Effect of Cleavage of the Heavy Chain of Human Plasma Kallikrein on Its Functional Properties

By Robert W. Colman, Yanina T. Wachtfolg, Umberto Kucich, George Weinbaum, Steven Hahn, Robin A. Pixley, Cheryl F. Scott, Ariane de Agostini, Danielle Burger, and Marc Schapira

Human plasma kallikrein consists of an N-terminal heavy chain of molecular weight (mol wt) 52,000, linked by disulfide bonds to two light chain variants (mol wt 36,000 or 33,000). Although the active catalytic site of kallikrein resides on the C-terminal light chain, the role of the N-terminal heavy chain is less clear. We therefore studied an enzyme designated β-kallikrein, containing a single cleavage in the heavy chain (mol wt 28,000 + 18,000) and compared it to the enzyme, α-kallikrein, with an intact heavy chain. The rates of inactivation by C1 inhibitor of plasma α- and β-kallikreins were kinetically identical, as measured by residual amidolytic activity, after various times of incubation with the inhibitor. Both enzymes reacted completely with C1 inhibitor after 18 hours and formed identical C1 inhibitor-kallikrein complexes of mol wt 195,000. The rate of activation of factor XII by α-kallikrein and β-kallikrein was similar. In contrast, the rate of cleavage of high molecular weight kininogen (HMWK) by α-kallikrein was at least fivefold faster and the ratio of coagulant activity to amidolytic activity was fourfold greater than for β-kallikrein. Plasma α-kallikrein, at concentrations potentially achievable in plasma, induced aggregation of neutrophils, but β-kallikrein failed to elicit this response. In addition, human neutrophils pretreated with cytochalasin B released 2.46 ± 0.10 μg/10^7 cells of elastase antigen, but β-kallikrein released only 0.25 ± 0.10 μg/10^7 cells. These observations suggest that cleavage of the heavy chain influences the rate of cleavage of HMWK and decreases its coagulant activity. Moreover, an intact heavy chain appears to be requisite to support the ability of kallikrein to aggregate neutrophils and release elastase.

© 1985 by Grune & Stratton, Inc.
with Cl inhibitor and factor XII, β-kallikrein cleaves HMWK, displays coagulant activity, and decreases neutrophils to a much lesser degree than α-kallikrein. Thus, cleavage of the heavy chain of kallikrein appears to decrease its maximum ability to release bradykinin and to stimulate neutrophils, thereby impairing the possible participation of this enzyme in the inflammatory response.

MATERIALS AND METHODS

Reagents

Sodium dodecyl sulfate, methylene bis-acrylamide, acrylamide, and high and low molecular weight standards (200,000; 116,000; 93,000; 66,500; 45,000; 31,000; 21,000; 14,000) were purchased from Bio-Rad, Richmond, Calif. Soybean trypsin inhibitor, β-mercaptoethanol, and cytochalasin B were purchased from Sigma Chemical, St Louis. Glass tubes (6 × 50 mm) were obtained from Fisher Scientific Co, King of Prussia, Pa. Inositol was purchased from American Concentrates, New York. Coomassie brilliant blue R-250 was purchased from LKB, Rockville, Md. D-Pro-Phe-Arg-p-nitroaniline (S-2302) and B2-Ile-Glu-Gly-Arg-p-nitroaniline (S-2222) were obtained from Helena Laboratories, Beaumont, Tex. Hanks’ balanced salt solution was purchased from GIBCO Laboratories, Grand Island, NY. Flat-bottomed polystyrene microtiter plates (96 wells, immlunon No. 2) were purchased from Dnatech Laboratories, Alexandria, Va. Goat anti-rabbit IgG (heavy and light chain), conjugated to horseradish peroxidase, was obtained from Cappel Laboratories, West Chester, Pa. Dimethylpolysiloxane was obtained from Siefried Aj, Zofinjen, Switzerland. All other reagents were reagent grade or better.

Plasma

Normal pooled plasma, used as a reference standard, was purchased from George King, Biomedicals, Overland Park, Kan. Factor XII-deficient plasma was kindly supplied by Dr Margaret Johnson, Wilmington, Del. Prekallikrein-deficient plasma was graciously supplied by Dr Charles Abildgaard, Davis, Calif. Kininogen-deficient plasma was donated directly to us by Mrs M. Williams, Philadelphia.

Purified Proteins

Kallikrein was prepared by activation of purified prekallikrein by XII at a molar ratio of 1:200, enzyme to substrate. The activation was stopped with purified trypsin inhibitor (50 µg/mL), which was a kind gift of Dr Edward Kirby and Patrick McDevitt of this institution. The preparation contained no measurable factor XI, Xla, XII, XIIIa, plasminogen, or plasmin as determined by coagulant and amidolytic assays. A published photograph of the reduced SDS-PAGE indicates that the preparation contains two chains of mol wt 52,000 and 36,000 (33,000), corresponding to α-plasma kallikrein.

The unreduced molecule demonstrates both variants (mol wt 88,000 and 85,000) as seen below in Fig 3a. Plasma kallikrein was also directly purified from acetone-activated plasma using the method of Nagase and Barrett, without modification.

On reduced SDS-PAGE (Fig 1), the preparation contained the same two light chain variants (mol wt 37,000 and 32,000) and the polypeptides corresponding to the cleaved heavy chain (mol wt 28,000 and 18,000), similar to that previously described and designated by us as β-plasma kallikrein. The unreduced molecule (Fig 4a below), similar to α-kallikrein, contains polypeptides of mol wt 88,000 and 85,000. The specific activity of both α- and β-kallikrein was 72 µmol S2302 hydrolyzed/min/mg protein.
sodium phosphate buffer, pH 7.6, containing 127 mmol/L NaCl. Details of the kinetic analysis have been published.14

Assay of Amidolytic Activity of Kallikrein

Kallikrein was assayed for amidolytic activity using the substrate S-2302.25 Ten microliters of sample was added to a prewarmed plastic cuvette (37°C) containing 280 μL 0.1 mol/L sodium phosphate, pH 7.6, plus 0.15 mol/L NaCl, and 1 mmol/L EDTA plus 50 μL of 4 mmol/L S-2302 (final concentration 0.6 mmol/L). The change in optical density was recorded on a Gilford No. 240 spectrophotometer or a Cary 210 double beam spectrophotometer. One amidase unit is defined as 1 μmol of substrate hydrolyzed per minute under these conditions. The stock solutions of α- and β-kallikrein were adjusted to 9 amidase U/mL for use in most of these experiments.

Activation of Factor XII by Kallikrein

Factor XII (2 μL/mL or 0.74 μmol/L) in a Tris buffer (0.02 mol/L Tris, 0.02 mol/L NaCl, pH 7.5) was incubated with either α- or β-kallikrein (0.11 amidase U/mL or 19 nmol/L) at 37°C in a polypropylene tube. The generation of factor XIIa activity with time was determined by initial velocity measurements using the chromogenic substrate S-2222.15 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12 At the concentration used, the kallikrein did not contribute to the release of p-nitroaniline (pNA) from S-2222.12

Cleavage of HMWK by α- and β-Kallikrein

HMWK in 0.015 mol/L Tris-acetate, pH 8.0, was incubated with either α- or β-kallikrein at 37°C, which was previously determined to have similar amidolytic activity on S-2302.26 At various times, 5-μL aliquots were removed from each polypropylene tube and added to glass tubes of 6 x 50 mm, containing 65 μL H2O, and 25 μL buffer (0.4 mol/L Tris-Cl, pH 6.8) 8% SDS, and 5 μL 2-mercaptoethanol (0.8 mol/L). The samples were incubated overnight at 37°C prior to electrophoresis. SDS-PAGE was performed on 10% gels with Pharmacia mini-gels with mol wt markers of 200,000, 100,000, 66,000, 45,000, 30,000, and 20,000. The gels were stained with Coomassie blue R-250. Silver staining was used to quantitate HMWK separated by SDS-PAGE. Smaller HMWK fragments were not stained by Coomassie blue R-250 and were therefore not detectable. Stained gels for selected experiments were scanned using a SD 3,000 Spectrophotodensitometer (Schoeffe Instrument Division, Kratos, Inc., Westwood, NJ). The relative peak areas were quantified by weighing tracings on standard paper.

Assay of Factor XII Coagulant Activity

The assay was performed by a modification of the partial thromboplastin time26 using congenitally Factor XII-deficient plasma. One hundred microliters of deficient plasma, 100 μL of 200 mmol/L Tris-Cl, pH 7.4, containing 0.15 mol/L NaCl, 100 μL of kaolin (5 mg/mL in 0.15 mol/L NaCl), and 100 μL of 0.2% inosinithin in buffer were mixed. Normal plasma (10, 5, 2, or 1 μL) was added and incubated at 37°C for eight minutes. One hundred microliters of 30 mmol/L CaCl2 was added, and the coagulation time was measured. This procedure was used to generate a standard curve (log-log relationship). Samples for analysis were assayed under the same conditions using 5 or 10 μL of sample, and the data were expressed as percentage of normal pooled plasma. One unit is the amount of activity in 1 mL of normal pooled plasma.

Release of Neutrophil Elastase in a Purified System

Ten million isolated neutrophils were incubated in 1 mL of Hanks' balanced salt solution containing 2 mmol/L CaCl2 at 37°C, while stirring at 1,200 rpm in the presence of 5 μg/mL cytochalasin B.27 After 20 minutes, 100 μL of α- or β-kallikrein was added to this incubation mixture. Upon centrifugation at 13,000 g for five minutes at 23°C in a Micro-Centrifuge (model 235A, Fisher Scientific Co., Pittsburgh), the supernatants were recovered and frozen at -20°C until elastase determinations could be performed. An indirect competitive enzyme-linked immunosorbent assay was used to quantify neutrophil elastase, as previously described.11 Briefly, antibody and human neutrophil elastase were preincubated at 16°C overnight in glass tubes before their addition to 96-well flat-bottomed polystyrene microtiter plates, which were previously coated with 20 ng of antigen per well. The time of competition between insoluble antigen in the fluid phase for the limiting amount of primary antibody sites was one hour at 16°C. Goat anti-rabbit IgG, conjugated with horseradish peroxidase, was then added, the plate washed, the substrate added, and the absorbance was measured at 450 nm. The addition of increasing amounts of diisopropylfluorophosphate (DFP)-inactivated neutrophil elastase decreased the amount of color development, as compared with control, and was directly proportional to the amount of DFP-human neutrophil elastase added.11

RESULTS

Inactivation of Plasma α- or β-Kallikrein by C1 Inhibitor

The rates of inhibition of plasma α-kallikrein and β-kallikrein by C1 inhibitor were compared using similar amounts of the two kallikreins, as determined
Fig 2. Inactivation of plasma kallikrein by C1 inhibitor. α-Kallikrein (O) or β-kallikrein (□) was incubated with various concentrations of C1 inhibitor and then assayed, at various times, for residual amidolytic activity, α- and β-kallikrein final concentrations: 50 nmol/L, C1 inhibitor final concentrations: (a) 0, (b) 0.15 μmol/L, (c) 0.28 μmol/L, (d) 0.45 μmol/L.

Complex Formation of Plasma α- or β-Kallikrein With C1 Inhibitor

Incubation of equal amounts of either plasma α-kallikrein (Fig 3) or plasma β-kallikrein with C1 inhibitor (Fig 4) was carried on for 18 hours at 22 °C in order to allow the reaction to proceed to completion. Both plasma α-kallikrein and β-kallikrein appeared as a doublet of mol wt 88,000 and 85,000 on SDS-PAGE in the absence of reducing agent, as previously reported, and C1 inhibitor was primarily (90%) a species of mol wt 105,000, with a minor component of 95,000, which probably represents a fragment derived from protease digestion. After 18 hours, all of the kallikrein had disappeared in both cases, indicating that both plasma α- and β-kallikrein had intact active sites. A small amount of C1 inhibitor, mol wt 105,000, remained. There was an increase of the mol wt 95,000 species, which probably represents a proteolytic breakdown fragment of C1 inhibitor by kallikrein, since the enzyme is in slight molar excess, an observation that has been described previously. The major product of the interaction of plasma α- and β-kallikreins with C1 inhibitor was a species of mol wt 195,000, which is known to represent the enzyme–inhibitor complex. No major differences were observed between the results obtained with plasma α-kallikrein (Fig 3) and plasma β-kallikrein (Fig 4).

Activation of Factor XII by α- or β-Kallikrein

Factor XII was incubated with either α- or β-kallikrein and the rate of formation of activated factor

Fig 3. Formation of α-kallikrein–C1 inhibitor complex. SDS-PAGE (7.5%, unreduced) of plasma α-kallikrein (a), C1 inhibitor (b), and of the mixture resulting from the incubation of α-kallikrein and C1 inhibitor for 18 hours at 22 °C (c). Lanes a and b contained 20 μg of protein, while lane c contained 20 μg of α-kallikrein and 20 μg of C1 inhibitor.

Fig 4. Formation of β-kallikrein–C1 inhibitor complex. SDS-PAGE (7.5%, unreduced) of plasma β-kallikrein (a), C1 inhibitor (b), and of the mixture resulting from the incubation of β-kallikrein and C1 inhibitor for 18 hours at 22 °C (c). Lanes a and b contained 20 μg of protein, while lane c contained 20 μg of β-kallikrein and 20 μg of C1 inhibitor.
Comparison of HMWK Cleavage by Plasma α- and β-Kallikrein

HMWK was incubated with either plasma α- or β-kallikrein at a 1:60 molar enzyme-substrate ratio. The cleavage was analyzed by SDS-PAGE in the presence of a reducing agent and quantified by densitometric scanning. HMWK was cleaved to a heavy chain, mol wt 65,000, and a light chain, mol wt 45,000. When the rate of formation of the 45,000 mol wt light chain by α- and β-kallikrein was compared, we observed that by ten minutes of incubation, α-kallikrein produced five times more 45,000 mol wt light chain than was generated by β-kallikrein (Fig 6). Thus, α-kallikrein is a more efficient enzyme than β-kallikrein for cleaving HMWK in solution.

Comparison of the Coagulant Activity of Plasma α- and β-Kallikrein

Because the amidase activity of α-kallikrein and the light chain of kallikrein are identical, we used this information as a reference to compare the coagulant activity of the two enzymes. Representative preparations of α- and β-kallikrein were assayed in duplicate for amidase and coagulant activity. The α-kallikrein had an activity of 4.20 amidase U/mL and 11.0 coagulant U/mL. The β-kallikrein had an activity of 3.03 amidase U/mL, but only 2.00 coagulant U/mL. Thus, the ratio of coagulant to amidase activity for α-kallikrein was 2.6 as compared to 0.66 for β-kallikrein. Therefore, α-kallikrein has fourfold greater coagulant activity than β-kallikrein.

Aggregation of Neutrophils by α-Kallikrein and β-Kallikrein

When plasma α-kallikrein at 0.45 μmol/L (2.5 amidase U/mL final concentration) was incubated with neutrophils, full aggregation was observed, which is consistent with our previous observations where as little as 0.18 μmol/L gave a full response. In contrast, plasma β-kallikrein (2.5 amidase U/mL, 0.45 μmol/L) failed to elicit any response (Fig 7). Therefore, only intact α-kallikrein can aggregate neutrophils.
Release of Elastase From Human Neutrophils by $\alpha$- and $\beta$-Kallikrein

When $10^7$ isolated human neutrophils, primed with cytochalasin B, were exposed to $\alpha$-kallikrein (0.45 $\mu$mol/L final concentration), 2.46 ± 0.10 $\mu$g of human neutrophil elastase was released, as measured by the enzyme-linked immunosorbent assay (ELISA). However, under the same conditions, $\beta$-kallikrein (0.45 $\mu$mol/L) only released 0.28 ± 0.16 $\mu$g of human neutrophil elastase. Therefore, consistent with the aggregation of neutrophils (Fig 7), $\alpha$-kallikrein releases elastase from cytochalasin B-primed human neutrophils at least one order of magnitude greater than does $\beta$-kallikrein.

DISCUSSION

Mammalian serine proteases usually consist of two disulfide-linked chains. The C-terminal chains of all the enzymes contain the active catalytic site (Ser, His, Asp) and demonstrate extensive homologies and virtually the same molecular weight. Although the N-terminal chain of chymotrypsin and trypsin are very short, plasma proteases have an N-terminal chain that may exceed mol wt 60,000 and thus contain over 500 residues. The N-terminal chains appear to determine the specific functions of the enzyme. For example, activated factor XII, containing an N-terminal heavy chain (mol wt 60,000) and a C-terminal light chain (mol wt 28,000), exhibits coagulant activity in addition to amidolytic activity as well as the ability to cleave prekallikrein to an active enzyme kallikrein. The isolated C-terminal polypeptide (XIIa) exhibits both amidolytic and prekallikrein activating activity, but has less than 1% of the coagulant activity of factor XIIa. Similar results were found with the isolated light chain of kallikrein, which, although it exhibited amidolytic activity and factor XII activating ability similar to that of the intact enzyme, it had only 0.6% of the coagulant activity of the intact kallikrein.

In this study, we explored the effect of limited proteolysis of the heavy chain of kallikrein (Fig 1) on several of the functions of this enzyme. The inhibition of $\alpha$- and $\beta$-kallikrein by Cl inhibitor is kinetically (Fig 2) as well as structurally identical (Figs 3 and 4). This result is consistent with the observation that the coagulant activity of kallikrein by DFP, a reaction localized to the light chain of kallikrein, was fourfold greater than that of $\beta$-kallikrein, supporting the conclusion that cleavage of the heavy chain decreases, but does not abolish, the coagulant activity. The similar changes in the ability of kallikrein to cleave HMWK and express coagulant activity is not unexpected, since both depend on the conversion of the procofactor HMWK to an active cofactor.

The most profound effect of the cleavage of the heavy chain of kallikrein is the loss of its ability to aggregate neutrophils (Fig 7) and to release elastase. Our previous studies demonstrate that the active
site of kallikrein, located in the light chain, is necessary for activation of neutrophils, since neither kallikrein incubated with DFP or soybean trypsin inhibitor nor prekallikrein can stimulate neutrophils. The present study is the first evidence that the heavy chain of kallikrein is involved in the interaction with neutrophils. This finding is consistent with our previous finding\(^\text{10}\) that trypsin, an analogue of the light chain, has less than 10% of the ability of kallikrein to aggregate neutrophils. However, the hypothesis that the heavy chain contains the binding site for the neutrophil membrane and that such binding is required prior to a proteolytic event must be tested by direct binding studies.

Precedent for limited proteolysis resulting in decreased activity is offered by the decrease in factor XIIa coagulant activity as it is degraded from mol wt 80,000 to mol wt 60,000 prior to formation of factor XII,\(^\text{36}\) as well as conversion of two-chain factor VIIa to a three-chain species.\(^\text{37}\) The origin of β-kallikrein remains unclear, although Nagase and Barrett\(^\text{15}\) have postulated that the cleavage is autolytic. Mandle and Kaplan\(^\text{1}\) have observed a form of prekallikrein of mol wt 75,000 produced by cleavage of kallikrein at a high enzyme-substrate ratio. Kallikrein appears to have the potential to hydrolyze a bond in its heavy chain to form a molecule with reduced activity toward certain substrates, a reaction that might serve as a negative feedback to help control kallikrein stimulation of the inflammatory process.

**REFERENCES**

27. Scott CF, Silver LD, Schapira M, Colman RW: Cleavage of human high molecular weight kinogen markedly enhances its
coagulant activity: Evidence that this molecule exists as a procofactor. J Clin Invest 73:954, 1984


Effect of cleavage of the heavy chain of human plasma kallikrein on its functional properties

RW Colman, YT Wachtfogel, U Kucich, G Weinbaum, S Hahn, RA Pixley, CF Scott, A de Agostini, D Burger and M Schapira