Human High Molecular Weight Kininogen

STUDIES OF STRUCTURE-FUNCTION RELATIONSHIPS AND OF PROTEOLYSIS OF THE MOLECULE OCCURRING DURING CONTACT ACTIVATION OF PLASMA*

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Human high M, kininogen was purified from normal plasma in 35% yield. The purified high M, kininogen appeared homogeneous on polyacrylamide gels in the presence of sodium dodecyl sulfate and mercaptoethanol and gave a single protein band with an apparent M, = 110,000. Using sedimentation equilibrium techniques, the observed M, was 108,000 ± 2,000. Human plasma kallikrein cleaves high M, kininogen to liberate kinin and gives a kinin-free, two-chain, disulfide-linked molecule containing a heavy chain of apparent M, = 65,000 and a light chain of apparent M, = 44,000. The light chain is histidine-rich and exhibits a high affinity for negatively charged materials. The isolated alkylated light chain quantitatively retains the procoagulant activity of the single-chain parent molecule.

I-Human high M, kininogen undergoes cleavage in plasma during contact activation initiated by addition of kaolin. This cleavage, which liberates kinin and gives a two-chain, disulfide-linked molecule, is dependent upon the presence of prekallikrein and Factor XII (Hageman factor) in plasma. Addition of purified plasma kallikrein to normal plasma or to plasmas deficient in prekallikrein or Factor XII in the presence or absence of kaolin results in cleavage of high M, kininogen and kinin formation.

Human plasma contains substances that release the vasoconstrictor, bradykinin (1). This substance, a nonapeptide that is able to diminish blood pressure (1, 2), induce smooth muscle contraction (1, 2), and provoke pain (3), is released after cleavage of precursor protein molecules, kininogens, by proteolytic enzymes (4, 5). Human plasma contains at least two distinct forms of kininogen that differ in molecular weight (6-10) and in susceptibility to various kallikreins (6, 8).

Exposure of plasma to negatively charged surfaces initiates intrinsic coagulation (11), the kinin-forming pathway (12), fibrinolysis (13), and the generation of vasoactive peptides (14). It has been recently shown that human plasmas deficient in high M, kininogen (15, 16) exhibit abnormalities in contact activation reactions, including the kinin-forming, intrinsic coagulation, and fibrinolytic pathways (15-20). High M, kininogen isolated from bovine plasma is able to correct the contact activation abnormalities of such plasmas (21-22). The functional role of high M, kininogen as a nonenzymatic cofactor in contact activation of intrinsic coagulation and fibrinolysis has been demonstrated (23, 24).

Bovine high M, kininogen is a single-chain molecule, which has been partially sequenced by Iwanaga and his colleagues (25, 26), who also analyzed the relationships between structure and function of the molecule (21, 22, 27, 28). In this study we describe a purification procedure for human high M, kininogen that yields a highly purified single-chain molecule. We demonstrate that the two-chain, kallikrein-cleaved, kinin-free high M, kininogen as well as the light chain isolated from this cleaved molecule quantitatively retain the procoagulant activity of the native single-chain molecule. We also demonstrate that human high M, kininogen undergoes cleavage in plasma during contact activation and that this cleavage, which liberates kinin and gives a two-chain, disulfide-linked molecule, is dependent upon the presence of prekallikrein and Factor XII (Hageman factor). A preliminary report of this work has been presented (29).

MATERIALS AND METHODS

Purification of High M, Kininogen—High M, kininogen was isolated from citrated fresh human plasma. The plasma (2.2 liters) was obtained as previously described (30) and dialyzed at 20°C against the starting buffer (40 mM Tris, 10 mM succinic acid, pH 8.2, containing 1 mM EDTA, 1 mM benzamidine, 50 µg/ml of Polybrene (Aldrich Chemicals), 0.02% NaN3, and 13 mM NaCl) for chromatography on a column (10 x 40 cm) containing 140 g of DEAE-Sephadex A-50 (Pharmacia). The protein fraction that did not adhere to the resin contained prekallikrein and Factor XI and flowed through the column in the starting buffer. The column was then washed with a buffer containing the same components, but made 121 mM Tris, 44 mM in succinic acid, pH 7.7, and 103 mM NaCl (15 mmoi conductivity). This buffer effected the elution of plasminogen and Factor XII. After extensive washing with this buffer, the high M, kininogen was eluted at 480 ml/h with a buffer containing the same components as above but made 193 mM Tris, 75 mM succinic acid, pH 7.4 and 182 mM NaCl (24 mmoi). Fractions containing high M, kininogen were pooled and brought to 50% saturation of (NH4)2SO4 by addition of solid (NH4)2SO4 at 4°C. After stirring for 90 min, the precipitate was collected by centrifugation for 1 h at 2000 × g. The precipitate was dissolved in 130 ml of buffer containing 50 mM sodium acetate, 75 mM NaCl, 0.5 mM EDTA, 0.5 mM benzamidine, and 0.01% NaN3 at pH 5.3. After extensive dialysis in this buffer, a slight precipitate was eliminated by centrifugation for 20 min at 12000 × g, and the sample was then applied to an SP-Sephadex C-50 column (2.5 x 13 cm). After the sample entered the gel, the column was washed at 30 ml/h with 450 ml of a buffer containing 0.1 M sodium acetate, 150 mM NaCl, 1 mM EDTA, 1 mM benzamidine, and 0.02% NaN3, pH 5.3. A linear gradient elution was then performed with 150 ml of the latter buffer but containing 500 mM NaCl in the distal chamber.

Clotting Activity of High M, Kininogen—High M, kininogen clotting activity was measured by two-stage kaolin-activated partial
thromboplastin times (31) using high M, kininogen-deficient plasma (Fitzgerald trait plasma obtained from George King Biomedical). One unit of high M, kininogen clotting activity is defined as the amount of activity present in 1 ml of a standard pool of normal citrated human plasma.

Protein concentrations were determined by the Lowry method (32) using bovine serum albumin (Sigma) as reference protein. Polyacrylamide Gel Electrophoresis—Electrophoresis was carried out in the presence of SDS on 7.5% or 10% polyacrylamide gels (6 mm × 8 cm) according to Weber et al. (33). The gels were stained for proteins with Coomassie blue G-250.

Analytical ultracentrifuge equipped with a scanner, ultraviolet optics, and a multiplexer. The samples were centrifuged using 12-mm filled Epon double-sector cells equipped with a siliconized glass tube with 87 μl containing 16 mg/ml of kaolin, 5 times diluted rabbit brain cephalin (Sigma), and 20 mM CaCl2 in Tris-buffered saline. Ten-microliter aliquots were withdrawn as a function of time and rapidly added to siliconized glass tubes in a boiling water bath containing 100 μl of 1% SDS, 8 mM urea, and 1% β-mercaptoethanol. The samples were boiled for 8 min and electrophoresed on 10% SDS-polyacrylamide gels. The gels were then sliced and the radioactivity of the slices determined. Data were recorded on punchtape and analyzed on a Hewlett-Packard 9810A calculator and plotter. In order to determine the kinetics of kinin liberation in plasma during contact activation, identical experiments without the radiolabeled high M, kininogen were performed in a total volume of 2 ml. As a function of time, 100-μl aliquots were withdrawn and rapidly added to 200 μl of boiling 0.9% NaCl (12). This heat treatment was sufficient to quench totally the liberation of kinin. After the samples were held in a boiling water bath for at least 5 min, each sample was brought to room temperature and assayed in the kinin bioassay.

RESULTS

Preparation of Human High M, Kininogen—The purification steps and the yield for a typical preparation of human high M, kininogen are shown in Table I. The purification steps are described under "Materiale and Methods." The DEAE-Sephadex chromatography step allowed the separation of high M, kininogen from Factor XI and prekallikrein. Factor XII and plasminogen were subsequently eluted with the 15-mM buffer. High M, kininogen was eluted from the DEAE-Sephadex at a conductivity of 24 mmosm. The bulk of the remaining contaminating proteins was then eluted during the sulfopropyl-Sephadex chromatography. At pH 5.3, high M, kininogen bound to the sulfopropyl-Sephadex column, while the other proteins did not, and was subsequently eluted with a NaCl gradient at 23-mmosm conductivity. This purification procedure gave high M, kininogen in 35% yield with a 1000-fold purification. Based on the observed specific activity of 16 clotting units/mg, the concentration of high M, kininogen in plasma is estimated to be 61 μg/ml. Over a 2-year period, the activity was measured by the latent period between administration of the fluid being tested and the onset of contraction. The sensitivity of the response was calibrated with a standard solution of Bradykinin (Sandoz Pharmaceuticals) by adding 0.3 to 5 ng to the bath.

Kinin Liberation and Clotting Activity of High M, Kininogen Inhbitured With Kallikrein—High M, kininogen (final concentration 230 μg/ml) was incubated at 37°C with plasma kallikrein (final concentration 0.65 μg/ml) in 1.6 ml of 10 mM Tris-HCl buffer, pH 8.1, and 50-μl aliquots were withdrawn as a function of time and mixed with a 25-μl solution containing 75 μg/ml of soybean trypsin inhibitor (Sigma) and 2.4 mM orthophenanthroline. Under these conditions the soybean trypsin inhibitor blocked kallikrein activity immediately. One to 5 μl of each sample containing 0.13 to 0.66 μg of high M, kininogen were then tested for kinin content in the rat uterus bioassay, and 10 μl of each sample was diluted 1 to 60 into Tris-buffered saline containing 1 mg/ml of bovine serum albumin for analysis of high M, kininogen procoagulant activity.

Kinin Liberation and Cleavage of High M, Kininogen in Plasma During Contact Activation—Seventy microliters of 125I-high M, kininogen (6 μCi, 0.2 clotting unit/ml) in Tris-buffered saline were mixed with the same volume of either normal human plasma or prekallikrein-deficient or Factor XII-deficient plasmas in plastic tubes. To initiate contact activation, the 140-μl sample was mixed in a siliconized glass tube with 87 μl containing 16 mg/ml of kaolin, 5 times diluted rabbit brain cephalin (Sigma), and 20 mM CaCl2 in Tris-buffered saline. Ten-microliter aliquots were withdrawn as a function of time and rapidly added to siliconized glass tubes in a boiling water bath containing 100 μl of 1% SDS, 8 mM urea, and 1% β-mercaptoethanol. The samples were boiled for 8 min and electrophoresed on 10% SDS-polyacrylamide gels. The gels were then sliced and the radioactivity of the slices determined. Data were recorded on punchtape and analyzed on a Hewlett-Packard 9810A calculator and plotter. In order to determine the kinetics of kinin liberation in plasma during contact activation, identical experiments without the radiolabeled high M, kininogen were performed in a total volume of 2 ml. As a function of time, 100-μl aliquots were withdrawn and rapidly added to 200 μl of boiling 0.9% NaCl (12). This heat treatment was sufficient to quench totally the liberation of kinin. After the samples were held in a boiling water bath for at least 5 min, each sample was brought to room temperature and assayed in the kinin bioassay.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Purification of human high M, kininogen</th>
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<tr>
<td>Volume</td>
<td>Total protein</td>
</tr>
<tr>
<td>ml</td>
<td>units</td>
</tr>
<tr>
<td>Plasma</td>
<td>185.6</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>1340</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>160</td>
</tr>
<tr>
<td>precipitation</td>
<td>SP-Sephadex</td>
</tr>
</tbody>
</table>

1 The abbreviation used is: SDS, sodium dodecyl sulfate.

2 B. N. Bouma, L. A. Miles, G. Beretta, and J. H. Griffin, manuscript in preparation.
determinations of the specific clotting activity of four different preparations of high $M_r$ kininogen using different samples of deficient plasma have given values ranging from 13 to 19 clotting units per mg.

**Molecular Weight of High $M_r$ Kininogen**—In SDS-polyacrylamide gels in the absence of reducing agents, high $M_r$ kininogen migrated as a single protein band, as shown in Fig. 1, corresponding to an apparent $M_r$ of 110,000. The presence of reducing agents did not modify the apparent $M_r$, showing that the molecule was isolated as a single polypeptide chain.

The $M_r$ of native high $M_r$ kininogen determined by sedimentation equilibrium methods was 108,000 ± 2,000.

**Cleavage of Human High $M_r$ Kininogen by Human Plasma Kallikrein**—The proteolytic cleavage of high $M_r$ kininogen in solution by purified kallikrein was studied. A mixture of unlabeled and $^{125}$I-labeled high $M_r$ kininogen at 2.5 clotting units/ml was incubated in the presence of 0.19 µg/ml of kallikrein at pH 8.1. Aliquots were withdrawn at a function of time and rapidly added to an excess of boiling hot SDS to quench the reaction. The samples were then analyzed by electrophoresis on SDS-polyacrylamide gels in the presence and absence of mercaptoethanol. As seen in Fig. 2 on unreduced gels, the protein corresponding to the 110,000 $M_r$ species disappeared with increasing time, while two other species of slightly faster mobility were progressively formed. The analysis of the samples on reduced SDS gels showed that the cleaved molecules were made up of disulfide-linked polypeptide chains. After 165-min incubation (Fig. 2), the 110,000 form completely disappeared, showing that all the high $M_r$ kininogen single-chain molecules were cleaved, and two molecular species with apparent $M_r$ values of 65,000 and 44,000 were seen on reduced SDS gels.

In order to quantitate the kinetics of cleavage of $^{125}$I-high $M_r$ kininogen by kallikrein, the stained gels were sliced and the radioactivity profiles on reduced gels were analyzed as a function of time. The results indicated that under the conditions used 50% of the single-chain form of high $M_r$ kininogen was cleaved in 17 min and that >90% of the radioactive molecules at 110,000 $M_r$ was cleaved in 90 min. In these experiments the cleavage of $^{125}$I-high $M_r$ kininogen as judged in the radioactivity profiles showed a good correlation to cleavage as judged by gel staining.

In order to analyze kinin liberation in relation to proteolysis of high $M_r$ kininogen by kallikrein, the presence of kinin in the incubated samples was examined. A parallel experiment was performed in which the kallikrein activity was stopped as a function of time by adding 50-µl aliquots of the reaction mixture to 25 µl of soybean trypsin inhibitor and orthophenanthroline (see under “Materials and Methods”). The samples were then tested for procoagulant activity and for the presence of kinin. Kallikrein progressively liberates kinin from high $M_r$ kininogen, and the amount of kinin maximally reached 1% of the weight of incubated high $M_r$ kininogen. The clotting activity of the kinin-free high $M_r$ kininogen molecule remained...
invariably the same, showing that kinin release does not result in any loss of procoagulant activity.

Isolation of a Kinin-containing Cleaved High M, Kininogen—In approximately half of our preparations of high M, kininogen a disulfide-linked, two-chain form of high M, kininogen copurified with the single-chain molecule. This species migrated on unreduced SDS gels as a single protein band exhibiting a migration slightly faster than the single-chain molecule. This two-chain form of high M, kininogen contained kinin. The intermediate form of the high M, kininogen observed during kallikrein cleavage (Fig. 2) exhibited an identical mobility on SDS-polyacrylamide gels in the absence of reducing agent.

Purification of the Heavy and Light Chains of Kinin-free High M, Kininogen—High M, kininogen (12 mg) was cleaved by human plasma kallikrein under conditions described under “Materials and Methods” to provide material for isolation of the polypeptide chains of the kinin-free molecule. As determined by a rat uterus bioassay, kinin was entirely liberated from high M, kininogen, while the clotting activity of this molecule remained unchanged. The kinin-free high M, kininogen migrated as a single band on an SDS-polyacrylamide gel in the absence of reducing agent. Analysis of this sample on a reduced SDS-polyacrylamide gel verified (Fig. 3) that the cleaved high M, kininogen was a disulfide-linked, two-chain molecule made up of a light chain and a heavy chain with apparent M, values of 44,000 and 65,000. For the isolation of the heavy and light chains, kinin-free high M, kininogen was reduced and alkylated as described under “Materials and Methods.” The alkylated light chain and heavy chain were then separated by sulfopropyl-Sephadex chromatography. At a 13-mmho conductivity at pH 5.8, the heavy chain did not bind to the resin, while the light chain was retained. A NaCl gradient was then applied to elute the light chain. The elution peak appeared at a conductivity of 40 mmho. As seen in Fig. 3, the isolated alkylated heavy and light chains migrated as single bands on SDS-polyacrylamide gels and exhibited the respective apparent M, values of 65,000 and 44,000.

Activities of the Isolated Heavy and Light Chains of High M, Kininogen—The clotting activities of the isolated alkylated heavy and light chains were determined and compared to the activities of native and kinin-free high M, kininogen. The results (Table II) show that the isolated light chain exhibited a clotting of 31 units per mg or 1.4 units per nmol, a specific clotting activity identical with that of the native molecule. Thus, the light chain accounts for the entire activity of the native and of the kinin-free molecules. The heavy chain did not show any detectable activity. These quantitative results indicate that the procoagulant activity of high M, kininogen resides exclusively in the light chain region of the molecule without any apparent participation of the heavy chain region in this activity.

Amino Acid Composition of Human High M, Kininogen and of Its Heavy and Light Chains—The amino acid compositions of high M, kininogen and of the isolated heavy and light chains were determined and are shown in Table III. In general, the amino acid compositions of the molecules are very close to those of bovine high M, kininogen and of the light and heavy chains isolated after cleavage of this molecule by human urinary kallikrein (26). In particular, the 11.2% histidine content of the human light chain is very high, and it reflects most of the histidine content of the native molecule. Interestingly, this proportion of histidine is the same as that reported for the bovine fragment that contains an unusual histidine-rich region named Fragment 1-2 (26).

**Table II**

<table>
<thead>
<tr>
<th>Form of high M, kininogen</th>
<th>Native high M, kininogen</th>
<th>Kinin-free high M, kininogen</th>
<th>Light chain</th>
<th>Heavy chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotting units per mg</td>
<td>13</td>
<td>12</td>
<td>31</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Clotting units per nmol</td>
<td>1.4</td>
<td>1.3</td>
<td>1.4</td>
<td>&lt;0.06</td>
</tr>
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</table>

**Table III**

<table>
<thead>
<tr>
<th>Amino acid composition of human high M, kininogen</th>
<th>Residues per 100 residues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
</tr>
<tr>
<td>Lysine</td>
<td>7.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.5</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>10.9</td>
</tr>
<tr>
<td>Threonine</td>
<td>7.9</td>
</tr>
<tr>
<td>Serine</td>
<td>7.2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>12.5</td>
</tr>
<tr>
<td>Proline</td>
<td>6.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.7</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.0</td>
</tr>
<tr>
<td>Valine</td>
<td>4.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.95</td>
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<tr>
<td>Isoleucine</td>
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<tr>
<td>Leucine</td>
<td>6.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.6</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.0</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>2.7</td>
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Cleavage of High M, Kininogen in Plasma—The functional role of high M, kininogen as a cofactor in the activation of surface-bound Factor XII by kallikrein as well as in the activation of Factor XI or prekallikrein by surface-bound activated Factor XII has been previously demonstrated (23, 24). It has also been shown that prekallikrein is very rapidly activated to kallikrein during the contact activation process. Since purified high M, kininogen is susceptible to cleavage by purified human plasma kallikrein, the question arose of whether or not the newly generated kallikrein cleaves the single-chain high M, kininogen during contact activation. To analyze this question, 125I-high M, kininogen was added to normal plasma or to plasmas deficient to prekallikrein or Factor XII. As described under "Materials and Methods," contact activation of plasma was induced by kaolin, and aliquots withdrawn as a function of time were analyzed on reduced SDS-polyacrylamide gels and in kinin bioassays. As seen in Fig. 4, the radioactivity profiles of the gels indicate that the single-chain high M, kininogen was rapidly cleaved. Maximum cleavage was reached in less than 100 s after kaolin was added. The kinetics of cleavage shown in Fig. 5 indicate that following an initial lag period half of the high M, kininogen molecules were cleaved at 36 s. Kinin appearance very closely paralleled the cleavage of high M, kininogen. The maximum amount of kinin liberated was 0.19 ng/ml (Fig. 5). Assuming kinin represents 1% of the mass of high M, kininogen and correcting for the dilution of plasma in these experiments, it is calculated that the kinin released reflects cleavage of 62 ng/ml of high M, kininogen in the original plasma. In prekallikrein-deficient plasma or Factor XII-deficient plasma, no cleavage of high M, kininogen and no kinin release were observed. Addition of purified plasma kallikrein to plasmas deficient in either prekallikrein or Factor XII resulted in cleavage of the 125I-high M, kininogen and a release of kinin. This indicates that kallikrein was sufficient for high M, kininogen cleavage and kinin release and that Factor XII is not necessary if prekallikrein has been converted to kallikrein.

Structure of Kinin-free High M, Kininogen Cleaved in Plasma Compared To That Cleaved by Purified Kallikrein in Solution—A comparative analysis of the structure of 125I-high M, kininogen cleaved in plasma to that of the kinin-free molecule generated by purified kallikrein in solution was performed to determine whether or not the single chain protein was cleaved identically under each set of conditions. To generate the molecular species arising from the cleavage of high M, kininogen in plasma, 15 µl of normal human plasma containing 125I-high M, kininogen was incubated for 2 min at 37 °C in the presence of 5 mg/ml of kaolin in 60 µl of Tris-buffered saline. The kaolin in this reaction mixture bound 55% of the 125I-high M, kininogen. This kaolin was washed extensively by Tris-buffered saline containing 1 mg/ml of bovine serum albumin. Then the proteins were eluted from the surface and electrophoresed on reduced 10% SDS-polyacrylamide gels. A sample of 125I-high M, kininogen cleaved in a purified system by plasma kallikrein was similarly analyzed on reduced 10% SDS-polyacrylamide gel. As seen in Fig. 6, high M, kininogen obtained from plasma incubated with kaolin was a cleaved molecule made up of two polypeptide chains that exhibited the same apparent M, values as the heavy and light chains formed when high M, kininogen was cleaved in solution by plasma kallikrein. In gel profiles not shown, the 125I-high M, kininogen found in the plasma supernatant at the end of the incubation was also entirely cleaved. Moreover, as seen in Fig. 6, the light chain was poorly labeled using the chloramine-T method. This is probably due to the fact that the tyrosine content of the light chain is low (0.7%) compared to that of the heavy chain (3.7%).

The Mobility of Kinin-free High M, Kininogen on Reduced SDS-Polyacrylamide Gels is Influenced by Plasma Pro
ble clotting unit/ml) was incubated for 35 min at 37°C, either in plasma kallikrein, 125I-high M, kininogen (15 μCi/ml, 0.28 clotting units/ml) was incubated for 30 min at 37°C, or in prekallikrein-deficient plasma diluted 1 to 3 with Tris-buffered saline in a total volume of 55 μl in 0.01 M Tris-HCl buffer, pH 8. The incubated sample was boiled for 8 min in presence of 50 μl of 1% SDS, 8 mM urea, 1% β-mercaptoethanol, and 0.1 M EDTA and analyzed on SDS-polyacrylamide gels. The '*' labeled samples incubated under the conditions described in the text were mixed with SDS under reducing conditions and analyzed on 7.5% SDS-polyacrylamide gels. A, mixture of 50 μl of 125I-high M, kininogen (20 μCi/ml, 2.8 clotting units/ml) was incubated for 30 min at 37°C in the presence of 25 μg/ml of plasma kallikrein in a total volume of 55 μl in 0.01 M Tris-buffered saline, pH 7, or in prekallikrein-deficient plasma. The '*' labeled samples and the 125I-labeled samples incubated under the conditions described in the text were mixed with SDS under reducing conditions and analyzed on 7.5% SDS-polyacrylamide gels. A, mixture of 50 μl of 125I-high M, kininogen (20 μCi/ml, 2.8 clotting units/ml) was incubated for 30 min at 37°C in the presence of 25 μg/ml of plasma kallikrein in a total volume of 55 μl in 0.01 M Tris-HCl buffer, pH 8. The incubated sample was boiled for 8 min in presence of 50 μl of 1% SDS, 8 mM urea, 1% β-mercaptoethanol, and 0.1 M EDTA and analyzed on SDS-polyacrylamide gels. B, mixture of 50 μl of 131I-high M, kininogen cleaved by kallikrein in prekallikrein-deficient plasma and of 50 μl of 131I-high M, kininogen cleaved by purified kallikrein in solution.

**DISCUSSION**

Human high M, kininogen purified as described here is a single polypeptide chain of M, 110,000. Previously, highly purified high M, kininogen composed of two disulfide-linked chains of 70,000 and 50,000 M, was isolated (8) although Nagasawa and Nakayasu reported the isolation of a single-chain molecule (10). Initially, we were able to isolate a two-chain form (23). Other authors reported obtaining a mixture of two species of high M, kininogen (36, 39). The two species were kinin-containing molecules in either a one-chain or a two-chain form (38, 39). Thompson et al. reported the isolation of high M, kininogen mainly as a single polypeptide chain (40). Taken together, these results suggest that human high M, kininogen is originally a single chain 110,000 M, molecule that is proteolytically converted either in vivo or during the isolation procedure into a two-chain species.

The major kininogenase of plasma circulates as a zymogen, prekallikrein, that is activated to kallikrein by limited proteolysis by activated Factor XII (41). Incubation of high M, kininogen with purified human plasma kallikrein was performed and showed, in agreement with other reports, that kinin is progressively liberated, and that a two-chain, disulfide-linked high M, kininogen is formed (10, 38, 40). In the kallikrein cleavage studies, a form of high M, kininogen appeared on unreduced SDS-polyacrylamide gels that had a mobility that was intermediate between the native single-chain molecule and the final two-chain product. This intermediate form of the molecule may be identical with the two-chain form of high M, kininogen that contains kinin. Following cleavage by plasma kallikrein, high M, kininogen is made up of a heavy chain of apparent M, 65,000 and a light chain of apparent M, 44,000 that are disulfide linked. Following reduc-
tion and alkylation, the heavy and light chains can be isolated using chromatography on a sulfopropyl-Sephadex column at pH 5.8. Analysis of the procoagulant activity of the two-chain, kinin-free molecule and of the alkylated isolated heavy and light chains indicates that the alkylated light chain quantitatively retains the procoagulant activity of the native molecule and of the two-chain disulfide-linked molecule. The alkylated heavy chain does not show any detectable procoagulant activity. This quantitative estimation of the respective activities of high M₉ kininogen and its fragments is consistent with the previous qualitative observations that human kinin-free high M₉ kininogen as well as alkylated light chain shorten the clotting time of high M₉ kininogen-deficient plasma (40).

Suzuki and his colleagues purified bovine high M₉ kininogen as a single-chain molecule (42, 43) and demonstrated that human urinary kallikrein cleaves the molecule resulting in kinin liberation and giving a two-chain molecule containing an NH₂-terminal heavy chain and a COOH-terminal light chain (26, 44). Waldmann et al. (27) and Wuepper et al. (28) showed that this kinin-free high M₉ kininogen and the carboxymethylated light chain are as procoagulant as the native bovine molecule when measured in human high M₉ kininogen-deficient plasma. However, when bovine high M₉ kininogen is cleaved by bovine plasma kallikrein, the procoagulant activity is entirely lost (21, 22). This loss of activity is correlated with the release, along with kinin, of a histidine-rich glycopeptide fragment (25, 26, 43, 44). Sequence studies indicated that this histidine-rich fragment is situated in the NH₂-terminal end of the light chain region of bovine high M₉ kininogen (25).

This report presents the amino acid composition of human high M₉ kininogen and of its fragments formed by cleavage with human plasma kallikrein. In general, the amino acid compositions of the human polypeptide chains are very similar to those of the corresponding bovine polypeptide chains (26). The histidine content of the isolated human light chain is 11.9% and reflects most of the histidine content of the native molecule. This proportion of histidine is the same as that observed in the bovine polypeptide chain obtained by cleavage of that molecule with human urinary kallikrein (26). Histidine-rich polypeptide fragments have been isolated from proteolytic digests of human high M₉ kininogen (45). It appears, therefore, that the active human light chain contains the unusual histidine-rich region. It is particularly noteworthy that human plasma kallikrein does not readily liberate the histidine-rich region from human high M₉ kininogen as shown here and elsewhere (39), whereas bovine plasma kallikrein readily releases the histidine-rich fragment from bovine high M₉ kininogen with a consequent loss of procoagulant activity (vide supra).

When contact activation is induced by addition of kaolin to plasma, rapid cleavage of 125I-human high M₉ kininogen and liberation of kinin occur (Fig. 5). The time course of kinin appearance closely parallels the cleavage of 125I-high M₉ kininogen. This cleavage in plasma gives a disulfide-linked molecule similar to the molecule cleaved by kallikrein in purified reaction mixtures as judged by reduced SDS-polyacrylamide gel electrophoresis. In prekallikrein-deficient or Factor XII-deficient plasma, no cleavage of high M₉ kininogen was observed upon kaolin addition. However, addition of purified plasmin kallikrein to plasmas deficient in either prekallikrein or Factor XII resulted in cleavage of high M₉ kininogen and in the release of kinin. Thus, it appears that the plasma kallikrein generated during contact activation is responsible for the rapid cleavage of high M₉ kininogen in plasma during contact activation and for the release of kinin.

The mechanisms by which high M₉ kininogen contributes to contact activation reactions have been recently clarified. In kinetic studies of surface-dependent reactions, it was shown that high M₉ kininogen functions as a stoichiometric cofactor in the activation of Factor XI as well as of prekallikrein by surface-bound activated Factor XII (23, 24). Moreover, it was observed that high M₉ kininogen also facilitated the limited proteolysis of surface-bound Factor XII by kallikrein (25, 46). It was, therefore, suggested that Factor XII and high M₉ kininogen associate with each other on a negatively charged surface and that this association facilitates the action of activated Factor XII on its substrates. It was shown that high M₉ kininogen associates in solution with prekallikrein (47) or with Factor XI (48) and that high M₉ kininogen is indeed necessary for the binding of prekallikrein or Factor XI to negatively charged surfaces in the milieu of plasma (49). As demonstrated here, kaolin added to plasma containing 125I-high M₉ kininogen binds the labeled molecule. Moreover, a region of high M₉ kininogen, the light chain, was isolated that is rich in histidine and that binds firmly to negatively charged resins. Thus, it appears that high M₉ kininogen binds to negatively charged surfaces through this histidine-rich region and acts as a surface-bound receptor for Factor XI and prekallikrein where these molecules are activated by surface-bound activated Factor XII (49, 50). Factor XI, molecules remain bound at the surface sites of activation, in contrast to kallikrein molecules that readily dissociate into the surrounding space (49, 51). Using immunological techniques we have recently shown that the isolated light chain is by itself able to bind prekallikrein. This emphasizes the essential and sufficient role of the light chain moiety of high M₉ kininogen for the contact activation cofactor activities of the parent molecule.

Since high M₉ kininogen is rapidly cleaved in plasma during contact activation, the question arises of whether or not the molecule has to be cleaved to be procoagulant. Kato et al. (52) recently reported that the rate of kallikrein generation in a purified system containing bovine Factor XII, bovine plasma prekallikrein, and kaolin is more effectively accelerated in the presence of a nicked bovine high M₉ kininogen than in the presence of the native single chain form of the molecule. Thus, these authors suggested that limited proteolysis of high M₉ kininogen during the early phase of contact activation digestion may transform this molecule into an "active" high M₉ kininogen. Further studies will be required to establish whether conformational changes in human high M₉ kininogen due to limited proteolysis are essential or relevant for the activity of this molecule as a cofactor in contact activation.

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