New and Rapid Functional Assay for C1 Inhibitor in Human Plasma

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C1 inhibitor (C1-INH) and α2-macroglobulin (α2M) account for over 90% of the inactivation of purified plasma kallikrein by normal human plasma. The rate of kallikrein inactivation is also dependent on the presence of high molecular weight kininogen (HMWK), which forms a reversible complex with kallikrein protecting the active site of the enzyme against inhibitors. By selectively inactivating α2M with methylamine, and eliminating the protective effect of HMWK by dilution, the inactivation of kallikrein by plasma became almost exclusively dependent on C1-INH. Functional C1 inhibitor was assessed by measuring the pseudo-first-order rate constant for the inactivation of kallikrein by diluted methylamine-treated plasma in 29 individuals, including 11 controls, 11 oral contraceptive users, 5 patients with classical hereditary angioedema (HAE), and 2 patients with variant HAE. Over a wide range of concentrations, an excellent correlation (r = 0.90) was observed between functional and antigenic C1-INH among controls, oral contraceptive users, and patients with classical HAE. This new functional assay for C1-INH can be performed in less than 3 hr with commercially available reagents. Therefore, this assay will be helpful for the diagnosis and management of conditions associated with the deficiency of C1-INH, such as HAE.

We have recently defined the variables controlling the inactivation of kallikrein in plasma. The inactivation of kallikrein is dependent almost exclusively on the presence of two protease inhibitors, C1-INH and α2-macroglobulin (α2M). Indeed, C1-INH and α2M account for over 90% of the inactivation of kallikrein by normal plasma. However, the rate of kallikrein inactivation is also dependent on the presence of HMWK, both in plasma and in purified systems, since HMWK forms a reversible complex with kallikrein, which protects the enzyme from inactivation.

In this article, we describe a new functional assay for C1-INH, based on the determination of the rate constant for the inactivation of purified kallikrein by plasma. We use conditions that selectively inactivate α2M, as well as greatly reduce the effect of HMWK. Under these conditions, the rate of inactivation of kallikrein by plasma becomes almost exclusively dependent on the presence of functional C1-INH.

MATERIALS AND METHODS

Reaction Vessels

Glass tubes (6 x 50 mm) were obtained from Fisher Scientific Co. (Pittsburgh, Pa.). The tubes were coated with silicone (Dri-Film SC-87, Pierce, Rockford, Ill., in a mixture of hexane isomers, Fisher Scientific Co., Pittsburgh, Pa.). Silicone-coated tubes were used only to increase the reaction rate. We have recently defined the variables controlling the inactivation of kallikrein in plasma.
once, within a week of preparation. Alternatively, polypropylene microcentrifuge tubes, 0.75-ml capacity (Walter Sarstedt Inc., Princeton, N.J.), which were preincubated with buffer containing 2 mg/ml BSA for 5 min before the addition of other reactants, were employed.

**Plasma Kallikrein**

Kallikrein was prepared by activating 1.0 ml purified prekallikrein, (1.4 mg), which is a single component on sodium dodecyl sulfate polyacrylamide gel electrophoresis, with 10 μl factor XII fragments (0.45 μg). Using 0.6 mM H-D-Pro-Phe-Arg-p-nitroanilide (S-2302, Kabi Group Inc., Greenwich, Conn.) as a substrate in a volume of 330 μl, the catalytic efficiency, kcat/Km, of this preparation was 4.5 x 106 M⁻¹ s⁻¹ and the stock kallikrein (1.4 mg/ml) could be calculated to give A₅₀₄₀ = 21.6/min. In contrast, 10 μl of the stock solution of factor XII fragments gave A₅₀₄₀ = 0.20/min. Therefore, a 1:100 dilution of factor XII fragments added to the prekallikrein preparation would give a A₅₀₄₀ = 0.002/min or less than 0.01% of the kallikrein amidolytic activity. Therefore, factor XII fragments were not removed from the kallikrein preparations. In some experiments, kallikrein was produced by the activation of the prekallikrein obtained after the second SP-Sephadex step of the preparation. This material contained 30 mg IgG/mg kallikrein as the only contaminant. In other experiments, kallikrein was purchased from Kabi Group Inc., Greenwich, Conn. Moreover, all three preparations were stable for at least 6 mo when kept at 4°C.

**Plasma Samples**

Plasma samples, prepared as described, were obtained after written and informed consent from 11 normal controls and from 11 women using oral contraceptive agents. Kininogen-deficient plasma was generously donated to us by M. Williams. Three plasma samples from patients with classical HAE were generously donated to us. Two additional plasma samples from patients with classical HAE were kindly provided by Dr. V. H. Donaldson, Children's Hospital Research Foundation, Cincinnati and Dr. M. M. Frank, NIAID, NIH, Bethesda. In addition, two plasma samples from patients with variant HAE were kindly provided by Dr. J. G. Curd, Department of Molecular Immunology, Scripps Clinic and Research Foundation.

**Cl-Inhibitor Antigen**

Cl-inhibitor antigen was assayed by radial immunodiffusion using antiserum to Cl-inhibitor, obtained from Behring Diagnostics, Somerville, N.J. The plates were calibrated with purified Cl-Inhibitor Antigen was determined by radial immunodiffusion.

**Treatment With Methylamine**

Selective inactivation of α2M in the plasma samples was achieved by preincubation of 100 μl of plasma with 25 μl of 0.2 M methylamine (final concentration 40 mM) for 2 hr at 23°C. Methylamine (40% solution, w/v) was obtained from Fisher Scientific Co., King of Prussia, Pa.

**Kinetic Studies**

Ten microliters of various dilutions of methylamine-treated plasma were mixed with 50 μl sodium phosphate buffer (0.1 M), pH 7.6, containing 0.15 M NaCl in a silicone-coated glass tube at 23°C. Then, 10 μl of kallikrein stock solution were mixed with the diluted methylamine-treated plasma at 23°C (kallikrein final concentration 50 nM) so that the final volume of the preincubation mixture was 70 μl. The final dilution of plasma was calculated including the dilution of 4:5 introduced by treatment with methylamine. At precise time intervals, 10-μl aliquots of the preincubation mixture were transferred to a cuvette containing 330 μl of S-2302 (0.6 mM) in sodium phosphate buffer (85 mM), pH 7.6, and 127 mM NaCl at 37°C. The change in absorbance at 405 nm was continuously recorded using a Cary 210 double-beam spectrophotometer (Varian, Palo Alto, Calif.). The half-life (t½) of enzyme activity was determined (min) after addition of diluted methylamine-treated plasma and the pseudo-first-order rate k constant for the inactivation of plasma was obtained by dividing 0.693 by t½. The k values were expressed in min⁻¹. Under these conditions, the hydrolysis rate of S-2302 by 1 nM kallikrein was 1.84 nmole/min.

**Statistical Methods**

The significance of difference between means was assessed by unpaired Student’s t tests. Linear regression, as well as the coefficient of correlation and its deviation from zero, were calculated as described.

**RESULTS**

**Kinetics of Inactivation of Kallikrein by Plasma Depleted of α2M: Influence of HMWK**

The inactivation of kallikrein amidolytic activity by two concentrations of normal plasma or kininogen-deficient plasma followed pseudo-first-order kinetics, provided that α2M in each plasma had been inactivated by prior incubation with methylamine (Fig. 1). Dilution of the enzyme–plasma mixture with substrate terminated the inactivation reaction, resulting in a linear hydrolysis rate of the substrate. Both plasma samples contained an identical concentration of Cl-INH antigen as determined by radial immunodiffusion. The pseudo-first-order rate constants, k', for the inactivation of purified kallikrein by normal plasma were 0.31 (Fig. 1, open triangles) and 0.99 min⁻¹ (Fig. 1, open circles), when the final dilutions of the plasma samples in the preincubation mixtures were, respectively, 1/875 and 1/146. When kallikrein was inactivated by kininogen-deficient plasma, at a final dilution of 1/875, k' was also 0.31 min⁻¹ (Fig. 1, closed triangles), while it was 1.65 min⁻¹ when the final dilution of the kininogen-deficient plasma was 1/146 (Fig. 1, closed circles). Thus, the presence of HMWK, at a final concentration of 0.5 μM (Fig. 1, open circles), resulted in a 40% reduction in k', when compared to k' observed in the absence of HMWK (Fig. 1, closed circles). In contrast, when HMWK was present at a final concentration of 0.08 μM (Fig. 1, open triangles), its effect was not detectable, since the k' for the inactivation of kallikrein by either normal plasma or kininogen-deficient plasma at that dilution were identical. Therefore, a 1/8.75 dilution of plasma seems to be appropriate to minimize the effect of HMWK on the inactivation rate of purified kallikrein. At this plasma dilution, Cl-INH is still present at a concentration that is a five-fold molar excess over kallikrein, resulting in
FUNCTIONAL ASSAY FOR PLASMA C\textsubscript{1} INHIBITOR

Fig. 1. Kinetics of inactivation of kallikrein amidolytic activity. Both methylamine-treated normal plasma (open symbols) or methylamine-treated kininogen-deficient plasma (closed symbols) were employed. Kallikrein (0.3 \( \mu \)g) was preincubated at 23\(^\circ\)C with the plasma in a final volume of 70 \( \mu l \) and then assayed at various times for residual amidolytic activity. The final dilutions of the plasma were 1/1.46 (circles) or 1/8.75 (triangles). The result displayed is representative of three replicate experiments.

pseudo-first-order inactivation kinetics. All three preparations of kallikrein gave comparable results.

Influence of the Concentration of Kininogen-Deficient Plasma on the Inactivation of Kallikrein

To assess the relationship between \( k' \) for the inactivation of purified kallikrein and the concentration of plasma in the preincubation mixture, purified kallikrein was inactivated by various concentrations of methylamine-treated kininogen-deficient plasma. A linear relationship was observed between \( k' \) and various dilutions of methylamine-treated kininogen-deficient plasma, ranging from 1/87.5 to 1/5.8, i.e., 1–15 \( \mu l \) of plasma in a final volume of 70 \( \mu l \) (Fig. 2). Therefore, samples containing between 10% and 150% of the kallikrein inhibitory activity present in methylamine-treated normal plasma can be accurately studied using experimental conditions where the final dilution of the plasma to be tested is 1/8.75. At a dilution less than 1/5.8, the increase in \( k' \) is less than proportional to the increase in plasma volume due to the protective effect of HMWK.

Relationship Between \( k' \) and C\textsubscript{1}-INH Antigen

The \( k' \) value for the inactivation of purified kallikrein by a 1/8.75 dilution of methylamine-treated plasma as well as the C\textsubscript{1}-INH antigen concentration was determined in 29 individuals, including 11 controls, 11 women using oral contraceptives, 5 patients with classical HAE, and 2 patients with variant HAE (Table I). The mean \( k' \) value among controls was found to be 0.35 ± 0.07 min\(^{-1}\) (mean ± SD), while the mean C\textsubscript{1}-INH antigen concentration was 253 ± 41 \( \mu g/ml \) (mean ± SD). No differences in \( k' \) values and C\textsubscript{1}-INH antigen concentrations were observed between male and female controls. The mean value for \( k' \) and C\textsubscript{1}-INH antigen levels in oral contraceptive users was decreased to 60% (\( p < 0.001 \)) and 63% (\( p < 0.001 \)), respectively, of the values observed.
Table 1. Kallikrein Inactivation Rate Constants $k'$ and C1-INH Antigen Levels in 29 Individuals

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>$k'$</th>
<th>$p$ Value</th>
<th>C1-INH Antigen</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>11</td>
<td>0.35 ± 0.07</td>
<td></td>
<td>235 ± 41</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>0.35 ± 0.07</td>
<td></td>
<td>230 ± 35</td>
<td></td>
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<tr>
<td>Female</td>
<td>3</td>
<td>0.35 ± 0.06</td>
<td></td>
<td>251 ± 60</td>
<td></td>
</tr>
<tr>
<td>Oral contraceptive users</td>
<td>11</td>
<td>0.21 ± 0.04</td>
<td>&lt;0.001</td>
<td>159 ± 23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Classical HAE</td>
<td>5</td>
<td>0.08 ± 0.03</td>
<td>&lt;0.001</td>
<td>31 ± 13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Variant HAE</td>
<td>2</td>
<td>0.06 ± 0.04</td>
<td>&lt;0.001</td>
<td>233 ± 10</td>
<td>NS</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± SD. Significance of differences between means was assessed by unpaired Student's t test. Oral contraceptive users were compared to female controls, while patients with HAE were compared to all normal controls.

among female controls. Moreover, the mean values for $k'$ and C1-INH antigen in patients with classical HAE was reduced to 23% ($p < 0.001$) and 13% ($p < 0.001$) of the values observed in controls. In two patients with variant HAE, the $k'$ value was low ($p < 0.01$), similar to what was observed in the patients with classical HAE. However, these two patients had normal C1-INH antigen concentrations. A positive correlation ($r = 0.90, p < 0.001$) was observed between C1-INH antigen concentration and $k'$ using the data obtained from control individuals, oral contraceptive users, and classical HAE patients (Fig. 3). Therefore, since the $k'$ for the inactivation of kallikrein by methylamine-treated plasma was negligible in the absence of C1-INH, it appeared that the rate constant was almost exclusively dependent on the concentration of C1-INH.

We now show that methylamine-treatment of plasma does not inactivate HMWK. Indeed, the rate of kallikrein inactivation by a 1/1.46 dilution of methylamine-treated kininogen-deficient plasma was 67% faster than the rate observed when kallikrein was inactivated by a 1/1.46 dilution of methylamine-treated normal plasma (Fig. 1). This difference in inactivation rates was apparently dependent only on HMWK, since C1-INH antigen concentration was

![Fig. 3. Correlation of C1-INH antigen and pseudo-first-order rate constant for the inactivation of kallikrein. Methylamine-treated plasma from 29 individuals was studied. For $k'$ value determinations, the final dilution of methylamine-treated plasma in the preincubation mixtures was 1/8.75. Otherwise, the conditions of kallikrein inactivation were the same as described in Fig. 1. C1-INH antigen was assayed as described in Materials and Methods. Open circles: controls. Closed circles: oral contraceptives users. Open triangles: patients with classical HAE. Closed triangles: patients with variant HAE. The line drawn is a least-squares fit of the experimental points ($r = 0.90$).](http://www.bloodjournal.org by guest on April 14, 2017. For personal use only.)
identical in both plasma samples. Furthermore, this difference can be accurately described by an equation\[ k' = k'/1 + [1 + ([HMWK]/Kd)] \] where \( k' \) is the pseudo-first-order rate constant in the presence of HMWK and Kd is the dissociation constant of the kallikrein–HMWK complex. It should be noted that in this equation the concentration of HMWK is the only variable. To circumvent the protective effect of HMWK, the plasma was diluted to 1/8.75, thereby reducing the concentration of HMWK to 0.11 U/ml or 0.08 \( \mu M \). At this concentration of HMWK, \( k' \) in the presence of HMWK should be only 10% slower than \( k' \) observed in the absence of the cofactors.\[19,20\] Such a small difference was not detectable with our experimental conditions (Fig. 1) and may represent the error of the method. Alternatively, identical \( k' \) may be the result of higher concentrations of minor kallikrein inhibitors such as \( \alpha_2 \)-plasmin inhibitor, antithrombin III, and \( \alpha_1 \)-antitrypsin in the normal plasma, as compared to their concentration in kininogen-deficient plasma.\[20\] In either case, when normal plasma was diluted to 1/8.75, the effect of HMWK became negligible. Moreover, under these conditions, a linear concentration dependence was observed between \( k' \) and kallikrein inhibitor concentrations ranging from 10% to 150% of normal plasma levels (Fig. 2).

The mean \( k' \) value among control individuals was found to be 0.35 min \(^{-1} \) using a 1/8.75 dilution of methylamine-treated plasma. Since C1-INH accounts for 90% of the inactivation of kallikrein in plasma depleted of \( \alpha_2 \)-M,\[17\] \( k' \) for the inactivation of kallikrein by C1-INH at normal plasma concentration of this inhibitor should be 2.76 min \(^{-1} \). The concentration of functional C1-INH can be obtained by dividing \( k' \) by the second-order rate constant for the reaction of kallikrein and C1-INH, which is 1.02 \times 10^6 M^{-1}.\[19\]

Thus, the mean functional concentration of C1-INH in the plasma of the controls appears to be 2.7 \( \mu M \) or 283 \( \mu g/ml \), in close agreement with the mean value determined for C1-INH antigen, which is 253 \( \mu g/ml \) (Table 1).

To test the validity of our assay for the determination of functional C1-INH in plasma, \( k' \) and C1-INH antigen values were then determined in oral contraceptive users, which have been recently reported to have reduced antigenic and functional C1-INH levels,\[32\] as well as in patients with HAE. The \( k' \) and C1-INH antigen values were significantly reduced in both groups when compared to normal controls (Table 1). Moreover, there was an excellent correlation between C1-INH antigen and \( k' \) values (Fig. 3). Thus, the determination of \( k' \) for the inactivation of kallikrein by methylamine-treated plasma gives an appropriate estimate of the concentration of functional C1-INH in plasma. This assay is rapid, since it can be completed in less than 3 hr, including methylamine-treatment. Furthermore, all of the reagents employed are commercially available. Therefore, this assay will facilitate the rapid assessment of functional C1-INH in human plasma. This rapid assessment is important for identification and management of patients with C1-inhibitor deficiency, such as is seen in HAE, especially since replacement therapy is now available.\[33\]

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