Characterization of the Procoagulant Chain Derived From Human High Molecular Weight Kininogen (Fitzgerald Factor) by Human Tissue Kallikrein

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Human high molecular weight kininogen (HMWK), a single-chain protein with mol wt 120,000, is cleaved by human urinary kallikrein (HUK) to release kinin from within a disulfide loop and form a two-chain protein that retains all the procoagulant activity of the native molecule. Cleavage of HMWK by HUK is associated with a reduction in size to mol wt 115,000, as assessed by SDS-PAGE of unreduced protein, whereas the two chains of the reduced protein present together as a single broad band with mol wt 64,000. The 64,000 chain with procoagulant activity was chromatographically separated from the nonfunctional chain of similar size. The homogeneous procoagulant chain had an amino acid composition similar to that of smaller procoagulant (“light”) chains isolated by others upon cleavage of HMWK with plasma kallikrein and elicited an antiserum that was monospecific by Ouchterlony analysis and inhibited the procoagulant function of HMWK. Thus, the limited proteolysis of HMWK by HUK has permitted, for the first time, the isolation of a stable procoagulant chain that is equal in size to the nonfunctional chain. The common terminology of “heavy” and “light” chain for kinin-free kininogen obtained with plasma kallikrein reflects the continued degradation of the procoagulant carboxy-terminal chain and is not appropriate for the initial two-chain product formed when kinin is released from HMWK. It is proposed that the initial cleavage products of HMWK be designated the A-chain, the B-fragment, and the C-chain, representing the amino-terminal chain, the released vasoactive peptide containing the bradykinin sequence, and the carboxy-terminal procoagulant chain, respectively. Thus, intact HMWK would contain, in sequence, A, B, and C regions.

Kininogens are plasma proteins that contain the amino acid sequence of the vasoactive kinin polypeptides. Bradykinin or lysyl-bradykinin are released from kininogens by limited proteolysis by enzymes known as kallikreins (EC 3.4.21.8). Human plasma contains two distinct forms of kininogen, designated high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK). The former may be complexed with prekallikrein in plasma and is thought to be the preferred substrate for plasma kallikrein. Human HMWK has been shown not only to be a source of kinin but also to serve, together with plasma prekallikrein or with factor XI, as an important component in the initial step of contact activation of the Hageman-factor-dependent pathways. Plasmas deficient in HMWK have a prolonged in vitro partial thromboplastin time, as well as diminished kaolin-activatable kinin generation and fibrinolysis. Purified HMWK, which is able to correct all the abnormalities in such plasmas, consists of a single polypeptide chain with mol wt 120,000. HMWK is cleaved by purified plasma kallikrein at two sites in the interior of the molecule to liberate the kinin moiety and yield a two-chain disulfide-linked kinin-free protein. The amino-terminal chain, or “heavy chain,” shares extensive immunologic determinants with LMWK, while the carboxy-terminal chain, or “light chain,” is antigenically unique and quantitatively retains the procoagulant activity of the intact HMWK. Plasma kallikrein appears to cleave the carboxy-terminal chain of HMWK at a second site, diminishing the size by approximately 10,000. Without accompanying reduction of procoagulant activity.

Glandular (tissue) kallikreins, which are found in kidney, pancreas, and salivary glands and in their secretions, are structurally, functionally, and antigenically different from plasma kallikrein, but are related to each other. Glandular kallikrein has also been identified antigenically in human and rat plasma and the glandular kallikrein, extracted from human plasma by a procedure that included immunoaffinity chromatography, cleaved both synthetic substrates and HMWK. A single study of the interaction of a glandular kallikrein, namely, human salivary kallikrein, with human plasma HMWK substrate described limited time-dependent proteolysis but did not assess residual procoagulant activity. Highly purified HMWK has been employed in the present study as a substrate for highly purified human urinary kallikrein to examine functionally and structurally the kinin-free kininogen formed by this tissue kallikrein.
MATERIALS AND METHODS

Reagents

SP Sephadex-C50, QAE Sephadex A-50, protein sizing standards, and Sephadex G-100 were from Pharmacia Fine Chemicals, Piscataway, NJ; acrylamide, N,N'-methylene-bisacrylamide, sodium dodecyl sulfate (SDS), dithiothreitol, ammonium persulfate, and anti-human α₂-macroglobulin came from BioRad Laboratories, Richmond, CA. Kunitz bovine basic pancreatic trypsin inhibitor (aprotinin) was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN; α-N-p-tosyl-L-arginine methylester (TAME), benzamidine, anti-human antithrombin III, and Coomassie Brilliant Blue were from Sigma Chemical Co., St. Louis, MO. Disisopropyl-fluorophosphate (DFP), anti-α1-antitrypsin inhibitor and anti-α₂, anti-α₂-antitrypsin were from Calbiochem-Behring, San Diego, CA; Polybrene (hexadimethrine bromide) from Aldrich Chemical Co., Milwaukee, WI; and bradykinin from New England Nuclear Co., Boston, MA. Hageman-factor-deficient and factor-XI-deficient plasma were obtained from Seratec, New Brunswick, NJ; Fletcher factor (plasma prekallikrein) deficient and Fitzgerald factor (HMWK) deficient plasmas from George King Biomedical (plasma prekallikrein) deficient and Fitzgerald factor from Seratec, New Brunswick, NJ; Fletcher factor (plasma prekallikrein) deficient and Fitzgerald factor (HMWK) deficient plasmas from George King Biomedical Inc., Overland Park, KS; and antiserum against the inhibitor of the first component of complement (C1Inh) from Miles Laboratories, Inc., Elkhart, IN. Iodoacetamide was purchased from Eastman Organic, Rochester, NY.

Assays

During kininogen purification, 100–500 μl samples of column fractions were incubated with 10 μl of purified human urinary kallikrein for 5 min at 37°C, and the kinin generated was measured on the guinea pig ileum28 standardized with synthetic bradykinin. In studies with purified HMWK and purified urinary kallikrein, the reactants were separately incubated for 5 min at 37°C, mixed, and incubated for another 5 min at 37°C, unless otherwise stated, and the generated kinin was assayed immediately.

The procoagulant activity of column fractions and purified kininogen was examined in a modified kaolin-activated clotting time assay.27 Equal volumes (usually 25 μl) of HMWK-deficient plasma, column fractions, and a kaolin-phospholipid mixture were incubated at 37°C for 2 min. After addition of 25 μl of 0.05 M CaCl₂, incubation was continued at room temperature until an adherent clot formed. A standard curve was developed with serial dilutions of pooled normal plasma (1:15–1:100), assuming that undiluted plasma contains 1 U/ml of HMWK procoagulant activity.27,28 Negative controls included Hageman-factor-deficient, factor-XI-deficient, and plasma prekallikrein-deficient plasmas.

Alkaline polyacrylamide gel electrophoresis (PAGE) was performed according to the directions supplied by Bichler Instruments (Fort Lee, NJ). For SDS-PAGE,29 samples containing 18–32 μg of protein were made 6.6 M in urea and 1% in SDS, incubated with or without 0.1 M dithiothreitol for 20 min at 60°C, followed by incubation with 0.2 M iodoacetamide for 15 min at 60°C, and then electrophoresed in 7.5% gels containing 0.5 M urea.

For amino acid analysis, a 680-μg sample of HMWK procoagulant chain was dialyzed against distilled water prior to lyophilization and hydrolysis in 6 M HCl at 110°C for 24 hr. The sample was lyophilized, redissolved in citrate buffer, pH 2.2, and subjected to analysis on a Durrum D500 analyzer.

Protein Purification

Human urinary kallikrein (HUK) was purified from pooled fresh urine by affinity chromatography on aprotinin-CH-Sepharose and gel filtration on Sephadex G-100.26 The final product revealed a single band with a mol wt of 48,000 on SDS-PAGE with and without prior reduction; a single stained protein band on alkaline PAGE having the same mobility as kallikrein function and antigen identified in eluates from a sliced gel run in parallel; and the same amino acid composition and single amino terminal residue sequence as reported by others for HUK.30–32 The kinetic constants with TAMe used as substrate at 25°C and pH 8.0 were Vₘₐₓ = 1.0 × 10⁻⁴ M, Vₜₐₜ = 0.61 × 10⁻³ mole/min/mg enzyme, and kₘₐₓ = 5.08 sec⁻¹ as determined kinetically in the direct spectrophotometric assay27; they agree with those reported by others.33 The purified enzyme released 925.9 μg BK equivalents/min/mg enzyme protein from an excess of heat-inactivated human plasma.34

HMWK was isolated from citrated human plasma that was made 0.02 M in benzamidine, 0.01 M in disodium ethylenediaminetetra-acetic acid (Na₂EDTA), 0.01 M in DFP, and 50 mg/liter in Polybrene prior to storage at −70°C for no more than 3 wk. A total of 3,000 ml was thawed, dialyzed at room temperature against 0.01 M Tris-HCl, pH 8.0, containing 0.001 M benzamidine, 0.001 M Na₂EDTA, 0.001 M DFP, and 50 mg/liter Polybrene, and applied at room temperature to a 2-liter column of Sephadex QAE A50 equilibrated with the above buffer. Step-wise elution employed the same buffer containing 0.075 M, 0.12 M, 0.18 M and 0.35 M NaCl.1 Each milliliter samples of 500 ml column fractions were dialyzed at 4°C against 0.05 M Tris-HCl, pH 8.0, with 0.1 M NaCl and screened for protein concentration in a Lowry assay with rabbit IgG as a standard,27 for capacity to serve as a substrate for purified human urinary kallikrein, and for ability to correct the clotting defect in HMWK-deficient plasma. Fractions exhibiting both functional activities eluted with the 0.35 M NaCl buffer and were pooled and brought to 55% ammonium sulfate saturation by the addition of solid (NH₄)₂SO₄ at 4°C. The pool was stirred for 90 min and centrifuged for 1 hr at 2,000 g; the precipitate was dissolved in 0.05 M sodium acetate buffer, pH 5.3, containing 0.075 M NaCl, 0.5 M Na₂EDTA, 0.5 M benzamidine, and 1.0 mM DFP. After dialysis against the same buffer, the resolubilized precipitate was applied to a 2.5 × 25 cm column of SP-Sephadex C50 and eluted with a linear salt gradient from 0.15 M to 0.5 M NaCl in the same buffer.30 One milliliter portions of 4.9-ml column fractions were dialyzed and analyzed as described for the QAE column. Fractions containing HMWK were pooled, dialyzed extensively against 0.01 M Tris-HCl, pH 8.0, containing inhibitors as indicated for the first QAE Sephadex column, and applied to a 10-ml column of QAE Sephadex A50 equilibrated with the same buffer. After extensive washing, the column was eluted with a linear salt gradient from 0.0 M to 0.4 M NaCl, and 0.5-ml samples of 2.5-ml column fractions were dialyzed and assayed as above. HMWK functions appeared at a conductivity of 175 to 20 mS. These fractions were pooled, concentrated to 740 μg/ml, dialyzed against 0.003 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, aliquoted and stored at −70°C. Samples were concentrated in dialysis bags (mol wt cutoff 3,500) covered with sucrose.

The overall recovery based on kinin-generating activity was 33.9% (Table 1). The purified HMWK had a specific clotting activity of 18.1 U/mg of protein and contained 5.2 μg kinin/mg HMWK as assessed with purified HUK. At 37°C and the optimum pH (8.8), the Kₘ for HUK on HMWK was 5.0 × 10⁻⁴ M, the Vₜₐₜ = 5.2 × 10⁻⁴ mole/min/mg enzyme, the kₘₐₓ = 0.43 sec⁻¹, and the second-order rate constant (k₃/k₄) was 8.05 × 10⁶ M⁻¹ sec⁻¹. The purified HMWK migrated on SDS-PAGE as a single major protein band of apparent mol wt 120,000, with or without prior reduction (Fig. 1). The minor HMW band present in the unreduced samples was no longer seen after reduction (Fig. 1) or after incubation with urinary kallikrein and is considered to represent aggregates of HMWK. No plasma prekallikrein, Hageman factor, or factor XI was detected by clotting assay with the deficient plasma samples; no C1Inh, α₂-macroglobulin, α₂-antitrypsin, antithrombin III, or inter-α-tryp-
sin inhibitor was detected by Ouchterlony analysis of 20 μg of HMWK protein.

RESULTS
Purification and Characterization of the Procoagulant Chain by Function and Size

After incubation of 12 μg of purified HMWK with 80 μg of HUK for 30 min at 37°C, analytical SDS-PAGE of the unreduced product yielded a major band with mol wt 115,000 and a minor band at mol wt 120,000. After reduction and alkylation before electrophoresis, a major broad band with an apparent mol wt of 64,000 was observed together with the minor band, which remained at mol wt 120,000 and most likely represents a small amount of uncleaved HMWK. Incubation of HMWK with HUK for 2 hr failed to reveal any smaller products. For preparation of the cleavage products, 6 mg of purified HMWK was incubated with 61 μg of purified HUK for 6 hr at 37°C and pH 8.1. After incubation, the mixture was passed directly over an aprotinin-CH-Sepharose column equilibrated at pH 8.0 to remove the HUK. The HMWK was recovered quantitatively in the column effluent and was free of HUK as indicated by the fact that 100 μl of the effluent failed to generate any kinin from 250 μl of heat-inactivated plasma used as a source of kininogen substrate.

Reduction and alkylation of the HMWK and chromatography on SP-Sephadex C50 with a linear salt gradient were performed as described. The effluent from the column yielded a protein peak that contained 25% of the total procoagulant activity recovered, and contained minimal kinin as assessed after incubation with HUK. Seventy-five percent of the procoagulant activity was eluted in the gradient at 25 mS in a protein peak that contained no detectable kinin, as assessed after incubation with HUK. On a weight basis, this material had approximately twice the procoagulant activity of the starting material (40.2 U/mg versus 18.1 U/mg). Analysis of the effluent by SDS-PAGE revealed a major stained band with mol wt 64,000 and faint bands with mol wt 120,000, 89,000, and 74,000, whereas the material eluting at 25 mS contained only a protein with mol wt 64,000 (Fig. 2). The effluent apparently contained a small amount of intact and degraded kininogen as well as free “heavy” chain, while the 25-mS eluate consisted only of a single molecular weight species containing the procoagulant chain.

Amino Acid Analysis

Because the amino acid content of HMWK “heavy” and “light” chains generated with plasma kallikrein varies by 2–8-fold for 6 residues, namely histidine, glycine, alanine, valine, tyrosine, and half-cysteine, the procoagulant chain with mol wt 64,000 generated with HUK was subjected to amino acid analysis for comparison with the data from two studies. The amino acid content of the mol wt 64,000 chain obtained with HUK is similar to that previously reported for human HMWK “light” chain of smaller size generated with human plasma kallikrein (Table 2).
Table 2. Comparison of the Amino Acid Composition of Procoagulant High Molecular Weight Kininogen Chains Generated by Human Tissue and Plasma Kallikreins

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Human Urinary Kallikrein (64,000 mol wt)</th>
<th>Human Plasma Kallikrein (44,000 mol wt)*</th>
<th>Human Plasma Kallikrein (56,000 mol wt)t</th>
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<tbody>
<tr>
<td>Asp</td>
<td>11.3</td>
<td>12.5</td>
<td>11.2</td>
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<tr>
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<td>6.0</td>
<td>8.4</td>
<td>6.2</td>
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<td>Gly</td>
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<tr>
<td>Ala</td>
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<td>0.6</td>
<td>0.6</td>
<td>ND</td>
</tr>
<tr>
<td>Val</td>
<td>2.0</td>
<td>1.9</td>
<td>3.9</td>
</tr>
<tr>
<td>Met</td>
<td>0.3</td>
<td>1.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Ile</td>
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<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Leu</td>
<td>5.0</td>
<td>6.0</td>
<td>5.1</td>
</tr>
<tr>
<td>Tyr</td>
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<td>0.7</td>
<td>1.2</td>
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<tr>
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<td>2.8</td>
<td>2.1</td>
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<tr>
<td>His</td>
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<td>11.2</td>
<td>9.7</td>
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<td>7.9</td>
</tr>
<tr>
<td>Arg</td>
<td>2.8</td>
<td>1.4</td>
<td>2.9</td>
</tr>
</tbody>
</table>

*From reference 38.
†From reference 14.

and unlike that reported for the “heavy” chain.38 The minimum molecular weight calculated for the HUK-generated procoagulant chain was 32,800, which is essentially half the size determined by SDS-PAGE.

Characterization of Antibody Raised Against the HUK-Generated Procoagulant Fragment of HMWK

A 250-µg sample of the purified procoagulant chain was emulsified in complete Freund’s adjuvant and used to immunize a goat by multiple dorsal subcutaneous injections. Three weeks after boosting with the same amount of protein in incomplete Freund’s adjuvant, antiserum was collected. The antiserum yielded a single precipitin line of complete identity against whole normal human plasma, purified HMWK, and purified HMWK procoagulant fragment; it failed to recognize LMWK or an antigen in HMWK-deficient plasma (Fig. 3). An IgG fraction of either antiserum or preimmune serum, subjected to heat inactivation (56°C for 1 hr), was obtained by (NH₄)₂SO₄ fractionation and DEAE-cellulose chromatography.39 Fifty-microliter portions of 10% normal plasma were incubated for 1 hr at room temperature with serial dilutions of IgG. After centrifugation at 1,000 g, 35 µl were removed and assessed for the residual capacity to correct the clotting defect in HMWK-deficient plasma.

Fig. 2. Determination of the apparent molecular weight of the purified procoagulant chain generated when HMWK was cleaved by HUK. In the upper gel and standard curve are the sizing standards myosin (MYO), β-galactosidase (βG), phosphorylase B (PB), bovine serum albumin (BSA), and ovalbumin (OA). The purified procoagulant chain is shown in the lower gel and on the plot (O).
DISCUSSION

The purification of HMWK and urinary kallikrein, each to apparent homogeneity, has permitted the demonstration that kinin-free HMWK is composed of two stable chains of equal size, one of which possesses the full procoagulant activity of the native protein. The purified HMWK presents as a single chain molecule of mol wt 120,000 on SDS-PAGE of reduced and unreduced protein (Fig. 1), as reported by others. The specific procoagulant activity of 18.1 U/mg of native protein and of 40.2 U/mg of isolated procoagulant chain yield essentially identical activities per mole. These activities are comparable to those reported for the native protein, for the kinin-free protein, and for the isolated “light” chain generated with plasma kallikrein. The purified HUK presented as a single 48,000 mol wt protein in reduced and unreduced SDS-PAGE and yielded a single amino acid residue at each step through 25 sequencing cycles.

HUK cleavage of the 120,000 mol wt single-chain HMWK, so as to release kinin, yielded a protein which, without reduction, migrated at a mol wt of 115,000 on SDS-PAGE. With reduction, the cleaved kininogen presented as a broad band with a mol wt of 64,000, indicating that when kinin was released from within a disulfide bonded region by HUK, the residual chains were of approximately equal size. After reduction and chromatographic resolution as for the isolation of “heavy” and “light” chains of HMWK formed by plasma kallikrein, the procoagulant chain generated by HUK cleavage was obtained as a homogeneous 64,000 mol wt protein (Fig. 2) under conditions where the “heavy” chain presented at a molecular weight similar to that reported by others. Its specific procoagulant activity was identical to that of the native molecule, and its amino acid composition was strikingly similar to that of the “light” chain obtained by cleavage of HMWK with plasma kallikrein (Table 2). Further, this procoagulant chain elicited an antiserum that was shown to be monospecific for it by Ouchterlony analysis (Fig. 3) and which neutralized the capacity of normal plasma to correct the clotting defect of HMWK-deficient plasma (Table 3).

| Table 3. The Ability of Anti-HMWK Procoagulant Chain to Prevent Normal Plasma From Correcting the Clotting Defect in HMWK-Deficient Plasma |
|-------------|-----------------|-----------------|-----------------|
| Normal Human Plasma Incubated With | Clotting Time (min) | Residual Activity (U/0.1 ml) | Percent Inhibition |
| IgG, neat (35 mg/ml) | 23.0 | <0.0015 | >98 |
| 1:2 | 29.0 | <0.0015 | >98 |
| 1:4 | 29.0 | <0.0015 | >98 |
| 1:8 | 26.0 | <0.0015 | >98 |
| 1:16 | 22.0 | <0.0015 | >98 |
| 1:32 | 10.0 | 0.005 | 92 |
| 1:64 | 6.0 | 0.023 | 60 |
| Buffer | 4.5 | 0.057 | 0 |
| Plasma-free control | 54.0 | — | — |

*All dilutions were made with 0.05 M Tris-HCl, pH 8.0, with 0.2 M NaCl.
Thus, plasma kallikrein degrades the “light” chain in a manner not seen with HUK during the release of kinin and the conversion of the substrate to a two-chain protein. Human urinary kallikrein has also been shown to have more limited sites of cleavage in the heterologous bovine HMWK substrate as compared to bovine plasma kallikrein, which not only further degrades the substrate but, in contrast to human plasma kallikrein, also markedly reduces the procoagulant activity of the bovine substrate. The literature indicating that the cleavage of human HMWK by plasma kallikrein to a kinin-free protein composed of a “heavy” and a “light” chain reflects the continued cleavage of the procoagulant carboxyterminal chain and obscures the structure of the initial two-chain product after kinin release. The limited proteolysis of HMWK by HUK has defined the kinin-free HMWK as being composed of two stable chains of equal size. It is proposed that the initial cleavage products of HMWK be designated the A-chain, the B-fragment, and the C-chain, representing the amino-terminal chain, the released vasoactive peptide containing the bradykinin sequence, and the carboxy-terminal procoagulant chain, respectively. Thus, intact HMWK would contain, in sequence, A, B, and C regions (Fig. 4).

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