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Research Article

Patients lacking high molecular weight (HMW) kininogen have profound abnormalities of the Hageman factor-dependent pathways of coagulation, kinin formation, and fibrinolysis. The ability of HMW kininogen to potentiate the Hageman factor fragments (HFf) activation of prekallikrein and Factor XI in plasma was studied. HFf only partially converted Factor XI to XIa and prekallikrein to kallikrein in plasma deficient in HMW kininogen (Williams trait), while enhanced activation of Factor XI and prekallikrein by HFf resulted after reconstitution with HMW kininogen. In a system using highly purified components, HMW kininogen increased the initial rate of prekallikrein activation whether the kallikrein formed was assayed by arginine esterase activity or kinin-forming ability. The potentiation of prekallikrein activation occurred over a 12-fold range of enzyme (HFf) concentration and was nonhyperbolic with respect to substrate (prekallikrein). HMW kininogen exerted its effect even in the absence of prekallikrein since the hydrolysis of acetylglycyl-lysine methyl ester by HFf was increased by HMW kininogen. These results suggest that one of the functions of HMW kininogen is to augment the catalytic action of HFf.

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Potentialiation of the Function of Hageman Factor Fragments by High Molecular Weight Kininogen

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ABSTRACT Patients lacking high molecular weight (HMW) kininogen have profound abnormalities of the Hageman factor-dependent pathways of coagulation, kinin formation, and fibrinolysis. The ability of HMW kininogen to potentiate the Hageman factor fragments (HF_f) activation of prekallikrein and Factor XI in plasma was studied. HF_f only partially converted Factor XI to XI_a and prekallikrein to kallikrein in plasma deficient in HMW kininogen (Williams trait), while enhanced activation of Factor XI and prekallikrein by HF_f resulted after reconstitution with HMW kininogen. In a system using highly purified components, HMW kininogen increased the initial rate of prekallikrein activation whether the kallikrein formed was assayed by arginine esterase activity or kinin-forming ability. The potentiation of prekallikrein activation occurred over a 12-fold range of enzyme (HF_f) concentration and was nonhyperbolic with respect to substrate (prekallikrein). HMW kininogen exerted its effect even in the absence of prekallikrein since the hydrolysis of acetylglycyl-lysine methyl ester by HF_f was increased by HMW kininogen. These results suggest that one of the functions of HMW kininogen is to augment the catalytic action of HF_f.

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INTRODUCTION

The adsorption and activation of Factor XII (Hageman factor [HF])¹ on a negatively charged surface is known to initiate the coagulation (1), fibrinolytic (2), and kinin-forming pathways (3). Initially, it was suggested that only activated Hageman factor (HF_a) was necessary for the subsequent activation of each of these pathways and it was assumed that activation of HF occurred upon binding to certain negatively charged surfaces. Subsequently, the abnormal rate of activation of these three plasma proteolytic pathways in Fletcher trait plasma (4) was attributed to a deficiency of prekallikrein (5). This absence of prekallikrein was shown to result in a diminished rate of formation of HF_a (6). However, other observations (7-9) suggested that yet another factor was required for normal activation and function of the HF-dependent pathways.

The unravelling of this conundrum was made possible by the investigation of several unusual but asymptomatic patients (Williams, Fitzgerald, Flaujeac) whose plasma samples exhibited profound abnormalities of all the Hageman-dependent pathways (10-12). Assays for the known coagulation factors were normal except for prekallikrein which while diminished in four cases

¹ *Abbreviations used in this paper:* AGLMe, acetylglycyl lysine methyl ester; HF, Hageman factor; HF_a, activated Hageman factor; HF_f, Hageman factor fragments; HMW, kininogen, high molecular weight kininogen; TAME, tosyl-L-arginine methyl ester.

(10, 11, 13, 14) was insufficient to account for the observed abnormalities. Since then, two other patients (13, 14) have been described with similar abnormalities of the Hageman-dependent pathways. Three of the patients (10, 12, 13) had absent or very low levels of both high and low molecular weight kininogens whereas Fitzgerald (11) had 50% low molecular weight kininogen. A high molecular weight (HMW) subfraction of kininogen was able to correct the abnormalities in each of these patients' plasma. HMW kininogen therefore appeared to be a critical factor required for the functioning of HF-dependent coagulation, and fibrinolysis and for the activation of prekallikrein.

HMW kininogen could accelerate Hageman factor catalyzed reactions by three mechanisms. It might facilitate the transformation of unactivated HF to HF_a, enhance the conversion of HF or HF_a to its fragments (HF_f) and/or potentiate the action of activated HF upon its substrates. This report deals with the third possibility by examining the effect of HMW kininogen upon the ability of HF_f to activate prekallikrein and Factor XI and cleave the synthetic substrate acetylglycyl-lysine methyl ester.

METHODS

Bradykinin triacetate (Sandoz Ltd., Basel, Switzerland) was used as the standard for native bradykinin. Hexadimethrine bromide (Aldrich Chemical Co., Inc., Milwaukee, Wis.); *N*- α -tosyl-L-arginine methyl ester (TAMe), acetylglycyl L-lysine methyl ester (AGLMe), bovine serum albumin five times recrystallized, and agarose (Sigma Chemical Co., St. Louis, Mo.); quaternary aminoethyl-Sephadex A-50, sulphopropyl-Sephadex C-25, carboxymethyl (CM)-Sephadex C-50, Sephadex G-150, and G-200 (Pharmacia Fine Chemicals Div. of Pharmacia Inc., Piscataway, N. J.); diethylaminoethyl (DEAE)-52 cellulose and CM-52 cellulose (Whatman Chemicals, Div. W. & R. Balston, Maidstone, Kent, England); Bio-Gel A 0.5 m, (Bio-Rad Laboratories, Richmond, Calif.), kaolin (Fisher Scientific Co., Pittsburgh, Pa.), inosithin (mixed soybean phospholipids, Associated Concentrates, Woodside, N. Y.), and cyanogen bromide (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) were obtained from the sources indicated.

HF-deficient and Factor XI-deficient plasma were prepared from the blood of severely deficient patients collected by George King Bio-Medical, Inc., Salem, N. H., in 0.1 vol 3.8% citrate, centrifuged at 5,000 g for 20 min, fresh-frozen and stored in 1-ml aliquots at -70°C. Prekallikrein-deficient plasma was a gift from Dr. C. Abildgaard (University of California, Davis, Calif.). The blood was drawn and handled as described below for normal donors. Anti-human plasma kallikrein antibody was prepared in rabbits and adsorbed with prekallikrein-deficient plasma as described previously (15). Sheep antibody to human low molecular weight kininogen was prepared as previously reported (16, 17).

Preparation of plasma proteins. To obtain normal human plasma for use in assays, nine volumes of blood from healthy donors was drawn directly into plastic syringes containing one volume of 3.8% citrate. The blood was transferred to plastic tubes and centrifuged at 5,000 g at 4°C for 15 min to remove the cells. Plasma was removed and a pool was

made from 20 normal male donors. 1-ml aliquots of the pool were stored at -70°C and used immediately after thawing. Any residual plasma was discarded. Plasma from Ms. Williams was collected and handled identically.

Plasma utilized for the isolation of prekallikrein, and kininogen was collected in plastic syringes with the same citrate anticoagulant as described previously. For each 10 ml of blood drawn, 3.6 mg of hexadimethrine bromide in 0.1 ml of 0.15 M NaCl was added. The blood was centrifuged at 900 g for 20 min at 4°C and the plasma was removed with plastic pipettes. Plastic columns and test tubes were utilized throughout all chromatographic procedures to minimize contact activation of HF. Samples were concentrated by ultrafiltration through a PM-10 membrane (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). Gel filtration Sephadex G-150, alkaline disc electrophoresis, and sodium dodecyl sulfate gel electrophoresis were performed as described previously (18). Protein was approximated by absorbance at 280 nm, with E₂₈₀^{1%} assumed to equal 10.

Hageman factor fragments. Plasma utilized for the isolation of HF_f was fresh frozen plasma collected in acid citrate dextrose generously supplied by the American Red Cross. The HF prealbumin fragments were prepared by sequential fractionation of plasma with ethanol, isoelectric precipitation, ion-exchange chromatography utilizing DEAE-cellulose (DE-52) and CM-cellulose (CM-52), and gel filtration utilizing Sephadex G-200, as described by Bagdasarian et al. (18). These fragments have been shown to be similar to those produced by trypsin, kallikrein, and plasmin proteolysis of HF (19) in regard to molecular weight, electrophoretic migration, immunoreactivity, reaction with inhibitors, and functional activity. The specific activity of the preparation used was 1,670 activator units/mg and it contained 60 μ g/ml protein. One activator unit is that amount of activator which will release an amount of kallikrein which will hydrolyze 1 μ mol of TAMe/min at 37°C from 1 ml of normal plasma (20). One activator unit of HF_f produced no significant hydrolysis of TAMe (0.008 μ mol TAMe hydrolyzed/min per ml) at 37°C. The molecular weight estimated by the calibrated G-200 Sephadex was 32,000 \pm 2,000 and no contamination with unfragmented HF (mol wt 90,000) or "large activators" (mol wt 70,000) was detected. There was no detectable contamination with any of the HF substrates, plasminogen, plasmin, kininogen, or kallikrein.

Prekallikrein. Highly purified prekallikrein was prepared by the method of Liu and Colman (21). 300 ml of human plasma was stirred for 30 min at 4°C with 300 ml of quaternary aminoethyl-Sephadex A-50 in 5 mM sodium phosphate buffer pH 8.0 containing 100 μ g/ml of hexadimethrine bromide and filtered through Whatman 1 filter paper. The filtrate, containing IgG and the HF substrates, was then stirred again with 300 ml quaternary aminoethyl-Sephadex A-50 and the second filtrate was passed through an arginine Sepharose column. Arginine was coupled to Sepharose 4B by the method of Axén et al. (22) using cyanogen bromide. After the reaction with arginine was complete the uncoupled activated Sepharose 4B was chemically blocked with ethanolamine. The effluent was concentrated by ultrafiltration to 5 ml, fractionated on Sephadex G-200, and the prekallikrein peak (mol wt 100,000) was further fractionated on CM-Sephadex (Fig. 1a). This final step of purification eliminated an additional major protein which eluted at tubes 18-23. A single peak of prekallikrein was identified in tubes 34-42 which contained arginine esterase activity and kinin-forming activity, only after incubation with HF_f, while prekallikrein antigen corresponded to the peak of functional activity. This highly purified prekallikrein yielded a single major band upon alkaline disc gel electrophoresis (Fig. 1b).

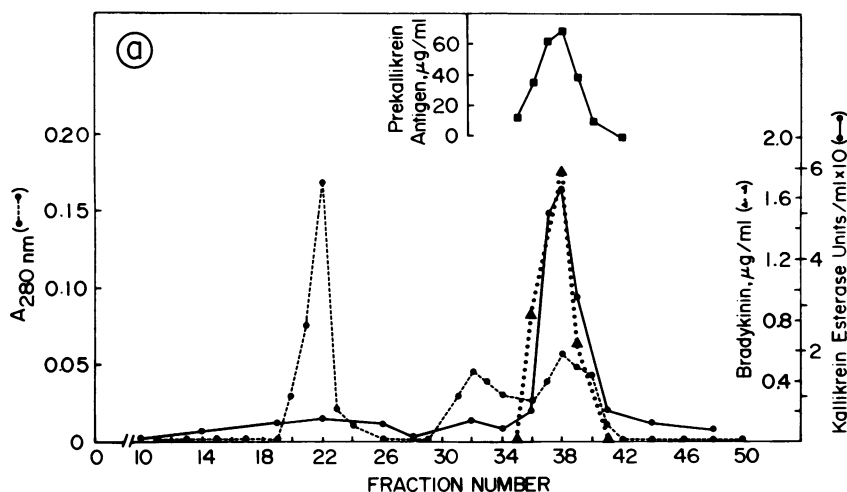


FIGURE 1a CM-Sephadex chromatography of prekallikrein. Prekallikrein (9 mg in 0.016 M phosphate, pH 6.5) was chromatographed on a CM-Sephadex column (2.5 × 24 cm) equilibrated with the same buffer at a flow rate of 21 ml/h. After washing for 12 fractions a linear gradient of NaCl was applied. The mixing chamber contained 200 ml of 0.016 M phosphate buffer, pH 6.5, in 0.1 M NaCl and the second chamber contained 200 ml of the same buffer in 0.6 M NaCl. 10-ml fractions were collected.

An identical unstained gel was sliced, and each slice was eluted with 0.2 ml of 0.05 M sodium phosphate buffer containing 0.15 M NaCl, pH 8.0. Upon incubation of the eluate for 18 min at 37°C with HF_r at a final concentration of 7.8 µg/ml, arginine esterase activity, was found in the slices that corresponded to the protein band seen. There was no preformed arginine esterase activity in the eluate incubated with buffer rather than HF_r. The concentration of prekallikrein was expressed as units of kallikrein esterase activity (see below) as determined after incubation with HF_r. The specific activity was 36 µmol TAME hydrolyzed/min per mg protein. One esterase unit of this prekallikrein concentration contained <0.01 U of HMW kininogen or Factor XI and displayed no measurable arginine esterase activity in the absence of HF_r.

Kininogen. Highly purified kininogens were prepared by the method of Pierce, Guimaraes, and Chenlu² described briefly in early reports (10, 23, 24).

Human plasma diluted 1:5 with distilled water containing 0.1 mg/ml hexadimethrine bromide was stirred with DE-23 cellulose at pH 6.0, 25°C. Elution was performed with 0.4 M Tris-HCl, pH 6.0. The eluate, adjusted to pH 7.1, was absorbed onto an anti-low molecular weight kininogen insolubilized by coupling to Sepharose 4B with cyanogen bromide. The kininogen was eluted with 8 M guanidine, dialyzed, and chromatographed on DEAE-cellulose at 4°C. Four main peaks of kininogen activity B1, B2, B3, and B4 were eluted by a gradient of 0.05–0.30 M sodium phosphate, pH 6.0. Gel filtration of the B4 peak on Bio-Gel A 0.5 m at 25°C yielded kininogens with apparent mol wt of 80,000, 160,000, and 225,000 called B4 α , B4 β , and B4 γ , respectively (24) which corrected the abnormalities in Williams trait plasma (10). The preparations of B4 γ and B4 β kininogen utilized in these experiments were both 0.1 mg/ml protein and contained 1.0 U/ml HMW kininogen as assayed by a functional clotting assay (see below). These two kininogens were used interchangeably and gave similar results and are

designated HMW kininogen. B4 γ kininogen yielded a single major band on alkaline disc gel electrophoresis (10). This HMW kininogen preparation at a concentration of 1.0 U/ml demonstrated <0.01 U/ml of Factor XI or prekallikrein, <0.3% of the Cl esterase inhibitor in normal plasma and zero kallikrein esterase units. No immunoprecipitates were demonstrable by radial immunodiffusion against antisera to Cl esterase inhibitor, α_2 -macroglobulin, α_1 -antitrypsin, α_1 -antichymotrypsin, and inter α_1 -antitrypsin. In addition, HMW kininogen failed to inhibit kallikrein in an amidolytic assay and plasmin in a fibrin plate assay. The low molecular weight kininogens B2 α and B3-2 α described previously (10)

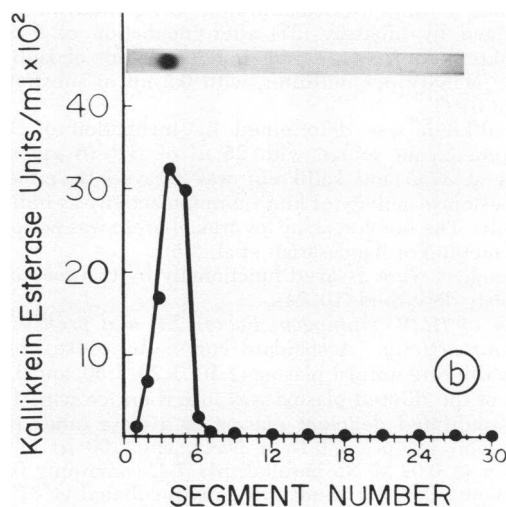


FIGURE 1b Disc gel electrophoresis of 50 µg purified prekallikrein from CM-Sephadex. Electrophoresis was performed as described in Methods. TAME esterase activity eluted from each segment was plotted.

² Manuscript in preparation.

TABLE I
Effect of HMW Kininogen on Factor XI Activation
by HF_f in Plasma

Plasma	HF _f μg/ml	HMW kininogen U/ml	XI _a formed U/ml
Normal	106	0	0.61
HF deficient	106	0	0.58
HMW kininogen deficient	53	0	0.04
	79	0	0.10
	106	0	0.20
	53	0.12	0.09
	79	0.12	0.22
	106	0.12	0.60

5 μl of HMW kininogen (1 U/ml) or 5 μl barbital buffer and 10–20 μl of HF_f (150 μg/ml) or buffer were incubated with 10 μl of plasma for 1 min at 25°C in a plastic test tube. The final volume was 35 μl. The assay for Factor XI_a was performed as described in the Methods. However, the incubation time was 37°C for 4 min. No coagulant was detected in the absence of HF_f.

also contained 0.1 mg/ml protein but had <0.01 U/ml HMW kininogen activity by a functional coagulation assay.

Assays of kinin-forming enzymes and substrates. Kallikrein activity was determined either by its arginine esterase activity or by its proteolytic activity. Esterase activity was determined with TAME as the substrate by a modification of the method of Siegelman et al. (25), which has been described in detail (26). Since HF_f was used as the activator of prekallikrein, kaolin was omitted. One unit of kallikrein esterase activity is the amount of kallikrein which will hydrolyze 1 μmol of TAME/min at 37°C. The proteolytic activity of kallikrein was measured by its ability to release bradykinin, a substrate consisting of heat-inactivated plasma, containing 3 mM *o*-phenanthroline (27). Bradykinin was quantitated by bioassay (27) after incubation of 5–25 μl of kallikrein source (15), containing one unit of kallikrein esterase activity per milliliter, with 0.2 ml of substrate for 2 min at 37°C.

Prekallikrein was determined by incubation of 25–50 μl of proenzyme source with 25 μl of HF_f (6 μg/ml) for 18 min at 37°C and kallikrein was assayed by either arginine esterase activity or kinin-forming activity as indicated in Results. The immunoassay for prekallikrein was performed by the method of Bagdasarian et al. (15).

Kininogens were assayed functionally by two methods as previously described (10, 24).

Assay of HMW kininogen, Factor XI, and prekallikrein coagulant activity. A standard curve was constructed by serially diluting normal plasma (1:10, 1:20, 1:50, and 1:100). 100 μl of the diluted plasma was mixed on ice with 100 μl of the undiluted deficient plasma in a glass tube. 100 μl of 5 mg/ml kaolin in 0.15 M NaCl, and 100 μl of 0.2% inosithin in 0.02 M Na barbital, pH 7.4, containing 0.15 M NaCl were added. The mixture was incubated at 37°C for 5 min except in the prekallikrein assay which was incubated for 2 min. The mixture was then recalcified with 100 μl of 0.03 M CaCl₂ in 0.15 M NaCl (28). A standard curve relating log clotting time to log dilution was plotted. A linear relationship was observed. Experimental samples were assayed by diluting

10 μl of the sample with 90 μl of the barbital buffer and performing the partial thromboplastin time in the same manner as the standard curve. The clotting time of the sample was then related to percent of normal pooled plasma by interpolation on the standard curve. One unit of Factor XI, prekallikrein, or HMW kininogen was defined as the amount present in 1 ml of normal plasma (pool of 20 normal individuals). Factor XI_a was assayed performing a partial thromboplastin time using Factor XI-deficient plasma in the absence of kaolin and in plastic tubes.

Assay of HF_f using AGLMe hydrolysis. 20 μl of HF_f (60 μg/ml) was incubated with 90 μl of various concentrations of AGLMe containing 2 mg/ml of bovine serum albumin at 37°C for time periods of 30–90 min, and the activity determined by a modification of the method described by Ulevitch et al. (29). The enzymatic activity of HF_f was linear with enzyme concentration for time periods extending to 90 min.

RESULTS

The effect of HMW kininogen on Factor XI and prekallikrein activation by HF_f in plasma

Factor XI activation. Previous studies have demonstrated (10, 12–14) or suggested that (11) plasma deficient in HMW kininogen also shows defective Factor XI activation by kaolin. To decide whether this coagulation abnormality was also attributable to an inability to potentiate the coagulant activity of HF_a, we used HF_f as the enzyme to attempt the conversion of Factor XI to Factor XI_a in plasma deficient in Factor XII and HMW kininogen (Table I). Activation of Factor XI in Factor XII-deficient plasma by HF_f was essentially the same as activation of Factor XI in normal plasma. In contrast, Factor XI_a formation in HMW kininogen-deficient plasma was diminished to about one-third of normal. This abnormality was fully corrected upon reconstitution of the plasma with HMW kininogen to 12% of normal pooled plasma, a level sufficient to correct the coagulation abnormality of HMW kininogen deficiency (10, 12, 13). When HMW kininogen was kept constant and the concentration of HF_f increased, an increase in the extent of Factor XI activation was observed. At each concentration of HF_f, a two to threefold increase in activation of Factor XI was observed.

Prekallikrein activation. Our previous study (10) demonstrated that plasma deficient in HMW kininogen does not support prekallikrein activation by kaolin despite adequate concentration of Factor XII and prekallikrein. This might be explained by abnormal surface activation and/or function of activated Factor XII in the absence of HMW kininogen. Therefore, activated HF in the form of HF_f was added to plasma congenitally deficient in Factor XII, prekallikrein, or HMW kininogen in the absence of kaolin (Table II). Prekallikrein activation was assessed by the generation of arginine esterase activity. HF_f generated 1.96 kallikrein esterase units upon incubation with normal plasma but generated only one-fourth of this value in

plasma deficient in HMW kininogen. When HMW kininogen was added to the HMW kininogen-deficient plasma half the kallikrein esterase units of normal plasma was generated. This level represents full correction since the prekallikrein content of this plasma has been shown to be 45–50% of normal whether assayed by coagulant activity, kinin release, or antigenically (10). The same formation of kallikrein was observed in the mixture of prekallikrein-deficient plasma with HMW kininogen-deficient plasma and HF-deficient plasma showed the same activation profile by HF_f as did normal plasma. In the absence of plasma, a combination of HMW kininogen and HF_f had no detectable arginine esterase activity.

Effect of HMW kininogen upon the activation of highly purified prekallikrein by HF_f

Generation of esterase activity. Prekallikrein was incubated at 37°C with HF_f in plastic tubes and the

TABLE II
Effect of HMW Kininogen on Prekallikrein Activation by HF_f in Plasma

Plasma	HF _f	HMW kininogen	Kallikrein esterase activity (units)
Normal	–	–	0
	+	–	1.96
HF deficient	–	–	0
	+	–	1.80
HMW kininogen deficient	–	–	0
	+	–	0.49
	+	+	0.98
Prekallikrein deficient	–	–	0
	+	–	0
	+	+	0
HMW kininogen deficient	+	–	0.98
	–	–	0
Prekallikrein deficient	+	–	0
	+	+	0

100 μl of plasma was mixed with 100 μl of 0.1 M sodium phosphate buffer, pH 7.6 containing 0.15 M NaCl. The mixture was incubated for 1 min at 25°C with 30 μl of HF_f in plastic tubes. The final concentration of HF_f was 1.8 μg/ml. The substrate TAME (0.05 M), dissolved in the same sodium phosphate buffer, was then added, the mixture was incubated at 37°C for 30 min, and assayed for kallikrein esterase activity. When HMW kininogen was added, it was used at a final concentration of 0.10 U/ml. A (+) indicates the presence of the reagent, a (–), the absence of the reagent. When HMW kininogen-deficient plasma was mixed with prekallikrein-deficient plasma, 100 μl of each plasma was used and the phosphate buffer was omitted. The results are means of duplicate experiments.

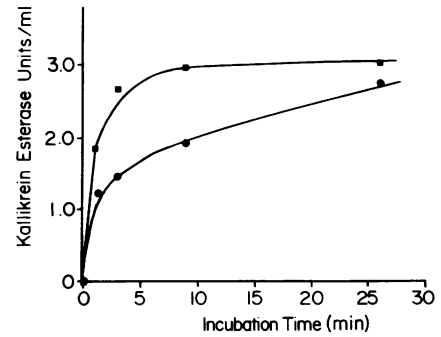


FIGURE 2 Prekallikrein (1.0 kallikrein esterase unit/ml) was incubated at 37°C with HF_f (3 μg/ml) in the presence (■) or absence of HMW kininogen (0.1 U/ml) (●). At appropriate time intervals, aliquots were removed and assayed for kallikrein activity as in the Methods. In this experiment and in all subsequent determinations of prekallikrein activation a “zero time” control (i.e. all reagents mixed but not incubated) was run and subtracted from all time points. The zero time control did not exceed 0.3 μmol TAME hydrolyzed/min per ml. The velocity in micromoles TAME hydrolyzed per minute per milliliter are plotted against time. The results are a mean of duplicate experiments.

formation of kallikrein was assayed by measuring its arginine esterase activity as a function of time (Fig. 2). Prekallikrein activation was rapid with 50% of the maximal activation occurring within the 1st min followed by a slower rate over the next 25 min. In the presence of HMW kininogen, which in all experiments was added before addition of HF_f, the initial rate increased about 1.5-fold without altering the maximum extent of activation. When the concentration of HF_f was decreased 5-fold (Fig. 3), the rate of conversion of

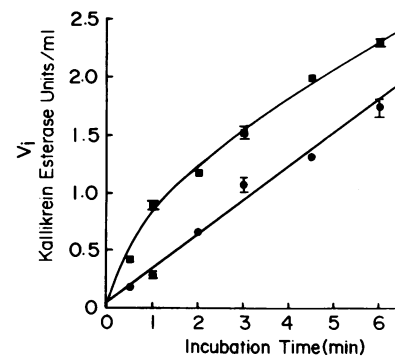


FIGURE 3 Prekallikrein (1.0 kallikrein esterase unit/ml) was incubated with HF_f (0.6 μg/ml) in the presence (■) or absence of HMW kininogen (●) at a concentration of 0.1 U/ml. At appropriate time intervals, aliquots were removed and assayed for kallikrein esterase activity as in Methods. The mean and SEM of four replicate experiments are shown. The equation of the line for the rate of kallikrein formation without HMW kininogen is $y = 0.021 + 0.298x$ ($r = 0.99$, $P < 0.001$). The significance of the differences at 1 min ($P < 0.001$) 3 min ($P < 0.01$) and 6 min ($P < 0.02$) were calculated by the unpaired Student's t test.

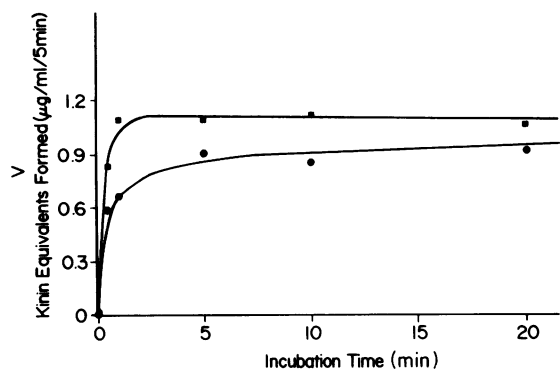


FIGURE 4 Prekallikrein (1.0 kallikrein esterase unit/ml) was incubated with HF_f (6 $\mu\text{g/ml}$) in the presence (■) and absence (●) of HMW kininogen (0.1 U/ml). At appropriate time intervals, aliquots were removed and assayed for release of kinin by bioassay as in Methods. A "zero time" control, which was subtracted from all values did not exceed 0.3 $\mu\text{g/ml}$ per 5 min kinin equivalents. The results are a mean of duplicate experiments.

prekallikrein to kallikrein over the first 6 min became linear. HMW kininogen augmented the initial rate of activation of prekallikrein threefold. When HMW kininogen (0.1 U/ml) was added to kallikrein (3 kallikrein esterase units/ml), the kallikrein esterase activity of the mixture was unchanged (3 U/ml). Thus, it ap-

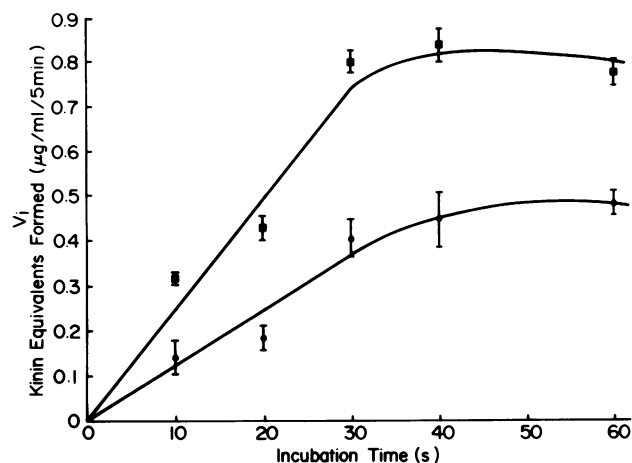


FIGURE 5 Prekallikrein (1.0 kallikrein esterase unit/ml) was incubated with HF_f (1.2 $\mu\text{g/ml}$) in the presence (■) and absence (●) of HMW kininogen at concentration of 0.1 U/ml. At appropriate time intervals, aliquots were removed and assayed for release of kinin from heated plasma by bioassay as in Methods. The mean and SEM of three replicate experiments are shown. The equation of the line for the rate of kallikrein formation without HMW kininogen is $y = -0.005 + 0.125x$ [$r = 0.92$, $P < 0.001$]. The equation for the line with HMW kininogen is $y = 0.007 + 0.25x$ [$r = 0.98$, $P < 0.001$]. The significance of the differences at 10 s. ($P < 0.02$), 20 s ($P < 0.01$), 30 s ($P < 0.01$), 40 s ($P < 0.05$), and 60 s ($P < 0.01$) were calculated by the unpaired Student's t test.

peared that HMW kininogen increased the rate of HF_f conversion of prekallikrein to kallikrein.

Generation of kinin-forming activity. HF_f was incubated with prekallikrein in the presence or absence of HMW kininogen and the kinin released from heated plasma was determined by bioassay (Fig. 4). An increased rate of formation of kallikrein proteolytic activity in the presence of HMW kininogen was observed similar to that seen with ester hydrolysis. HMW kininogen incubated with HF_f and prekallikrein generated 0.05 μg kinin/ml per 5 min which was 5% of the kinin released from the heated plasma by the same mixture (1.1 $\mu\text{g/ml}$ per 5 min). A mixture of prekallikrein (1.0 kallikrein esterase unit/ml) and HMW kininogen (0.1 U/ml) after incubation for 5 min released 0 $\mu\text{g/ml}$ kinin equivalents. Since the effect of HMW kininogen appeared to be upon the initial rate of prekallikrein activation rather than the yield of kallikrein, the kinetics of activation were followed over the first 60 s of the reaction after decreasing the HF_f concentration fivefold (Fig. 5). The initial rate of the reaction was linear with and without HMW kininogen for the first 30 s. Potentiation occurred at the earliest time measured (10 s) and was similar in magnitude (twofold) to the increase in initial rate observed when esterase activity was measured. Since no major differences in these experiments were evident whether kallikrein was assayed by esterase or kinin-releasing activity, only the esterase activity will be reported in the subsequent experiments.

Effect of HF_f concentration on acceleration of prekallikrein activation. Since HMW kininogen might affect the enzyme (HF_f) and/or the substrate (prekalli-

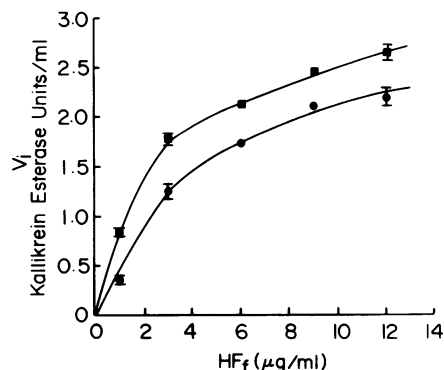


FIGURE 6 Prekallikrein (0.75 kallikrein esterase units) in 20 μl was incubated with various concentrations of HF_f for 1 min at 37°C in the presence (■) and absence (●) of HMW kininogen (final concentration 0.1 U/ml in total volume of 100 μl). The initial velocity (V_i) of kallikrein formation was determined by the arginine esterase assay after addition of 1 ml of 0.05 M TAME. The mean and SEM of four replicate experiments are shown. The significance of the differences at 1 μg ($P < 0.001$), 3 μg ($P < 0.001$), 6 μg ($P > 0.05$) and 12 μg ($P > 0.05$) HF_f/ml was determined using Student's t test.

krein) in the activation reaction, the initial rate of activation of prekallikrein by HF_t was measured as a function of the enzyme concentration (Fig. 6). Potentiation of prekallikrein conversion to kallikrein was observed in the presence of HMW kininogen. The percent enhancement was pronounced at low HF_t concentration, but was not significant at 6 and 12 $\mu\text{g/ml}$ HF_t . However the increase in kallikrein generated was similar for all concentrations of HF_t tested. A mixture of HF_t (12 $\mu\text{g/ml}$) and prekallikrein (0.75 kallikrein esterase units/ml) hydrolyzed 2.1 μmol TAME/min per ml while a mixture of the same concentration of prekallikrein and HMW kininogen (0.1 U/ml) hydrolyzed 0.003 μmol TAME/min per ml.

Effect of prekallikrein concentration on the rate of kallikrein formation. The initial rate of activation of prekallikrein by HF_t was next measured as a function of prekallikrein concentration (Fig. 7). The ability of HMW kininogen to augment the function of HF_t was apparent at low concentrations of prekallikrein. Under these conditions, there were no significant differences observed when prekallikrein concentrations were above 0.4 U/ml kallikrein esterase activity. At concen-

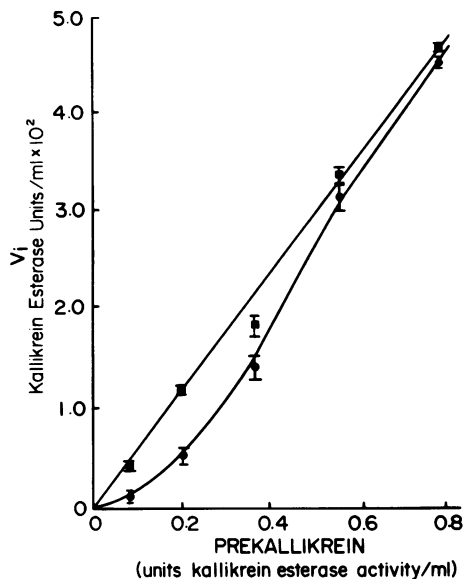


FIGURE 7 Prekallikrein (37.5 esterase units/ml) at various concentrations indicated on the abscissa was incubated with HF_t (6 $\mu\text{g/ml}$) in a total volume of 100 μl for 1 min at 37°C in the presence (■) and absence of HMW kininogen (●) (0.1 U/ml). The initial velocity (V_i) of kallikrein formation was determined by the arginine esterase activity. The mean and SEM of five determinations are shown. The significance of the differences at prekallikrein concentrations at kallikrein esterase units of 0.08 ($P < 0.01$), 0.20 ($P < 0.001$), 0.36 ($P < 0.05$), 0.56 ($P > 0.10$), 0.80 ($P > 0.10$) were determined using Student's t test. The equation of the line determined by linear regression for the rate of the presence of HMW kininogen is $y = 0.058x$ ($r = 0.99$, $P < 0.001$).

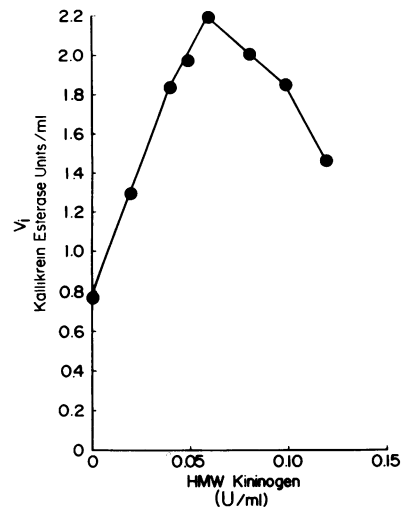


FIGURE 8 Prekallikrein (1.0 kallikrein esterase unit) was incubated with HF_t (0.6 $\mu\text{g/ml}$) for 3 min at 37°C in the presence of various concentrations of HMW kininogen. The results are a mean of triplicate experiments.

trations of 0.1 and 0.2 U/ml, a twofold potentiation by HMW kininogen was noted which was significant ($P < 0.01$) by Student's t test. The plot of initial velocity vs. substrate concentration does not describe a hyperbolic curve typical of Michaelis-Menten kinetics in the absence or presence of HMW kininogen. However, the kinetics approached a linear function in the presence of HMW kininogen.

Effect of HMW kininogen concentration on prekallikrein activation. The potentiation of HF_t conversion of prekallikrein to kallikrein is also a function of the concentration of HMW kininogen added. As shown in Fig. 8, augmentation of the activity of HF_t was maximal at 0.06 U/ml, of HMW kininogen (6 $\mu\text{g/ml}$ protein) and a further increase in HMW kininogen concentration yielded less potentiation. At the optimal concentration, a threefold augmentation of the initial rate was noted. Low molecular weight kininogens (B2 α and B3.2 α) (24) and bovine serum albumin all at concentrations ranging from 1 to 40 $\mu\text{g/ml}$ final concentration failed to increase prekallikrein conversion to kallikrein above that seen with HF_t alone. Thus, the effect is not merely one of preventing loss of kallikrein activity.

Effect of HMW kininogen on the esterase activity of HF_t . HMW kininogen might alter the substrate prekallikrein to increase its susceptibility to enzymatic attack or HMW kininogen might alter the enzyme HF_t to potentiate its function. In an attempt to distinguish between these two possibilities, the effect of HMW kininogen upon the ability of HF_t to hydrolyze the synthetic substrate AGLMe was examined (29). HMW kininogen was found to have no hydrolytic activity against this substrate. The concentration

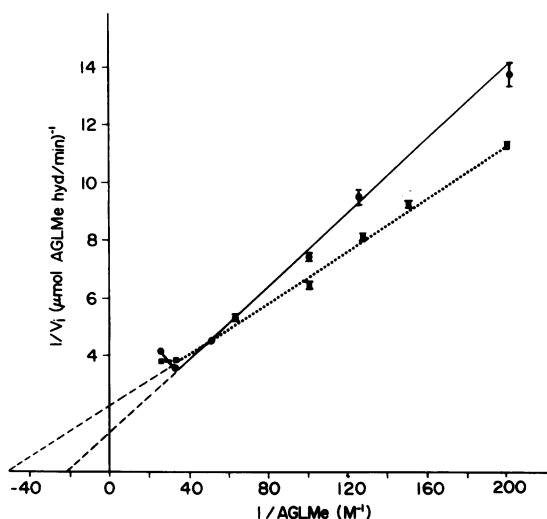


FIGURE 9 HF_f ($10.8 \mu\text{g/ml}$) was incubated with various concentrations of AGLMe in the presence (■) or absence of (●) HMW kininogen (0.09 U/ml). The mean and SEM of four determinations are shown.

of AGLMe was varied and its initial rate of hydrolysis by HF_f was measured (Fig. 9). A double reciprocal plot of the initial rate vs. the reciprocal of the substrate concentration was linear and the apparent K_m was 56 mM and the apparent V_{max} was $2.5 \mu\text{mol AGLMe hydrolyzed/min}$. Slight substrate inhibition was observed above 30 mM AGLMe . When HMW kininogen was added and AGLMe was below 25 mM , the initial rate (V_i) increases, and the V_{max} and K_m decrease. This experiment demonstrates potentiation of hydrolysis of AGLMe at low substrate concentrations and suggests that HMW kininogen potentiates the function of HF_f even in the absence of its natural substrates.

DISCUSSION

Earlier observations by Webster and Pierce (7) and Schiffman and Lee (8) have suggested that surface-bound Factor XII does not yield complete activation of Factor XI or prekallikrein, suggesting that at least one other factor is required for this interaction to proceed normally. The discovery of patients with severe defects of all the HF-dependent pathways, who had adequate levels of Factor XII and prekallikrein (10–14) has led to the identification of HMW kininogen (10, 12, 13) as a critical factor required for normal activation and/or function of HF. These patients fail to convert prekallikrein to kallikrein or release kinin upon incubation with kaolin. Although each plasma was subsequently shown to be deficient in HMW kininogen (10–13), three had little or no low

molecular weight kininogen (10, 12, 13) while Fitzgerald trait (11) had half normal levels of low molecular weight kininogen.

In this investigation we first examined the effect of HMW kininogen upon the ability of an activated form of Hageman factor (HF_f) to activate Factor XI and prekallikrein in plasma. From the data in Tables I and II, it appears clear that the action of HF_f to activate both Factor XI and prekallikrein was significantly augmented by the reconstitution of HMW kininogen-deficient plasma with HMW kininogen. It has been demonstrated that HF_f is a much less potent Factor XI activator than surface-activated Factor XII (19, 27, 30, 31). Thus in these experiments much higher concentrations of HF_f are needed for Factor XI activation (Table I) than for prekallikrein activation (Table II).

It appeared possible that the HMW kininogen enhanced the activity of the enzyme, HF_f , and/or altered the protein substrates prekallikrein and Factor XI to facilitate this interaction. The prekallikrein-activating pathway was then studied using highly purified components. Activation of prekallikrein occurred in the absence of HMW kininogen whether measured by arginine esterase activity (Figs. 1 and 2) or kinin formation (Figs. 3 and 4). However, in each case, the presence of HMW kininogen significantly potentiated the formation of kallikrein. This type of behavior is qualitatively analogous to that seen in the conversion of prothrombin to thrombin. Factor X_a cleaves prothrombin to form thrombin in the presence of phospholipid and calcium but the rate is slow (32). In the presence of a high molecular weight protein, Factor V, the rate is markedly enhanced (33). A similar situation is seen in the conversion of Factor X to Factor X_a by IX_a in which Factor VIII functions as a high molecular weight cofactor (34). Thus, HMW kininogen appears to be a cofactor which accelerates the conversion of prekallikrein to kallikrein by HF_f and the effect of HMW kininogen is on the initial rate of kallikrein formation (Figs. 2 and 4) while the extent of the reaction is not altered. Although HMW kininogen is a substrate of kallikrein in the concentrations used it did not affect the esterase or the kinin-forming activity of the enzyme.

To assess further the interrelationships of HF_f , prekallikrein, and HMW kininogen, we systematically varied the concentrations of enzyme, substrate, and cofactor, respectively. The relative potentiation of HF_f by HMW kininogen was greatest at low concentrations of HF_f . This is consistent with a role for HMW kininogen in augmenting the function of HF_f rather than a mechanism in which HMW kininogen functions independently of the enzyme. Thus, at a constant HF_f concentration, an increase in HMW kininogen

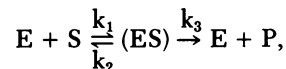
would increase the rate of prekallikrein activation. As shown in Fig. 8, such an effect of HMW kininogen was observed. In addition, the attainment of an optimal concentration of HMW kininogen for the augmentation of the function of HF_f is consistent with a stoichiometric relationship between the two proteins. Such a relationship of contact activation cofactor or HMW kininogen to surface-bound HF was suggested by Schiffman et al. (35), Meier et al. (36), and Griffin and Cochrane (37). The optimal concentration of HMW kininogen added in the purified system corresponds to the quantity which was required to fully reconstitute HMW kininogen-deficient plasma (10).

When the rate of prekallikrein activation was assessed as a function of prekallikrein concentration, a nonhyperbolic curve was obtained. Upon addition of HMW kininogen, this curve was shifted toward a linear relationship. These results suggest that HF_f is an enzyme with more than one binding site. One site (active site) may be more favorable for the interaction with prekallikrein and another site (effector site) may be more favorable for the interaction with HMW kininogen. When HMW kininogen is in an adequate concentration, it may stimulate catalysis by binding to the effector site. Saturation of this site would explain the stoichiometric relationship between HF_f and HMW kininogen.

All of these experiments suggest a direct interaction of HF_f with HMW kininogen. However, direct evidence comes from consideration of the effect of HMW kininogen on HF_f esterase activity. Like other proteolytic enzymes such as thrombin, plasmin, and kallikrein, HF_f and HF_a not only display proteolytic activity that converts prekallikrein to kallikrein but are also esterases which can hydrolyze AGLMe (29). Since HMW kininogen increased the activity of HF_f toward AGLMe in the same fashion as with its natural substrates, HMW kininogen probably has a direct effect on the enzyme HF_f. It should be emphasized that this is a model system and although it demonstrates a direct effect on HMW kininogen on HF_f, it does not rule out an additional effect on prekallikrein. Mandle et al. have recently reported that prekallikrein and HMW kininogen circulate in plasma as a complex (38) and it is therefore possible that HMW kininogen also facilitates the ability of prekallikrein to be cleaved by activated HF. HMW kininogen does not inhibit the activity of kallikrein in the fluid phase; interestingly, Ratnoff and Saito (39) have reported that kaolin inhibits the amidolytic activity of kallikrein and HMW kininogen reverses this inhibition. However, IgG and cytochrome *c* had the same effect thus, the specificity of this observation is questionable.

Both the apparent K_m and V_{max} were decreased

by HMW kininogen resulting in an increase in the initial rate at low AGLMe concentrations. In the Michaelis-Menten treatment, if



then

$$K_m = \frac{k_3 + k_2}{k_1}$$

and $V_{max} = k_3$ (E total). Since V_{max} decreases proportionately to K_m it is possible that only k_3 is decreased. An effect on enzyme-substrate complex dissociation was also found when the potentiation of Factor V upon Factor X_a esterase activated was analyzed (40). In the latter case, K_m and V_{max} increased so that Factor V promoted X_a arginine esterase activity only at high substrate concentration.

Thus it appears that HMW kininogen acts at multiple steps in the early phases of plasma proteolysis. First, it enhances the function of surface activated HF (36, 37, 41, 42) upon its substrates. This initial enhancement of HF_a generates sufficient kallikrein so that the more rapid enzymatic activation of HF by kallikrein can proceed (36, 37, 41). HMW kininogen also then facilitates this activation of HF by kallikrein (36, 37). Furthermore, HMW kininogen and prekallikrein circulate together as a complex (38), are presumably bound to surfaces together, and then interact with surface-bound HF. The present study demonstrates that HMW kininogen potentiates the activity of HF_f in the absence of a surface whether HF_f is assayed using its protein substrates prekallikrein and Factor XI or a synthetic ester. This finding suggests that HMW kininogen may augment the action of HF_a upon its natural substrates by an alteration of a HF catalytic site.

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