pm 0.1) \times 10^4$	$(1.7 \pm 0.1) \times 10^4$	$(1.7 \pm 0.1) \times 10^4$	$(1.7 \pm 0.1) \times 10^4$
	β	$(1.8 \pm 0.1) \times 10^4$	ND[b]	ND[b]	ND[b]
α_2-macroglobulin	α	$(3.4 \pm 0.3) \times 10^4$	$(3.4 \pm 0.3) \times 10^4$	$(4.9 \pm 0.4) \times 10^3$	$(4.9 \pm 0.4) \times 10^3$
	β	$(6.8 \pm 1.4) \times 10^3$	ND[b]	$(1.0 \pm 0.1) \times 10^3$ [c]	$(1.0 \pm 0.1) \times 10^3$ [c]
antithrombin	α	$(1.6 \pm 0.1) \times 10^2$	$(2.1 \pm 0.1) \times 10^3$	$(1.6 \pm 0.1) \times 10^2$	$(1.9 \pm 0.1) \times 10^5$
	β	$(1.6 \pm 0.1) \times 10^2$	$(1.9 \pm 0.1) \times 10^3$	ND[b]	$(3.6 \pm 0.9) \times 10^3$ [c]

[a] Second-order rate constants for the reactions of inhibitors with predominantly α-kallikrein were determined in the absence of heparin and H-kininogen from the slope of linear plots of observed pseudo-first-order rate constants (k_{obs}) against inhibitor concentration (0.5–2 μM), as detailed under Experimental Procedures. Rate constants for inhibitor reactions with β-kallikrein were obtained from k_{obs} determined at a single inhibitor concentration. The effect of heparin and/or H-kininogen on rate constants was determined from data in Figures 1–3 except for antithrombin reactions in the presence of heparin. Second-order rate constants in the latter case were determined from linear plots of k_{obs}, corrected for the free antithrombin reaction, against a range of antithrombin–heparin complex concentrations up to 1 or 0.07 μM in the absence or presence of 0.2 μM H-kininogen, respectively [see Olson et al. (1993)]. Reactions with the isolated kallikrein were biphasic in cases where k_{obs} for the α- and β-forms differed. In such cases, k_{obs} for the α-form was obtained from the predominant fast phase of the reaction. k_{obs} for the slow phase of such biphasic reactions was indistinguishable from k_{obs} measured for β-kallikrein reactions. [b] Not determined. Assumed to be the same as α-kallikrein values based on the monophasic inactivation curves observed in reactions with mostly α-kallikrein. [c] Determined from the slow phase rate constant for the reaction with the isolated kallikrein.

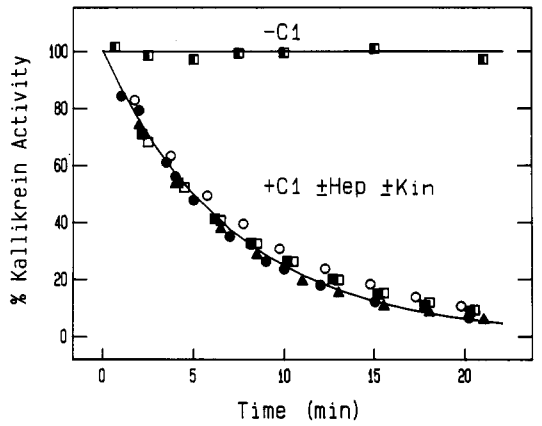


FIGURE 2: Effect of heparin and H-kininogen on the C1-inhibitor–kallikrein reaction. Rates of inactivation of 20 nM α-kallikrein by 0.14 μM C1-inhibitor in the absence of added components (●) or in the presence of 0.2 μM H-kininogen alone (○), 1 μM heparin alone (▲), or both 1 μM heparin and 0.2 (■) or 1 μM (□) H-kininogen, under the conditions in Figure 1. The solid line is a fit of the data by a single-exponential function. The half-solid squares depict a control incubation of enzyme in the absence of inhibitor.

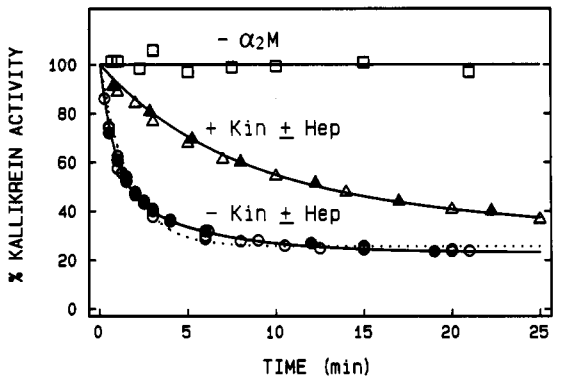


FIGURE 3: Effect of heparin and H-kininogen on the α_2-macroglobulin–kallikrein reaction. Rates of inhibition of 20 nM α-kallikrein by 0.5 μM α_2-macroglobulin in the absence of added components (○) or in the presence of 1 μM heparin alone (●), 0.2 μM H-kininogen alone (△), or both 1 μM heparin and 0.2 μM H-kininogen (▲), under the conditions of Figure 1. Control incubation of enzyme in the absence of inhibitor (□). Solid lines are fits of reaction curves by a double-exponential function as described under Experimental Procedures with observed fast and slow phase rate constants of 1.17 and 0.20 min^{-1} in the absence and 0.148 and 0.030 min^{-1} in the presence of H-kininogen. The dotted line shows a fit of data in the absence of H-kininogen by a single-exponential function.

H-kininogen (Figure 3). However, H-kininogen was found to reduce the rate of kallikrein inactivation, consistent with kininogen binding to kallikrein protecting the enzyme from α_2-macroglobulin inhibition, as has been reported previously (Schapira et al. 1982b; van der Graaf et al., 1984). A ~5-fold lower rate of inactivation of β-kallikrein by α_2-macroglobulin accounted for the reproducible deviations of progress curves for the reaction with the parent kallikrein preparation from a single exponential (Figure 3). Fitting of progress curves by a two-exponential function with the slower phase fixed at the value determined for the β-kallikrein reaction provided a more satisfactory fit. Reactions conducted at inhibitor concentrations from 0.5 to 1.5 μM yielded pseudo-first-order rate constants for the two exponential phases that increased proportionally with the inhibitor concentration from which second-order rate constants for α- and β-kallikreins were determined (Table I). These values somewhat exceed previously determined values (Schapira et al., 1982b; van der Graaf et al., 1984), possibly due to the failure to account for the slow phase and/or to temperature differences (Harpel et al., 1985).

Competition between Antithrombin, C1-inhibitor, and α_2-Macroglobulin for Kallikrein. The second-order rate constants measured for the reaction of kallikrein with antithrombin, C1-inhibitor, and α_2-macroglobulin in the absence or presence of fractionated heparin and H-kininogen (Table I), together with the plasma concentrations of these inhibitors, suggested that antithrombin should be an important inhibitor of kallikrein when both heparin and H-kininogen are present, but not when either or both of these effectors are absent. To test this prediction, we investigated the partitioning of ^{125}I-labeled kallikrein among these three inhibitors by quantitating the kallikrein–inhibitor complexes formed in a mixture of antithrombin, C1-inhibitor, and α_2-macroglobulin at relative concentrations equivalent to those in plasma in the absence or presence of the two effector components. Figure 4 shows the results of an autoradiogram of an SDS gel, run under nonreducing conditions, of the inhibitor–kallikrein complexes formed after complete inhibition of the added kallikrein. The right-hand portion of the gel (lanes 5–8) shows that the complexes formed with each one of the inhibitors alone were well resolved from each other and from free kallikrein. The multiple complexes formed between α_2-macroglobulin and kallikrein are due to covalent cross-linking of complexes by reactive thioesters (van der Graaf et al., 1984). Consistent with the predicted contributions of the three inhibitors to kallikrein inactivation (Table II), C1-inhibitor and α_2-macroglobulin were observed to be the primary inhibitors of

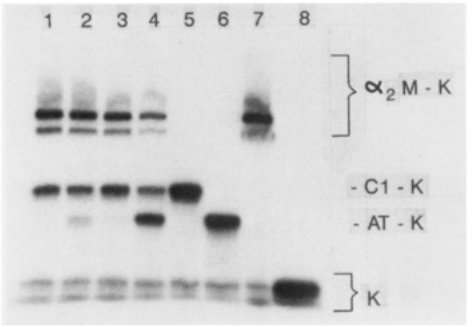


FIGURE 4: ^{125}I-Labeled kallikrein–inhibitor complexes formed in a simulated plasma mixture of kallikrein inhibitors. 2 nM ^{125}I-labeled kallikrein was reacted with a mixture of 0.17 μM C1-inhibitor, 0.38 μM α_2-macroglobulin, and 0.27 μM antithrombin [one-tenth the reported plasma concentrations of these inhibitors (Schapira et al., 1982a; van der Graaf et al., 1983a)] in I = 0.15, pH 7.4 Hepes buffer at 25 °C for 15 min. Complexes were resolved by SDS gel electrophoresis and visualized by autoradiography as described under Experimental Procedures. Reactions were done in the absence of added components (lane 1) or in the presence of 1 μM heparin alone (lane 2), 0.1 μM H-kininogen alone (lane 3), or both 1 μM heparin and 0.1 μM H-kininogen (lane 4). Lanes 5–8 are controls showing the kallikrein complexes formed with each purified inhibitor or kallikein alone.

Table II: Kallikrein–Inhibitor Complexes Formed in a Simulated Plasma Mixture of Kallikrein Inhibitors[a]

	% of total kallikrein–inhibitor complexes.			
inhibitor	–Hep – Kin	+Hep – Kin	–Hep + Kin[b]	+Hep + Kin[c]
C1-inhibitor	30 (25)	24 (24)	45 (56)	24 (21)
α_2-macroglobulin	70 (75)	68 (72)	55 (44)	22 (11)
antithrombin	0 (0.4)	9 (5)	0 (0.8)	54 (68)

[a] Relative amounts of kallikrein–inhibitor complexes in the autoradiogram of Figure 4 were determined by densitometric analysis of band intensities as described under Experimental Procedures and are expressed as a percentage of the sum of all band intensities. Values in parentheses are those predicted from relative pseudo-first-order rate constants calculated for each kallikrein–inhibitor reaction based on the data of Table I and assuming the parent kallikrein preparation was 80% α-form and 20% β-form. [b] H-kininogen was assumed to reduce the α_2-macroglobulin second-order rate constant by a factor of $1 + [\text{Kin}]/K_D$ in which K_D, the dissociation constant for the kininogen–kallikrein interaction, was assigned a value of 30 nM based on the data of Figure 3 (Schapira et al., 1982b). [c] k_{obs} for the antithrombin–α-kallikrein reaction was calculated from the kinetic parameters determined for the two-step inhibition reaction in the following paper with corrections for excess heparin inhibition (Olson et al., 1993).

kallikrein in the absence of heparin and H-kininogen (lane 1) or when just heparin (lane 2) or H-kininogen (lane 3) was present, with no (lanes 1 and 3) or little (lane 2) antithrombin–kallikrein complex detectable. Quantitation of the intensities of these complex bands confirmed these qualitative assessments (Table II). In contrast, antithrombin was the major kallikrein inhibitor when both kininogen and heparin were present, with C1-inhibitor and α_2-macroglobulin complexes with the enzyme being concomitantly reduced (lane 4, Table II). A reduction in α_2-macroglobulin complexes relative to C1-inhibitor complexes was also observed in the presence of H-kininogen alone (lane 3, Table II), consistent with the protective effect of kininogen on α_2-macroglobulin inhibition of kallikrein observed in kinetic studies. In all cases, the relative amounts of inhibitor–kallikrein complexes formed were in reasonable agreement with those predicted from the rate constants of Table I. These experiments therefore demonstrated that H-kininogen potentiation of the heparin-accelerated antithrombin–kallikrein reaction rate resulted in antithrombin effectively competing with C1-inhibitor and α_2-macroglobulin for kallikrein.

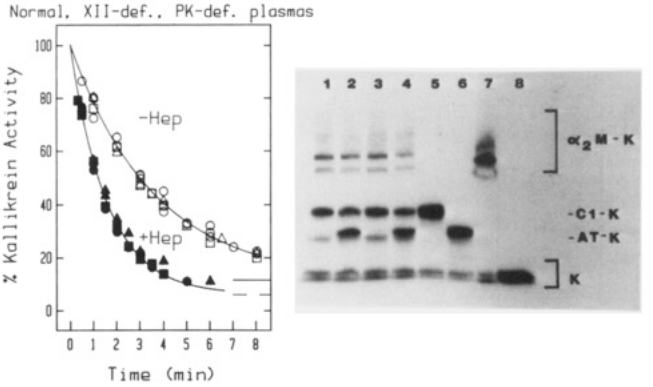


FIGURE 5: Effect of heparin on the rate of kallikrein inactivation and on the ^{125}I-labeled kallikrein–inhibitor complexes formed in normal, prekallikrein-deficient, and factor XII-deficient plasmas. (Left) Rates of inactivation of 12.5 nM kallikrein in normal (circles), factor XII-deficient (triangles), and prekallikrein-deficient (squares) plasmas diluted 10-fold in I = 0.15, pH 7.4 Hepes buffer (–EDTA) in the absence (open symbols) or presence (closed symbols) of 1 μM heparin. Kallikrein activity was measured as described under Experimental Procedures. Solid lines are fits of data by single-exponential decays with end points indicated by solid (–heparin) or dashed (+heparin) lines on the right-hand side of the graph. (Right) Autoradiogram of ^{125}I-labeled kallikrein–inhibitor complexes formed in 10-fold-diluted normal (lanes 1 and 2) or factor XII-deficient (lanes 3 and 4) plasmas in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 μM heparin after resolution by SDS gel electrophoresis as detailed under Experimental Procedures. Control lanes 5–8 are as indicated in Figure 4.

Effect of Heparin on Kallikrein Inactivation in Plasma. To determine whether antithrombin was a significant inhibitor of kallikrein in plasma in the presence of therapeutic levels of heparin (~1 unit/mL, Colman et al., 1989), the rates of kallikrein inactivation and formation of kallikrein–inhibitor complexes in normal and deficient plasmas were investigated. Figure 5 shows the results of experiments in 10-fold-diluted normal, factor XII-deficient, and prekallikrein-deficient plasmas. Addition of 1 μM fractionated heparin (0.8 unit/mL) resulted in a 2.3-fold enhancement in the pseudo-first-order rate constant for kallikrein inactivation in all cases, i.e., from 0.28 ± 0.01 to 0.64 ± 0.06 min^{-1}. Rates of kallikrein inactivation were first- order for ~90% of the reaction and the same in all plasmas either for heparin-enhanced or for unenhanced reactions, indicating that heparin or kallikrein was inducing no significant contact activation over the time frame of these experiments. These results indicated that a heparin-activatable inhibitor was responsible for at least 56% [i.e., 100[(0.64 – 0.28)/0.64]] of kallikrein inactivation in the heparinized plasma. The autoradiogram on the right-hand side of Figure 5 indeed confirmed the appearance of a prominent band corresponding in mobility to the antithrombin–kallikrein complex in the presence (lanes 2 and 4) but not in the absence (lanes 1 and 3) of heparin. Quantitation of the relative intensities of the complex bands (Table III) indicated that C1-inhibitor and α_2-macroglobulin were the predominant inhibitors of kallikrein in the absence of heparin while antithrombin made a major contribution of 50–53% in the presence of heparin, consistent with the prediction of the kinetic experiments.[2]

To ensure that the new complex band appearing in the presence of heparin was in fact antithrombin–kallikrein

[2] A greater contribution of C1-inhibitor to kallikrein inactivation was observed in plasma than in the simulated plasma mixture of inhibitors, suggesting that our assumed plasma concentrations of C1-inhibitor and α_2-macroglobulin are somewhat in error or that other unknown plasma factors affect these reactions.

Table III: Kallikrein–Inhibitor Complexes Formed in Normal and Deficient Plasmas[a]

plasma	additions	% α_2M	% C1	% AT	% other
normal	–Hep	36 ± 6	56 ± 9		8 ± 3
	+Hep	9 ± 3	31 ± 2	50 ± 3	
factor XII-deficient	–Hep	26 ± 7	61 ± 10		14 ± 3
	+Hep	16 ± 4	31 ± 3	53 ± 3	
H-kininogen-deficient	–Hep	37 ± 1	57 ± 1		6 ± 2
	+Hep	38 ± 5	51 ± 3	11 ± 3	
	–Hep + Kin	24 ± 2	66 ± 2		10 ± 2
	+Hep + Kin	16 ± 6	36 ± 3	48 ± 3	
antithrombin-deficient	–Hep	42 ± 3	52 ± 8		7 ± 3
	+Hep	36 ± 5	51 ± 7		13 ± 2
	–Hep + AT	41 ± 5	49 ± 7		10 ± 2
	+Hep + AT	20 ± 3	25 ± 3	55 ± 5	

[a] Average relative amounts of kallikrein–inhibitor complex bands in the autoradiograms of Figures 5–7 were determined by densitometric scanning of autoradiograms exposed for 4, 6, and 12 h as described under Experimental Procedures. Errors represent ± 2 SE.

complex rather than some other heparin-activatable inhibitor of similar molecular weight, e.g., heparin cofactor II (Tollefsen et al., 1983) or protein C inhibitor (Meijers et al., 1988; Espana et al., 1991), similar experiments were done in plasma specifically depleted of antithrombin. The results of these experiments, shown in Figure 6, indicated that heparin had no effect on the rate constant of 0.24 ± 0.01 min^{-1} for kallikrein inactivation in this deficient plasma and did not result in a significant complex band in the autoradiogram at a position corresponding to the antithrombin–kallikrein complex (lane 2). Correction of this plasma with purified antithrombin restored the heparin enhancement of the inactivation rate (from 0.24 ± 0.01 to 0.69 ± 0.05 min^{-1}) and the appearance of a predominant band corresponding in mobility to the antithrombin–kallikrein complex and comprising 55% of total kallikrein complexes, in the presence (land 4) but not in the absence (lane 3) of heparin (Table III). These results thus demonstrated that the heparin-activatable inhibitor responsible for the heparin rate enhancement and the new inhibitor–kallikrein complex band was antithrombin.

Finally, to prove that the major contribution of antithrombin to kallikrein inhibition in heparinized plasma was dependent on the potentiation of the heparin-accelerated antithrombin–kallikrein reaction by H-kininogen, similar experiments were performed in plasma deficient in H-kininogen, as shown in Figure 7. In this deficient plasma, kallikrein was inactivated with a 2-fold faster rate constant, i.e., 0.59 ± 0.02 min^{-1}, than that observed in normal plasma (see above), consistent with the faster inactivation of free kallikrein by α_2-macroglobulin than of the kallikrein–H-kininogen complex. In keeping with the predicted H-kininogen requirement for heparin enhancement of kallikrein inactivation in plasma, heparin had no effect on the enzyme inactivation rate in this deficient plasma. The autoradiogram substantiated the absence of a significant antithrombin–kallikrein complex band both in the absence (lane 1) and in the presence (lane 2) of heparin as well as the predicted greater contribution of α_2-macroglobulin to kallikrein inactivation in this plasma (Table III). Correction of the plasma with purified H-kininogen restored both the lower rate of kallikrein inactivation (0.35 ± 0.01 min^{-1}) and the ~2-fold heparin enhancement of the kallikrein inactivation rate (0.86 ± 0.08 min^{-1}) observed in normal plasma. The autoradiogram confirmed a decreased contribution of α_2-macroglobulin–kallikrein complexes in the corrected plasma comparable to that in normal plasma and the appearance of a predominant antithrombin–kallikrein complex band (48% of kallikrein complexes) in the presence (lane 4) but not in the absence (lane 3) of heparin (Table III).

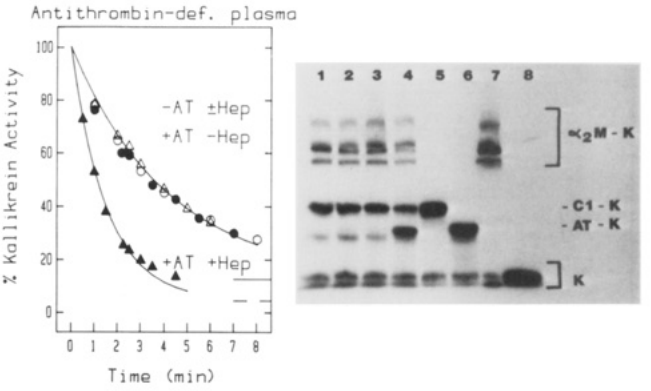


FIGURE 6: Effect of heparin on the rate of kallikrein inactivation and on kallikrein–inhibitor complex formation in antithrombin-deficient plasma. (Left) Rates of inactivation of 12.5 nM kallikrein in 10-fold-diluted antithrombin-deficient plasma (accounting for a 7% dilution during preparation) in the absence (open symbols) or presence (closed symbols) of 1 μM heparin, under the conditions of Figure 5. Reactions are shown before (circles) and after (triangles) correction with 0.27 μM antithrombin. Solid lines are single-exponential fits of data with end points given by the solid (upper curve) and dashed (lower curve) lines on the right-hand side of the graph. (Right) Autoradiogram of an SDS gel of separated ^{125}I-labeled kallikrein–inhibitor complexes formed in 10-fold-diluted antithrombin-deficient plasma in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 μM heparin before (lanes 1 and 2) and after (lanes 3 and 4) correction with 0.27 μM antithrombin. Experimental conditions are as in Figure 5.

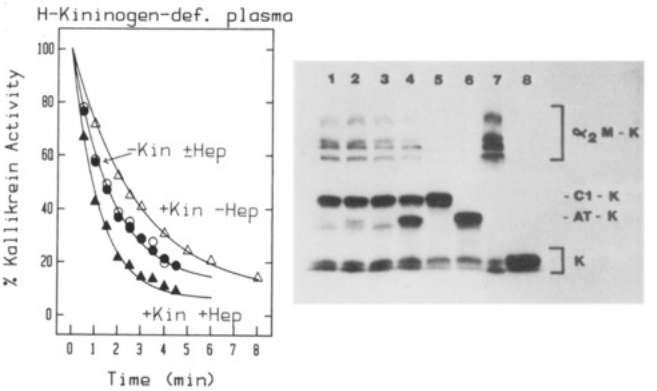


FIGURE 7: Effect of heparin on the rate of kallikrein inactivation and on kallikrein–inhibitor complex formation in H-kininogen-deficient plasma. (Left) Rates of inactivation of 12.5 nM kallikrein in 10-fold-diluted H-kininogen-deficient plasma in the absence (open symbols) or presence (closed symbols) of 1 μM heparin, under the conditions of Figure 5. Reactions are shown before (circles) and after (triangles) correction with 0.1 μM H-kininogen. Solid lines are single-exponential fits of data which yielded end points of 6.0 (upper curve), 12 (middle curve), and 8.1% (lower curve) residual activity. (Right) Autoradiogram of an SDS gel of the separated ^{125}I-labeled kallikrein–inhibitor complexes formed in 10-fold-diluted H-kininogen-deficient plasma in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of 1 μM heparin before (lanes 1 and 2) and after (lanes 3 and 4) correction with 0.1 μM H-kininogen. Experimental conditions are as in Figure 5.

Effect of Plasma Dilution, Heparin Activity, Metal Ions, and Temperature on Kallikrein Inactivation in Plasma. To show that the results obtained under the conditions of the above plasma experiments were representative of more physiological conditions, i.e., lower plasma dilution, unfractionated heparin, 37 °C, with divalent metal ions present, and that they provided the optimal heparin effect, the heparin enhancement of the rate of kallikrein inactivation in plasma was investigated as a function of the type (i.e., fractionated or unfractionated) and concentration of heparin, the plasma dilution, the temperature, and plasma levels of calcium and

Table IV: Effect of Temperature, Heparin Concentration, and Heparin Fractionation on the Acceleration by Heparin of Kallikrein Inactivation in Plasma[a]

	k_{obs} (min^{-1})		
	fractionated heparin		unfractionated heparin
[heparin] (μM)[b]	T = 25 °C	T = 37 °C	T = 25 °C
0	0.32	0.51	0.23
0.05			0.48
0.10	0.87	0.72	0.75
0.20	1.1	0.94	0.88
0.50	1.2	0.81	0.82
1.0	0.94	0.80	
2.0	0.67		

[a] Pseudo-first-order rate constants (k_{obs}) for kallikrein inactivation in the absence and presence of either molecular weight and antithrombin-affinity fractionated heparin or unfractionated heparin were determined in 10-fold-diluted factor XII-deficient plasma from the slopes of linear natural log plots of residual activity vs time after subtracting end-point velocities due to α_2-macroglobulin–kallikrein complexes. Plots were typically linear over the extent of the reaction examined (80–90%). [b] Concentrations based on antithrombin binding sites for the fractionated heparin and on dry weight for the unfractionated heparin assuming an average molecular weight of 15 000.

zinc ions. Table IV shows the kallikrein inactivation rate constants measured in 10-fold-diluted factor XII-deficient plasma as a function of fractionated or unfractionated heparin concentration and temperature. At 25 °C, a maximum rate enhancement of 3.8-fold was observed between 0.2 and 0.5 μM fractionated heparin (0.15–0.4 unit/mL), with higher concentrations producing decreased rate enhancements. The maximum heparin effect suggested a 74% contribution of antithrombin to kallikrein inactivation. An identical 3.8-fold maximum enhancement resulted when 3.8 μg/mL (0.6 unit/mL) unfractionated heparin was examined at the same temperature. When similar experiments with the fractionated heparin were performed at 37 °C, the maximum heparin enhancement was reduced to 1.8-fold, suggesting a smaller but still signficant 44% contribution of antithrombin to kallikrein inhibition.

The effect of plasma dilution on these results was first examined by quantitating the ^{125}I-labeled kallikrein–inhibitor complexes formed in the absence or presence of 1 μM fractionated heparin at decreasing dilutions of plasma. No significant effect of plasma dilution on the amount of antithrombin–kallikrein complex formed in the presence of heparin (51–60%) was observed between 10-fold and 3-fold dilution, whereas a somewhat reduced amount of complex (26%) was found with a smaller plasma dilution of 1.75-fold. The maximum heparin enhancement of the kallikrein inactivation rate constant was similarly reduced to 1.2-fold, reflecting ~20% antithrombin–kallikrein complexes, in 1.4-fold-diluted plasma (Table V). This reduced heparin effect in minimally diluted plasma was largely reversed, however, by dialysis of the plasma against reaction buffer containing Chelex (Table V). Such results suggested that sodium citrate added to the plasma for anticoagulation was responsible for the reduced heparin effect, possibly due to alterations in the plasma ionic strength or pH from normal physiological values.[3] Addition of calcium ions to the dialyzed plasma at their normal

Table V: Effect of Plasma Dilution and Calcium Ions on Heparin Acceleration of Kallikrein Inactivation in Plasma[a]

	k_{obs} (min^{-1})			
	undialyzed plasma		dialyzed plasma	
plasma dilution factor	–heparin	+heparin	–heparin	+heparin
10	0.23	0.70	0.20	0.55
1.4	1.3	1.6	0.91	2.4
1.4 + 5 mM Ca^{2+} [b]			0.94	1.6

[a] Pseudo-first-order rate constants (k_{obs}) for kallikrein inactivation in factor XII-deficient plasma were measured at 25 °C in the absence and presence of optimum levels of fractionated heparin (0.4 μM in 10-fold-diluted plasma and 2.8 μM in 1.4-fold-diluted plasma) as in Table IV. Plasma was used directly or after overnight dialysis against 200 volumes of I = 0.15, pH 7.4 Hepes buffer (without EDTA) containing 6 g of Chelex (Bio-Rad) per 100 mL. Experiments with dialyzed plasma were done in Chelex-treated and filtered Hepes buffer with no EDTA. [b] Ultrapure calcium chloride (≤0.01 ppm of Zn^{2+}, Merck) was used for these experiments.

plasma level reduced the maximum heparin enhancement of the kallikrein inactivation rate constant to 1.7-fold (Table V), indicating a smaller but still significant 41% contribution of antithrombin to kallikrein inactivation. Zinc ions added at 1 or 10 μM (as zinc chloride) to the dialyzed plasma had no detectable effect on k_{obs} measured under the same conditions in the absence or presence of heparin or calcium ions (not shown).

DISCUSSION

The present studies have shown that H-kininogen greatly enhances the rate of antithrombin inactivation of kallikrein in the presence of heparin but not in the absence of the polysaccharide. H-kininogen was found to increase the heparin rate enhancement of the antithrombin–kallikrein reaction about 100-fold, resulting in an overall 1200-fold rate enhancement. Such a value is comparable to the heparin rate enhancements observed for other antithrombin–proteinase reactions (Jordan et al., 1980; Olson et al., 1992b). The rate-enhancing effects of kininogen and heparin are specific for the reaction of antithrombin with kallikrein and are not observed for the reactions of kallikrein with its primary plasma inhibitors, C1-inhibitor and α_2-macroglobulin. Such specificity is understandable given the specific binding of antithrombin to heparin and the lack of binding of C1-inhibitor or α_2-macroglobulin to the polysaccharide.[4] Preliminary studies indicate that H-kininogen similarly enhances the rate of the heparin-dependent antithrombin–factor XIa reaction (Olson et al., 1992a), indicating similar actions of kininogen on the heparin-accelerated reactions of antithrombin with the two proteinases which form specific complexes with the protein. The stimulating effect of kininogen on the heparin-dependent reactions of antithrombin with kallikrein and factor XIa contrasts with the antagonizing effect of the protein on the heparin-enhanced antithrombin–thrombin reaction demonstrated in previous studies (Björk et al., 1989). The mechanism by which heparin accelerates the antithrombin–thrombin reaction involves the polysaccharide acting as a surface to bridge the interaction between antithrombin and thrombin bound to the same polysaccharide chain (Laurent et al., 1978; Olson & Björk, 1991). Thus, H-kininogen antagonizes this reaction most likely by competing with antithrombin or thrombin for binding to the heparin chain (Peterson et al., 1987). This mode of action is analogous to that of other

[3] The sodium citrate added to George King plasma (13 mM) would produce a net increase of ~0.063 M in the plasma ionic strength. This could account for the decreased heparin effect in minimally diluted, undialyzed plasma, since the H-kininogen and heparin-dependent acceleration of the antithrombin–kallikrein reaction is quite sensitive to ionic strength (Olson et al., 1993). The measured pH of the plasma (~7.7) could also contribute to the reduced heparin effect.

[4] C1-inhibitor and α_2-macroglobulin are not bound by heparin–agarose at physiological ionic strength, pH 7.4.

heparin binding proteins (Lane, 1989). In the case of kallikrein and factor XIa, however, specific complexes of H-kininogen with these enzymes are presumably responsible for facilitating rather than antagonizing their binding to the heparin surface where bridging of their interaction with surface-bound antithrombin can occur. It follows that free H-kininogen should similarly antagonize the binding of kininogen–enzyme complexes to heparin when present in sufficient excess over the enzyme and reduce the protein's potentiating effect. Evidence supporting such novel modes of action of kininogen is provided in the following paper (Olson et al., 1993).

The observations made in this study of a protective effect of H-kininogen on kallikrein inactivation by α_2-macroglobulin, but the absence of such an effect on the inactivation of the enzyme by C1-inhibitor and antithrombin, are in agreement with most previous reports (Schapira et al., 1982b; van der Graaf et al., 1983b, 1984; Silverberg et al., 1986). Such results can be rationalized by the different mechanisms of action of these inhibitors. In the case of α_2-macroglobulin, inhibition of proteinases results from the physical entrapment of the enzyme by a large cavity in the inhibitor whose finite dimensions limit the size of the enzyme that can be trapped (Barrett et al., 1979; Travis & Salvesen, 1983). Thus, the molecular weight of the H-kininogen–kallikrein complex is in the range of other proteinases such as factor XIa which are apparently too large to be accommodated in the α_2-macroglobulin cavity (Harpel, 1971). In the case of the serpin inhibitors, C1-inhibitor and antithrombin, proteinase inhibition results from a tight interaction of the inhibitor with just the active-site region of the enzyme (Travis & Salvesen, 1983), which is localized in the kallikrein light chain (van der Graaf et al., 1983b). Since H-kininogen binds to the heavy chain (Bock et al., 1985; Colman et al., 1985), bound kininogen may be far enough removed from the active-site region of the enzyme so as not to interfere with the interaction of kallikrein with serpin inhibitors.

On the basis of the large enhancement in the second-order rate constant for the antithrombin–kallikrein reaction produced by H-kininogen and heparin, the relative rate constants for the reactions of other plasma protein inhibitors of kallikrein, and the plasma concentrations of these inhibitors, antithrombin was predicted to be an important inhibitor of kallikrein in heparinized plasma. This prediction was confirmed both in a simulated plasma mixture of purified inhibitors and in plasma. The 1200-fold rate enhancement of the antithrombin–kallikrein reaction produced by H-kininogen and heparin in a purified system would seem to be inconsistent with the maximum ~4-fold rate enhancement of the kallikrein inactivation rate constant produced by heparin in plasma (Table IV). However, this apparent inconsistency results from the predominant contribution of C1-inhibitor and α_2-macroglobulin to the kallikrein inactivation rate in plasma in the absence of heparin, as shown in this and past studies (Schapira et al., 1982a; van der Graaf et al., 1983a). Thus, the contribution of antithrombin to the kallikrein inactivation rate constant in 10-fold-diluted plasma can be predicted to be only 0.003 min^{-1} in the absence of heparin (Table I), i.e., 1% of the observed inactivation rate constant of ~0.3 min^{-1} (Table IV). Since the rates of C1-inhibitor and α_2-macroglobulin reactions with the enzyme are unaffected by heparin, the contribution of antithrombin to the kallikrein inactivation rate in plasma in the presence of heparin is given by the increase in the inactivation rate constant produced by the polysaccharide, which at optimal heparin levels is $1.2 - 0.3 = 0.9$ min^{-1} (Table IV). Thus, the actual heparin rate enhancement of the antithrombin–kallikrein reaction in plasma is 0.9/0.003 or 300-fold. The magnitude of this rate enhancement is in keeping with the nearly absolute dependence of the formation of antithrombin–kallikrein complexes in plasma on heparin and H-kininogen (Table III, Figures 5–7). It follows that small apparent heparin rate enhancements of proteinase inactivation observed in plasma may reflect substantially larger true enhancements of specific heparin-activated inhibitor reactions and consequent marked contributions of such inhibitors to proteinase inactivation.

The reason why heparin rate enhancements observed in plasma do not attain the maximum preicted rate enhancement of 1200-fold is likely due to other heparin binding proteins in plasma which may compete with antithrombin for heparin (Lane, 1989), and thereby block the formation of productive quaternary complexes of antithrombin and H-kininogen–kallikrein complex bound to the same heparin chain (Olson et al., 1993). A reduced heparin rate enhancement in plasma may also result from deviations of the kininogen-stimulated reaction from second-order kinetic behavior due to the saturation of the productive quaternary complex at plasma concentrations of antithrombin–heparin complex (Olson et al., 1993).

Protein C inhibitor has also been shown to make a small but significant contribution to kallikrein inactivation in plasma (Meijers et al., 1988; Espana et al., 1991). The minor complex band with a mobility similar to that of the antithrombin–kallikrein complex, which appeared in autoradiograms of kallikrein–inhibitor complexes formed in plasma lacking heparin (Figures 5–7), may in fact represent the kallikrein–protein C inhibitor complex. This is suggested by the absence of any detectable antithrombin–kallikrein complex band in the autoradiogram of kallikrein complexes formed in a simulated plasma mixture of C1-inhibitor, α_2-macroglobulin, and antithrombin lacking heparin (Figure 4). Whether the contribution of protein C inhibitor to kallikrein inhibition in plasma is also enhanced by heparin by an H-kininogen-dependent mechanism is not clear from our data, but warrants further investigation.

Our demonstration that antithrombin is a major inhibitor of kallikrein in plasma containing therapeutic levels of heparin has important consequences for the regulation of kallikrein by clinically administered heparin as well as by endogenous heparin or heparan sulfate glycosaminoglycans on endothelial cell surfaces (Marcum & Rosenberg, 1984). Thus, endogenous heparin or heparan sulfate molecules would be expected to promote kallikrein inactivation *in vivo*, and possibly be important for controlling an enhanced rate of prekallikrein activation resulting from blood vessel injury, inflammation, or activation of the complement system (Colman, 1984). The involvement of cell-surface glycosaminoglycans in this promotion of kallikrein inactivation would further imply a localization of enzyme inactivation to such cell surfaces and the ability to modulate kallikrein activity through their accessibility. Our findings also provide a rational basis for early suggestions that heparin might be an effective clinical agent for treating patients with hereditary angioedema who lack C1-inhibitor and appear to ineffectively regulate kallikrein activity (Colman, 1976). The defective regulation of kallikrein activity in hereditary angioedema and association of this condition with uncontrolled inflammation have suggested that kallikrein may play a role in the inflammatory response (Colman, 1984). The anticipated reduction of the inflammatory activity of kallikrein by clinically administered heparin could thus contribute to the well-established antiinflammatory

action of heparin (Eckre et al., 1992). Finally, our results are significant with respect to the functional duality of H-kininogen in promoting both the activation of prekallikrein by factor XIIa and also the inactivation of kallikrein by antithrombin, which suggests a novel mode of regulation of this proenzyme–enzyme system. This aspect will be discussed more fully in the following paper where we provide evidence that the mechanism by which H-kininogen potentiates the heparin-dependent antithrombin–kallikrein reaction parallels the mechanism by which H-kininogen promotes the surface-dependent activation of prekallikrein by factor XIIa (Olson et al., 1993).

ACKNOWLEDGMENT

We thank Ingemar Björk for his helpful comments on the manuscript.

REFERENCES

Asakura, S., Hurley, R. W., Skorstengaard, K., Ohkubo, I., & Mosher, D. F. (1992) *J. Cell Biol. 116*, 465–476.
Barrett, A. J., Brown, M. A., & Sayers, C. A. (1979) *Biochem. J. 181*, 401–418.
Barrowcliffe, T. W., Mulloy, B., Johnson, E. A., & Thomas, D. P. (1989) *J. Pharm. Biomed. Anal. 7*, 217–226.
Björk, I., & Fish, W. W. (1982) *J. Biol. Chem. 257*, 9487–9493.
Björk, I. Olson, S. T., Sheffer, R. G., & Shore, J. D. (1989) *Biochemistry 28*, 1213–1221.
Bock, P. E., & Shore, J. D. (1983) *J. Biol. Chem. 258*, 15079–15086.
Bock, P. E., Shore, J. D., Tans, G., & Griffin, J. H. (1985) *J. Biol. Chem. 260*, 12434–12443.
Bock, P. E., Craig, P. A., Olson, S. T., & Singh, P. (1989) *Arch. Biochem. Biophys. 273*, 375–388.
Bouma, B. N., Vlooswijk, R. A. A., & Griffin, J. H. (1983) *Blood 62*, 1123–1131.
Cochrane, C. G., & Griffin, J. H. (1982) *Adv. Immunol. 33*, 241–306.
Colman, R. W. (1976) *Ann. Int. Med. 85*, 399.
Colman, R. W. (1984) *J. Clin. Invest. 73*, 1249–1253.
Colman, R. W., Wachtfogel, Y. T., Kucich, U., Weinbaum, G., Hahn, S., Pixley, R., Scott, C. F., de Agostini, A., Burger, D., & Schapira, M. (1985) *Blood 65*, 311–318.
Colman, R. W., Scott, C. F., Pixley, R. A., & De La Cadena, R. A. (1989) *Ann. N.Y. Acad. Sci. 556*, 95–103.
Dunn, J. T., & Spiro, R. G. (1967) *J. Biol. Chem. 242*, 5549–5555.
Eckre, H.-P., Naparstek, Y., Lider, O., Hydén, P., Hägermark, Ö., Nilsson, T., Vlodavsky, I., & Cohen, I. (1992) *Adv. Exp. Med. Biol. 313*, 329–340.
Espana, F., Estelles, A., Griffin, J. H., & Aznar, J. (1991) *Thromb. Haemostasis 65*, 46–51.
Ganrot, P. O. (1966) *Acta Chem. Scand. 20*, 2299–2300.
Griffin, J. H., & Cochrane, C. G. (1976a) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2554–2558.
Griffin, J. H., & Cochrane, C. G. (1976b) *Methods Enzymol. 45*, 56–65.
Hall, P. K., & Roberts, R. C. (1978) *Biochem. J. 171*, 27–38.
Han, Y. N., Komiya, M., Iwanaga, S., & Suzuki, T. (1975) *J. Biochem. (Tokyo) 77*, 55–68.
Harpel, P. C. (1971) *J. Clin. Invest. 50*, 2084–2090.
Harpel, P. C., Lewin, M. F., & Kaplan, A. P. (1985) *J. Biol. Chem. 260*, 4257–4263.
Harrison, R. A. (1983) *Biochemistry 22*, 5001–5007.
Heimark, R. L., & Davie, E. W. (1981) *Methods Enzymol. 80*, 157–172.
Hoogendoorn, H., Cerskus, A., Ofosu, F., Blajchman, M., & Hirsh, J. (1980) *Thromb. Res. 20*, 77–83.
Ikari, N., Sugo, T., Fujii, S., Kato, H., & Iwanaga, S. (1981) *J. Biochem. (Tokyo) 89*, 1699–1709.
Jordan, R. E., Oosta, G. M., Gardner, W. T., & Rosenberg, R. D. (1980) *J. Biol. Chem. 255*, 10081–10090.
Kato, H., Nagasawa, S., & Iwanaga, S. (1981) *Methods Enzymol. 80*, 172–198.
Kellerman, J., Lottspeich, F., Henschen, A., & Müller-Esterl, W. (1986) *Eur. J. Biochem. 154*, 471–478.
Kerbiriou, D. M., & Griffin, J. H. (1979) *J. Biol. Chem. 254*, 12020–12027.
Kerbiriou, D. M., Bouma, B. N., & Griffin, J. H. (1980) *J. Biol. Chem. 255*, 3952–3958.
Koide, T., Foster, D., Yoshitake, S., & Davie, E. W. (1986) *Biochemistry 25*, 2220–2225.
Kurecki, T., Kress, L. F., & Laskowski, M., Sr. (1979) *Anal. Biochem. 99*, 415–420.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lane, D. L. (1989) in *Heparin. Chemical and Biological Properties. Clinical applications*, (Lane, D. L., & Lindahl, U., Eds.) pp 363–391, Edward Arnold, London.
Latallo, Z. S., & Hall, J. A. (1986) *Thromb. Res. 43*, 507–521.
Laurent, T. C., Tengblad, A., Thunberg, L., Höök, M., & Lindahl, U. (1978) *Biochem. J. 175*, 691–701.
Lijnen, H. R., Hoylaerts, M., & Collen, D. (1983) *J. Biol. Chem. 258*, 3803–3808.
Mandle, R. J., Colman, R. W., & Kaplan, A. P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4179–4183.
Marcum, J., & Rosenberg, R. D. (1984) *Biochemistry 23*, 1730–1737.
Meijers, J. C. M., Kanters, D. H. A. J., Vlooswijk, R. A. A., van Erp, H., Hessing, M., & Bouma, B. N. (1988) *Biochemistry 27*, 4231–4237.
Melhado, L. L., Pelz, S. W., Leytus, S. P., & Mangel, W. F. (1982) *J. Am. Chem. Soc. 104*, 7299–7306.
Meloni, F. J., & Schmaier, A. H. (1991) *J. Biol. Chem. 266*, 6786–6794.
Mori, K., & Nagasawa, S. (1981) *J. Biochem. (Tokyo) 89*, 1465–1473.
Müller-Esterl, W., Fritz, H., Machleidt, W., Ritonja, A., Brzin, J., Kotnik, M., Turk, V., Kellerman, J., & Lottspeich, F. (1985) *FEBS Lett. 182*, 310–314.
Nakayasu, T., & Nagasawa, S. (1979) *J. Biochem. (Tokyo) 85*, 249–258.
Nordenman, B., Nyström, C., & Björk, I. (1977) *Eur. J. Biochem. 78*, 195–203.
Ohkuhbo, T., Kurachi, K., Takasawa, T., Shiokawa, H., & Sasaki, M. (1984) *Biochemistry 23*, 5691–5697.
Olson, S. T. (1985) *J. Biol. Chem. 260*, 10153–10160.
Olson, S. T. (1988) *J. Biol. Chem. 263*, 1698–1708.
Olson, S. T., & Shore, J. D. (1981) *J. Biol. Chem. 256*, 11065–11072.
Olson, S. T., & Björk, I. (1991) *J. Biol. Chem. 266*, 6353–6364.
Olson, S. T., Sheffer, R., & Shore, J. D. (1992a) *Agents Actions Suppl. 38*, 241–248.
Olson, S. T., Björk, I., Sheffer, R., Craig, P. A., Shore, J. D., & Choay, J. (1992b) *J. Biol. Chem. 267*, 12528–12538.
Olson, S. T., Francis, A. M., Sheffer, R., & Choay, J. (1993) *Biochemistry* (following paper in this issue).
Peterson, C. B., Morgan, W. T., & Blackburn, M. N. (1987) *J. Biol. Chem. 262*, 7567–7574.
Pratt, C. W., Whinna, H. C., & Church, F. C. (1992) *J. Biol. Chem. 267*, 8795–8801.
Reboul, A., Arlaud, G. J., Sim, R. B., & Colomb, M. G. (1977) *FEBS Lett. 79*, 45–50.
Retzios, A. D., Rosenfeld, R., & Schiffman, S. (1987) *J. Biol. Chem. 262*, 3074–3081.
Salvesen, G. S., Catanese, J. J., Kress, L. F., & Travis, J. (1985) *J. Biol. Chem. 260*, 2432–2436.
Schapira, M., Scott, C. F., & Colman, R. W. (1982a) *J. Clin. Invest. 69*, 462–468.

Schapira, M., Scott, C. F., James, A., Silver, L. D., Kueppers, F., James, H. L., & Colman, R. W. (1982b) *Biochemistry 21*, 567–572.
Scott, C. F., Schapira, M., James, H. L., Cohen, A. B., & Colman, R. W. (1982) *J. Clin. Invest. 69*, 844–852.
Shimada, T., Kato, H., Maeda, H., & Iwanaga, S. (1985) *J. Biochem. (Tokyo) 97*, 1637–1644.
Shore, J. D., Day, D. E., Bock, P. E., & Olson, S. T. (1987) *Biochemistry 26*, 2250–2258.
Silverberg, M., Longo, J., & Kaplan, A. P. (1986) *J. Biol. Chem. 261*, 14965–14968.
Sueyoshi, T., Enjyoji, K., Shimada, T., Kato, H., Iwanaga, S., Bando, Y., Kominami, E., & Katunuma, N. (1985) *FEBS Lett. 182*, 193–195.
Sugo, T., Ikari, N., Kato, H., Iwanaga, S., & Fujii, S. (1980) *Biochemistry 19*, 3215–3220.
Tait, J. F., & Fujikawa, K. (1986) *J. Biol. Chem. 261*, 15396–15401.
Tait, J. F., & Fujikawa, K. (1987) *J. Biol. Chem. 262*, 11651–11656.
Thaler, E., & Schmer, G. (1975) *Br. J. Haematol. 31*, 233–243.
Thompson, R. E., Mandle, R., & Kaplan, A. P. (1977) *J. Clin. Invest. 60*, 1376–1380.
Tollefesen, D. M., Pestka, C. A., & Monafo, W. J. (1983) *J. Biol. Chem. 258*, 6713–6716.
Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655–709.
van der Graaf, F., Koedam, J. A., & Bouma, B. N. (1983a) *J. Clin. Invest. 71*, 149–158.
van der Graaf, F., Koedam, J. A., Griffin, J. H., & Bouma, B. N. (1983b) *Biochemistry 22*, 4860–4866.
van der Graaf, F., Rietveld, A., Keus, F. J. A., & Bouma, B. N. (1984) *Biochemistry 23*, 1760–1766.
Virca, G. D., Travis, J., Hall, P. K., & Roberts, R. C. (1978) *Anal. Biochem. 89*, 274–278.