Hageman Factor Substrates

HUMAN PLASMA PREKALLIKREIN: MECHANISM OF ACTIVATION BY HAGEMAN FACTOR AND PARTICIPATION IN HAGEMAN FACTOR-DEPENDENT FIBRINOLYSIS*

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Two molecular forms of prekallikrein can be isolated from pooled normal human plasma. Their approximate molecular weights by sodium dodecyl sulfate-gel electrophoresis are 88,000 and 85,000. The two hands observed are shown to represent prekallikrein by functional, immunocromatographic, and structural criteria. Both forms are cleaved by activated Hageman factor, they appear to share antigenic determinants, they are not interconvertible upon incubation with activated Hageman factor or kallikrein, and the ratio of kinin-generating, and plasminogen-activating activities of the preparations is independent of the relative proportion of each band. Activated Factor XII converts prekallikrein to kallikrein by limited proteolysis and two disulfide-linked chains designated kallikrein heavy chain (M_r = 52,000) and kallikrein light chains (M_r = 36,000 or 33,000) are formed. The active site is associated with the light chains as assessed by incorporation of [H]diisopropyl fluorophosphate. No disassociable fragments were observed in the absence of reducing agents. However, kallikrein could digest prekallikrein to diminish its molecular weight by 10,000. In addition, two factors capable of activating plasminogen to plasmin have been isolated; one is identified as kallikrein. The second principle fractionates with Factor XI and is demonstrable in normal and prekallikrein-deficient plasma.

Prekallikrein is a γ-globulin proenzyme that circulates in plasma complexed to high molecular weight kininogen (1). Both prekallikrein (2-4) and HMW' kininogen (5-8) are required for the optimal activation and function of Hageman factor. Thus, plasmas deficient in prekallikrein or HMW kininogen have prolonged partial thromboplastin times as well as diminished rates of kaolin-activatable fibrinolysis.

Prekallikrein is converted by activated Hageman factor (9) to kallikrein and kallikrein cleaves HMW kininogen to liberate the vasoactive peptide bradykinin. However, published studies on the ability of kallikrein to convert plasminogen to plasmin are inconclusive. Colman first reported that kallikrein could activate plasminogen (10). Kaplan and Austen subsequently demonstrated a Hageman factor-activatable plasma factor called plasminogen proactivator, which upon activation, was able to convert plasminogen to plasmin (11). These authors proposed that plasminogen proactivator was responsible for the plasminogen-converting activity in prekallikrein samples. This postulate was challenged by Laake and Venneröd based upon their inability to separate prekallikrein from plasminogen proactivator activity and they concluded that kallikrein and plasminogen-activating activities are functions of the same molecule (12).

We have examined the mechanism of prekallikrein activation by HF and the ability of purified kallikrein to convert plasminogen to plasmin. Our findings can be summarized as follows. (a) Two molecular species of prekallikrein can be isolated from pooled normal human plasma. (b) Activated Hageman factor converts both forms of prekallikrein to kallikrein by limited proteolysis. The single chain of prekallikrein is cleaved at a single point and the two kallikrein chains formed are linked by one or more disulfide bonds. (c) Purified kallikrein directly converts plasminogen to plasmin. (d) An additional plasminogen-activating principle, which fractionates with Factor XI, and is distinct from prekallikrein, can be isolated from either normal plasma or prekallikrein-deficient plasma.

MATERIALS AND METHODS

Bradykinin standard (bradykinin triacetate, Sandoz Ltd., Basel, Switzerland); hexadimethrine bromide and diPFP, (Alrich Chemical Co., Inc., Milwaukee, Wisc.); Enzodiffusion fibrin plates and streptokinase (Hyland Division, Travenol Laboratories Inc., Costa, Mesa, Calif.), quaternary ammonium (QAE) Sephades A-50, sulfo- propyl (SP) Sephadex G-25, carboxymethyl (CM) Sepharose CL-6B, Sephadex G-100, Sephadex G-150, Sepharose 4B, and molecular weight standards (aldolase, ovalbumin, chymotrypsin, ribonuclease A, and blue dextran) (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.); homestatic phosphatase (cephalin) (ICN Nutritional Biochemicals Division, Cleveland, Ohio), kaolin (Fishier Chemical Co., Fair Lawn, N.J.); [*H]PrF,PF (3.9 Ci/mmol) (Amersham/Searle, Arlington Heights, Ill.); Aquasol, 2,5-diphenyloxazole (PPO), and sodium [*H]iodide (New England Nuclear Co., Boston, Mass.); acrylamide, N,N'-methylenebisacrylamide, Coomassie brilliant blue R-250, and bromphenol blue (Bio-Rad Laboratories, Richmond, Calif.); agarose, disodium, and tetrasodium ethylenediaminetetraacetate; transfer- rin, Chemical Co., St. Louis, Mo.); mercaptoethanol (Eastman Organic Chemical Co., Rochester, N.Y.); Bz-Pro-Phe-Arg-pNA HCl (Pentapharm LTD, Basel, Switzerland); and Trasylol (Calbiochem, La Jolla, Calif.) were obtained as indicated. Hageman factor-defi-
cient plasma containing 0.38% citrate was obtained from Sera Tox Biologicals, New Brunswick, N.J. Prekallikrein-deficient plasma was a gift from Dr. C. Albildgaard (University of California, Davis, Calif.). Factor XI-deficient plasma was obtained from George King Bio-medics, Inc. (Salem, N.H.). Purified Cl INH was kindly provided by Dr. Peter Harpel (Cornell Medical School, New York, N.Y.) and has been previously characterized (13). Purified β2-glycoprotein I was supplied by Behring Diagnostics (Somerville, N.J.).

Preparation of Plasma Proteins

Prekallikrein was isolated from 2 or 4 liters of fresh human plasma by a modification of a method previously described for the isolation of Factor XI (14). Platelet-poor human plasma containing 0.38% citrate and 0.36 mg/ml of hexadimethrine bromide was obtained within 3 h of collection. iPr2PF was added to achieve a final concentration of 10⁻⁴ M and the plasma was desalted on a 15-liter column of Sephadex G-25 equilibrated with pH 8.0 Tris/HCl buffer (0.01 M Tris, 10⁻⁴ M iPr2PF, 10⁻³ M EDTA). Fractions with a conductance less than 2 ms were pooled and applied to a column (19 x 25 cm) of QAE-Sephadex A-60 equilibrated with the same Tris buffer. This plasma collection as well as the chromatography to this point was done at room temperature (22-24°C). The γ-globulin fall-through from this column was applied at 4°C, without concentration or dialysis to a 1-liter column of CM-Sepharose CL-6B equilibrated in 0.003 M phosphate buffer, pH 6.0. Fractions with prekallikrein activity were pooled, concentrated by ultrafiltration through an Amicon UM-10 membrane, and fractionated on Sephadex G-150. The prekallikrein-containing fractions were then combined and passed over an immunoadsorbent prepared with sheep antibody to human IgG and β2-glycoprotein I coupled (15) to Sepharose 4B. All buffers were made 10⁻⁴ M in iPr2PF and iPr2PF was added to pooled fractions prior to concentration (19°C) (16). Protein concentration was estimated by absorbance at 280 nm with A₂₈₀ assumed to equal 10 or by the Lowry method (16). The color reaction was read at 700 nm and unknown samples were compared to a standard curve for human IgG. The final prekallikrein preparations contained no detectable Factor XI or β₂-glycoprotein I. Contamination with IgG was less than 2% by weight. The final prekallikrein contained 25 μg/ml Factor XI and 1% plasmin and gave a single band of molecular weight 95,000 upon SDS-gel electrophoresis after reduction and alkylation.

Prekallikrein samples were dialyzed into NaCl/P₄ and activated to kallikrein by incubation with an equal volume of Hageman factor (HF) (see below). Prekallikrein (0.25 to 1.0 mg/ml) was maximally activated in 10 to 15 min under these conditions. In experiments on the kinetics of prekallikrein activation by HF, a suboptimal concentration of HF, was chosen such that maximal activation occurred in greater than 30 min but less than 60 min.

Hageman Factor Fragments

Hageman factor fragments were prepared from Hageman factor which had activated during purification (9) or were isolated from aceton-activated human plasma (19) utilizing sequential chromatography on QAE-Sephadex, SP-Sephadex, and Sephadex G-100 as previously reported (9). A functionally pure reagent was obtained containing no detectable plasmin, plasminogen, prekallikrein, or Kallikrein. The sample was preincubated at room temperature with thrombin was detected; hirudin was added to these preparations to a final concentration of 2.0 units/ml which completely inactivated the thrombin and had no effect upon HF. Preparations were quantitated by bioassay on a guinea pig ileum (9) and adjusted such that 5 μl of Hageman factor fragment generated 10 ng of bradykinin following a 2-min incubation at 37°C with 0.2 ml of fresh EDTA plasma.

Plasminogen

Plasminogen was prepared by affinity chromatography using the procedure of Deutsch and Mertz (20). The preparation contained less than 1% plasmin and gave a single band of molecular weight 95,000 upon SDS-gel electrophoresis after reduction and alkylation.

Assays

Coagulation Assays — The PTT was measured by the method of Proctor and Rapaport (21). Hageman factor, prekallikrein, and Factor XI were determined by a modification of the PTT using congenitally deficient plasma (3). Assays of Kinin-forming Proteins — The proteolytic activity of kallikrein was routinely measured by its ability to release bradykinin from heat-inactivated plasma (8). Twenty-five microliters of kallikrein source was incubated with 0.2 ml of substrate at 37°C and the bradykinin quantitated by bioassay.

Prekallikrein was determined by incubation of 25 μl of proenzyme source with 25 μl of Hageman factor fragments (25 μg/ml) for 5 min at 37°C and determining the kallikrein generated.

Fibrinolytic assays — Plasmin was assayed with Hyland fibrin plates as previously described (8). A standard curve relating ring diameter to plasmin concentration was obtained by activating 0.2 ml of a reference preparation of purified plasminogen containing 200 μg/ml with 140 units of streptokinase for 30 min at 37°C; a linear plot relating log plasmin concentration to ring diameter was obtained between 6 and 100 μg of plasminogen/ml. Plasminogen activator was assayed by incubating 20 μl of plasminogen activator source with 20 μl of plasminogen (200 μg/ml) for 1 h at 37°C, and determining the plasmin generated in a fibrin plate.

Amidolytic Activity of Kallikrein

Buffer or sample (0 to 100 μl) was diluted with 1 ml of Tris/imidazole buffer, pH 7.9, and incubated at 37°C for 2 min. One hundred and fifty microliters of the prekallikrein substrate a-benzoyl-Pro-Phe-Arg-p-nitroanilide-HCl (1 mM) was added and the absorbance at 405 nm was recorded for 5 min in a Gilford spectrophotometer equipped with a thermostated (37°C) cuvette holder.

Autoradiography

125I-labeled samples were detected with medical x-ray film (Eastman Kodak, Rochester, N.Y.). 125I-labeled samples were processed according to the method of Bonner and Laskey (22).

Polyacrylamide Gel Electrophoresis

Alkaline disc gel electrophoresis was performed according to the method of Ornstein and Davis (23). SDS-polyacrylamide gel electrophoresis was run using the same buffer system as modified by King and Laemmli (24) with the exception that 4 M urea was added to the sample buffer. Electrophoresis was carried out in tubes (6 x 100 mm) or in a 1.5-mm vertical slab (Bio-Rad Laboratories, N.Y.). Molecular weights (Mₐ) of prekallikrein and kallikrein were estimated in reduced samples using reduced carbonic anhydrase (Mₐ = 30,000), ovalbumin (Mₐ = 43,000), transferrin (Mₐ = 90,000), and β-galactosidase (Mₐ = 130,000) as standards.

Immunoelectrophoresis, Ouchterlony Double Diffusion

Immunoelectrophoresis was performed on microscope slides in 1% agarose containing Veronal buffer, pH 8.6, 0.075 μ, and 0.01 M EDTA. Rabbit anti-human prekallikrein was prepared by injection of 20 to 50 μg of prekallikrein emulsified in complete Freund’s adjuvant into the popliteal lymph nodes of New Zealand rabbits. Intramuscular booster injections were given 2 and 8 weeks later with 100 μg of antigen emulsified in incomplete Freund’s adjuvant. Sheep antiprekallikrein was prepared as previously described (25). Both antisera were monospecific for prekallikrein after absorption with either purified human gamma globulin or prekallikrein-deficient sheep. Sheep anti-human IgG and anti-β₂-glycoprotein I was prepared by intramuscular injections with a mixture of 100 μg of each purified protein emulsified in incomplete Freund’s adjuvant followed by two intradermal booster injections with the same proteins emulsified in incomplete Freund’s adjuvant.

Statistical Methods

Straight lines were fitted to data points by the method of least squares and the fit judged by calculating correlation coefficients (r). Peak I samples were compared to other prekallikrein preparations (Fig. 12 and 13) by adding the distances of these points to the fitted line and applying Student’s t test.

RESULTS

Characterization of Prekallikrein and Kallikrein — Prekallikrein isolated from 2 liters of fresh human plasma was subjected to SDS-polyacrylamide gel electrophoresis in 9% acrylamide gels without reducing agents (Fig. 1). One major and
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FIG. 1 (left). SDS-polyacrylamide gel electrophoresis of 25 μg of purified human prekallikrein.

FIG. 2 (right). Sephadex G-150 (superfine) gel filtration of purified human prekallikrein. The ordinate is amidolytic activity generated after incubating column fractions with HF₆ and α-benzoyl-Pro-Phe-Arg-p-nitroanilide. The letter designations correspond to the SDS-polyacrylamide gel electrophoresis assay shown above the chromatogram.

FIG. 3. Immunoelectrophoresis of prekallikrein against two dilutions of rabbit antiserum to human prekallikrein absorbed with purified human IgG. The anode is to the right. The upper trough contains a 1:4 dilution of the antiserum and the lower trough undiluted antiserum.

only one minor band can be seen. These two bands were observed in all our prekallikrein preparations; however, the relative proportion of each band varied. Purified prekallikrein was next fractionated on a column (2.5 x 150 cm) of Sephadex G-150 (superfine) in order to determine whether these proteins could be resolved by an additional technique which reflects differences in molecular size. The prekallikrein was located by the amidolytic assay as described under "Materials and Methods." Samples were then taken across the peak and electrophoresed on SDS-polyacrylamide gel electrophoresis as shown in Fig. 2. The slower migrating band on SDS-polyacrylamide gel electrophoresis eluted earlier on gel filtration, again suggesting a difference in molecular size. However, both slower and faster species were contained within a single functional peak.

In order to investigate the antigenic relationship of the two proteins seen in our prekallikrein preparations, antibody to prekallikrein was reacted with a preparation having nearly equal amounts of the faster and slower migrating components. Only one precipitin arc was seen upon immunoelectrophoresis (Fig. 3) and a single line was obtained upon double diffusion against a wide range of antibody and antigen concentrations.

We next examined the change in SDS-polyacrylamide gel electrophoresis pattern when prekallikrein was activated to kallikrein by HF₆. In nonreduced samples there was no significant change in mobility when prekallikrein was converted to kallikrein. Both samples showed two bands and the relative proportion of these bands did not change. In the next experiment, 131I-prekallikrein and 125I-kallikrein were mixed and electrophoresed on the same SDS-gel. The gel was then sliced and counted. As shown in Fig. 4, 131I-prekallikrein and 125I-kallikrein counts were superimposable. Fig. 5 compares SDS-polyacrylamide gel electrophoresis patterns of prekallikrein and kallikrein in reduced and nonreduced samples. Reduced prekallikrein exhibited a slower relative mobility, presumably due to increased unfolding upon reduction of intrachain disulfide bonds. However, the standards exhibited this same change in mobility and the calculated molecular weights of reduced and nonreduced prekallikrein did not differ. Although it is not clear in this gel, the double-banded pattern of prekallikrein was not altered by reduction (see Fig. 7). Reduced kallikrein preparations had three new bands with faster mobility, while nonreduced kallikrein had two bands that were not distinguishable from prekallikrein. Thus prekallikrein is activated to kallikrein without significant change in molecular weight. It also follows that the double-banded pattern observed cannot be attributed to kallikrein contaminating our prekallikrein preparations. Fig. 6 shows a schematic diagram of this banding pattern including an estimated molecular weight and our nomenclature for each band. The molecular weight of prekallikreins I and II was accounted for the sum of the molecular weights of the heavy chain and light chains I and II, respectively. Thus, the molecular weight difference between prekallikreins I and II was reflected for by the sum of the molecular weights of the heavy chain and light chains I and II, respectively. Thus, the molecular weight difference between prekallikreins I and II was reflected for by the sum of the molecular weights of the heavy chain and light chains I and II. Thirty micrograms of kallikrein were then incubated with 10⁻³ M [3H]PrP, reduced, and subjected to SDS-polyacrylamide gel electrophoresis. When the stained gel was assessed by
radioautography (Fig. 7), both light chains incorporated the radiolabel and no [3H]Pr$_2$PF was incorporated into the heavy chain.

**Kinetics of Prekallikrein Activation by HF** — One-half milliliter of prekallikrein (0.8 mg/ml) plus $^{131}$I-prekallikrein were mixed with a suboptimal concentration of HF$_2$ (see "Materials and Methods") and incubated at 37°. Samples for polyacrylamide gel electrophoresis analysis were then withdrawn at time intervals up to 1 h and added to SDS buffer containing 2% SDS, 0.2% mercaptoethanol, 8 M urea, and 0.02 M EDTA. Samples for amidolytic assays (20 μl) were added to 1 ml of cold Tris/imidazole buffer, and assayed within 5 min of sampling. The results of SDS-polyacrylamide gel electrophoresis analysis are shown in Fig. 8. Controls included prekallikrein incubated at 4° or 37° with buffer for 60 min in the absence of HF, and a 0-min control in which HF, was added after the SDS-buffer and the mixture then incubated for 60 min. These controls were not different, indicating that prekallikrein was not cleaved in the absence of HF, and that SDS buffer immediately stopped the reaction. In prekallikrein samples incubated with HF, a progressive loss of protein from the prekallikrein region was accompanied by the appearance of kallikrein heavy and light chains. Both prekallikrein I and II appeared to be converted to kallikrein at the same rate and the proportion of stained protein appeared to be conserved between prekallikrein I and II and kallikrein light chains I and II. The gel was then sliced and counted for $^{131}$I. The weight of the gel was then calculated for each sample and this weight was divided by the value for the 60-min sample. Fig. 9 compares the rate of cleavage as reflected by the rate of the heavy chain formation with the genesis of amidolytic activity. Although similar, the kinetic curves are not identical. The generation of amidolytic activity appeared to precede cleavage.

We therefore examined the possibility that the observed bond cleavage is caused by the kallikrein generated rather than by HF, The kinetics of activation of prekallikrein were

![Fig. 5. SDS-polyacrylamide gel electrophoresis of 30 μg of prekallikrein and kallikrein. From left to right the gels are: reduced prekallikrein, nonreduced prekallikrein, reduced kallikrein, and nonreduced kallikrein.](image)

![Fig. 6. Diagrammatic representation of the SDS-polyacrylamide gel electrophoresis patterns from reduced samples of prekallikrein and kallikrein. Our nomenclature for the bands and estimates of their respective molecular weights are shown.](image)

![Fig. 7 (left). Autoradiogram of a reduced SDS-polyacrylamide gel electrophoresis of $^{[3H]}$Pr$_2$PF-treated light chains showing incorporation of Pr$_2$PF into both kallikrein light chains (left gel). An autoradiogram of a mixture of $^{[3H]}$-labeled kallikrein and prekallikrein is shown for comparison (right gel).](image)

![Fig. 8 (right). Kinetics of prekallikrein activation by HF, From left to right the gels contain the starting material followed by 0-, 2-, 5-, 10-, 15-, 25-, 40-, and 60-min time points after addition of HF, The final sample is a premixed control of SDS-buffer plus starting material and HF, incubated at 37° for 60 min.](image)
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Prekallikrein: Activation and Role in Fibrinolysis studied in the presence or absence of 2000 units/ml of Trasylol. Under these conditions, Trasylol did not inhibit HF, but completely inactivated kallikrein in less than 2 min as assessed by kinin generation or amidolytic activity. The SDS-polyacrylamide gel electrophoresis shown in Fig. 10 represents one such experiment. No significant difference in cleavage rate with or without Trasylol could be detected during a 1-h time course. Therefore the cleavage observed was not caused by the kallikrein generated.

To further investigate the ability of kallikrein to digest prekallikrein, 125I-labeled prekallikrein was incubated with HF, activated prekallikrein from which the HF, had been removed by ion exchange chromatography on QAE-Sephadex. The concentration of kallikrein chosen was twice the amount of activatable prekallikrein. As can be seen in the autoradiogram (Fig. 11), kallikrein digested prekallikreins I and II to yield prekallikreins whose molecular weights were diminished by 10,000 (last two gels). This cleavage was inhibited by addition of Trasylol to the incubation mixture.

Relationship of Prekallikrein to Plasminogen Proactivator - The prekallikrein utilized in the aforementioned studies corrected the functional abnormalities in prekallikrein-deficient plasma and upon activation not only digested HMW kininogen to liberate bradykinin, but also activated plasminogen to yield the fibrinolytic enzyme plasmin. Since plasminogen proactivator was previously reported to be similar to, but separable from prekallikrein (9), we attempted to ascertain whether the two bands observed on SDS-polyacrylamide gel electrophoresis might represent two different Hageman factor substrates or two molecular forms of prekallikrein. Preparations containing different quantities of prekallikrein at various stages of purification as well as purified prekallikrein with disproportionate amounts of prekallikrein I and II were assayed for amidolytic activity, bradykinin-generating ability, and plasminogen-activating activity. A linear relationship between bradykinin generation and p-nitroanilide liberation was observed in all preparations (Fig. 12). The correlation was highly significant (r = 0.78, p < 0.001) indicating that both assays are reflecting the same functional entity. When amidolytic activity or bradykinin-generating activity was compared to plasmin generation, a significant correlation was again observed (r = 0.99, p < 0.001) with the exception of the initial QAE-Sephadex effluent (Peak I) samples (Fig. 13). The data points from Peak I samples were pooled and compared to the data from the 12 remaining samples (see "Materials and Methods"). Peak I sample had significantly more (t = 8.78, p < 0.001) fibrinolytic activity relative to amidolytic activity, suggesting that it contained an additional fibrinolytic factor. All other samples tested had the same relative amount of p-nitroaniline, bradykinin, and plasmin-generating activity.

The ability of the inactivator of the first component of complement (Cl INH) to inhibit the amidolytic and plasminogen-activating activity of prekallikrein was assayed. The inactivator appeared to inhibit amidolytic activity but not the other two activities. The specificity of this inhibition is not known.

Fig. 9. Kinetics of prekallikrein activation by HF. All points are compared to the 60-min values which are defined as 100% activation. Open circles represent appearance of amidolytic activity. The closed circles are the rate of cleavage assessed by the increase in radiolabel in the kallikrein heavy chain position on SDS-polyacrylamide gel electrophoresis.

Fig. 10. Autoradiogram of 125I-labeled prekallikrein activated in the presence or absence of 2000 units/ml of Trasylol. The first five gels from left to right have prekallikrein plus HF, plus Trasylol incubated for 0, 5, 10, 20, and 45 min. The next five gels contain prekallikrein plus HF, plus buffer incubated 0, 5, 10, 20 and 45 min. The final sample is a prekallikrein control.

Fig. 11. Autoradiogram of 125I-labeled prekallikrein plus nonlabeled kallikrein. The samples from left to right are: prekallikrein plus kallikrein plus 2000 units/ml of Trasylol incubated for 0, 30, and 60 min; followed by prekallikrein plus kallikrein incubated for 0, 30, and 60 min without any Trasylol.
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In I I 1 .l .2 .3 KALLIKREIN UNITS/ml

FIG. 12. The relationship of bradykinin generation and liberation of p-nitroaniline from a-benzoyl-Pro-Phe-Arg-p-nitroanilide by kallikrein preparations at various stages of purification. The initial QAE-Sephadex effluent is shown by the open circles.

FIG. 14. Fibrin plate assay demonstrating the ability of Cl INH to inhibit the plasminogen-activating activity of kallikrein. Kallikrein was incubated for 30 min at 37° with either buffer or Cl INH and each sample was then passed over QAE-Sephadex. The upper plate shows the plasminogen-activating activity of fractions obtained with kallikrein and buffer and the lower plate shows the activity of the corresponding fractions obtained with kallikrein and Cl INH.

FIG. 13. The relationship of plasmin generation to amidolytic activity of kallikrein preparations at the initial stage of purification (QAE-Sephadex effluent, open circles) and later stages of purification (closed circles). The QAE-Sephadex effluent had a significantly greater ratio of plasmin generating to amidolytic activity.

The previous experiments (Fig. 13) suggest that kallikrein is one, but not the only factor in Peak I which functions as a plasminogen activator. Prekallikrein (G-150 stage) was activated with HF, and incubated for 30 min (37°) with buffer or Cl INH. The amount of Cl INH used in these experiments was determined in preliminary experiments to be at twice the concentration necessary to inhibit all the detectable bradykinin generation or amidolytic activity. The inactivated mixture and kallikrein control were then fractionated on 5-ml columns of QAE-Sephadex equilibrated in 0.003 M phosphate buffer, pH 8, to remove excess Cl INH. The effluents were assayed for amidolytic and plasminogen-activating activity. The initial fall-through of the control column had both plasmin-generating and p-nitroaniline-liberating activity while neither activity was demonstrable in the QAE-Sephadex fall-through of the Cl INH-kallikrein sample (Fig. 14).

The experiments (Fig. 13) suggest that kallikrein is one, but not the only factor in Peak I which functions as a plasminogen activator. We next fractionated 200 ml of normal and prekallikrein-deficient plasma on a column (18 x 25 cm) of QAE-Sephadex equilibrated in 0.003 M PO4, pH 8. The effluent of each column was concentrated to 50 ml and assayed for amidolytic, kinin-generating, and plasminogen-activating activity. No detectable amidolytic or kinin-generating activity was detectable in the effluent from prekallikrein-deficient plasma before or after activation with HF. Amidolytic or kinin assays were sensitive to less than 10% of the activity recovered in the effluent of normal plasma. However, the effluents obtained from the normal and prekallikrein-deficient plasma contained plasminogen-activating activity which could be increased by the addition of HF. The prekallikrein-deficient effluent contained approximately one-third of the plasminogen-activating activity of the normal plasma effluent.

The QAE-Sephadex effluent obtained from the prekallikrein-deficient plasma was applied to a column (5 x 40 cm) of SP-Sephadex equilibrated in 0.003 M phosphate buffer, pH 6, and the column eluted with a sodium chloride gradient (11, 26). The eluate was then assayed for prekallikrein, Factor XI, and plasminogen-activating activity. A single peak of plasminogen-activating activity which superimposed the Factor XI peak was observed (Fig. 15), while no peak of plasminogen-activating activity was found corresponding to the elution position of prekallikrein. The normal chromatogram had a
prekallikrein peak as well as a Factor XI peak and plasminogen-activating activity was associated with each peak.

**DISCUSSION**

Two forms of prekallikrein, designated prekallikrein I and II, could be isolated from pooled normal human plasma. Prekallikreins I and II had apparent molecular weights of 88,000 and 85,000 in SDS-polyacrylamide gel electrophoresis and were distinguished by reduced or nonreduced SDS-polyacrylamide gel electrophoresis as well as gel filtration under non-dissociating conditions. Activated Hageman factor converts prekallikrein to kallikrein by limited proteolytic digestion. The single chain of prekallikrein is cleaved and two disulfide-linked chains designated kallikrein heavy and light chains are formed. In the absence of reducing agents, the active enzyme did not differ from the proenzyme in terms of size or charge. Kallikreins I and II consisted of one heavy chain (M₀ = 52,000) and one light chain (M₀ = 36,000 or 33,000) demonstrable in reduced SDS-polyacrylamide gel electrophoresis. The difference in molecular weight of prekallikreins I and II was reflected in kallikrein light chains I and II. The evidence that both bands represent prekallikrein is indirect, but persuasive: (a) both forms are activatable by HF, and yield similar cleavage patterns; (b) they contain the same antigenic determinants; (c) they are not interconvertible upon activation with HF, or upon digestion of prekallikrein by kallikrein; (d) [³⁵S]HF-iPr-PF is incorporated into both light chains, indicating that each contains an active site serine; (e) plasminogen-activating activity is always proportional to the amount of kallikrein function (bradykinin generation or amidolysis) regardless of the ratio of prekallikreins I and II; (f) neither band is seen when prekallikrein-deficient plasma is fractionated and no plasminogen proactivator function is found in the usual position of prekallikrein on SP-Sephadex.

The cleavage of prekallikrein concomitant with activation was shown to be a direct interaction between HF, and prekallikrein. Neither the cleavage pattern nor the kinetics of cleavage were altered by the presence of high concentrations of Trasylol. However, kallikrein appeared able to digest prekallikreins I and II without activation to yield prekallikreins that were diminished by M₀ = 10,000. The rates of activation, when assayed by generation of an amidolytic site, were slightly faster than when cleavage was used as a measure of activation. Although this might suggest that cleavage occurs subsequent to activation, the addition of the SDS buffer to the sample for polyacrylamide gel electrophoresis analysis instantaneously stopped the reaction, while the functional assay required an additional incubation. The latter determination showed significant activity even at the earliest time point. It is also possible that the trace of [³⁵S]I-prekallikrein used to detect cleavage may not precisely reflect the cleavage rate of the nonradioabeled material; or that some fraction of our prekallikrein is cleaved at a slower rate by HF, and does not produce a functional molecule.

Although the mechanism of activation of human prekallikrein has not previously been reported, similar activation mechanisms of bovine and rabbit prekallikrein have been described. No change in molecular weight was seen upon conversion of bovine prekallikrein to kallikrein, and a single bond cleavage was identified such that upon reduction, a heavy and light chain were formed (27). Activation of rabbit prekallikrein was reported to occur with release of a small peptide of M₀ = 11,000. The resultant kallikrein was smaller and more acidic than prekallikrein (28). A later study, however, did not confirm such peptide formation as part of the activation mechanism and the product was a kallikrein composed of two disulfide-linked chains whose total molecular weight was the same as prekallikrein (29). It is possible that the peptide released was caused by kallikrein digestion of prekallikrein or kallikrein.

We have also reinvestigated the relationship of prekallikrein to plasminogen proactivator and have concluded that the Hageman factor-dependent plasminogen proactivator originally described by Kaplan and Austen (11) was prekallikrein or the prekallikrein derivative formed by kallikrein digestion, or both. We could not detect any antigenic difference in the various forms of prekallikrein and, although prekallikrein-deficient plasma does possess a plasminogen activator in the γ-globulin fraction, further fractionation revealed no plasminogen proactivator activity in the usual position of prekallikrein. The identity of prekallikrein and plasminogen proactivator was first suggested by Laake and Venneråk who were unable to separate kinin-generating and esterase activities from plasminogen-activating activity (12). We also found the activities to fractionate together, particularly when iPr-PF was incorporated into the isolation procedures. Plasminogen proactivator was reported to have a molecular weight of 10,000 less than prekallikrein and it is possible that the peak of fibrinolytic activity observed was a result of cleavage of prekallikrein by kallikrein (11) The identification of a peak of plasminogen-activating activity in the γ-globulin fraction of prekallikrein-deficient plasma also suggested that prekallikrein and plasminogen proactivator might not be identical (30). These results are at variance with other reports which indicated that the γ-globulin effluent obtained from prekallikrein-deficient plasma contains no plasminogen activator or proactivator activity (31). However, upon further investigation of normal plasma, a second peak of plasminogen-activating activity was identified which eluted in a position similar to Factor XI rather than prekallikrein (26). We have recently reported that SP-Sephadex chromatography of the γ-globulin effluent obtained from prekallikrein-deficient plasma yields only this second peak of fibrinolytic activity (32). As shown in Fig. 15, the activity was found superimposed upon Factor XI. It is not clear whether this activity is a property of Factor XI or represents an unrelated fibrinolytic factor. Further studies are in progress to distinguish these possibilities.

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