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## High Molecular Weight Kininogen or Its Light Chain Protects Human Plasma Kallikrein from Inactivation by Plasma Protease Inhibitors<sup>†</sup>

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**ABSTRACT:** Human plasma kallikrein is inactivated by C<sub>1</sub> inhibitor with a second-order rate constant of  $1.02 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ . When high molecular weight kininogen is present during the reaction, the rate constant for the inactivation is reduced, since kallikrein and high molecular weight kininogen form a noncovalent bimolecular complex,  $K_d = 0.75 \mu\text{M}$  [Schapira, M., Scott, C. F., & Colman, R. W. (1981) *Biochemistry* 20, 2738-2743]. The kinetics of the inactivation of kallikrein by  $\alpha_2$ -macroglobulin, antithrombin III, and  $\alpha_1$ -antitrypsin are now compared in the absence of high molecular weight kininogen. The second-order rate constants  $k_{+2}/K_i$  for the inactivation were respectively  $6.9 \times 10^5$ ,  $1.8 \times 10^4$ , and  $2.5 \times 10^2 \text{ M}^{-1} \text{ min}^{-1}$ . When high molecular weight kininogen was present during the reaction, the inactivation

rates by these plasma protease inhibitors were reduced as a result of the formation of a kallikrein-high molecular weight kininogen complex,  $K_d = 0.75 \mu\text{M}$ . When the light chain derived from reduction of kinin-free high molecular weight kininogen was substituted for the parent molecule, a more pronounced reduction in inactivation rates was observed due to the formation of a kallikrein-light chain complex ( $K_d = 0.14 \mu\text{M}$ ). These results demonstrate that the combining site for kallikrein on high molecular weight kininogen, responsible for complex formation and protection against inhibitors, resides in the light-chain region of the molecule. Moreover, kallikrein appears to bind more tightly to the light chain of high molecular weight kininogen than to the parent molecule.

**P**lasma kallikrein is a serine protease which converts factor XII to activated factor XII and factor XII fragments (Cochrane et al., 1973; Meier et al., 1977) and plasminogen to plasmin (Colman, 1969; Mandle & Kaplan, 1977) and liberates bradykinin from high molecular weight kininogen (Pierce & Guimaraes, 1976; Colman et al., 1975; Thompson et al., 1978; Kerbiriou & Griffin, 1979). Five plasma protease inhibitors are known to inactivate kallikrein, including C<sub>1</sub> inhibitor (Ratnoff et al., 1969),  $\alpha_2$ -macroglobulin (Harpel, 1970), antithrombin III (Lahiri et al., 1976),  $\alpha_1$ -antitrypsin (Fritz et al., 1972), and  $\alpha_2$ -plasmin inhibitor (Saito et al., 1979). The quantitative contribution of each inhibitor to the liquid-phase inhibition of kallikrein has not been determined, although C<sub>1</sub> inhibitor and  $\alpha_2$ -macroglobulin have been suggested as the major inhibitors of kallikrein in plasma (Gigli et al., 1970; Harpel, 1970; McConnell, 1972; Gallimore et al., 1979).

The role of high molecular weight kininogen must also be included in the variables affecting the inhibition of kallikrein in plasma. High molecular weight kininogen is a cofactor in the contact phase activation of blood coagulation, fibrinolysis, kinin formation, and the complement cascade (Kaplan, 1978; Davie et al., 1979). In the liquid phase, high molecular weight kininogen forms noncovalent bimolecular complexes with plasma prekallikrein, factor XI, and kallikrein (Mandle et al.,

1976; Thompson et al., 1977; Scott & Colman, 1980; Kerbiriou et al., 1980). Moreover, we have shown that the formation of a reversible complex between kallikrein and high molecular weight kininogen decreased the inactivation rate of kallikrein by C<sub>1</sub> inhibitor, soybean trypsin inhibitor, and diisopropyl fluorophosphate (Schapira et al., 1981). In this study, we describe the influence of the formation of a reversible complex between kallikrein and high molecular weight kininogen or its light chain on the inactivation rate of the enzyme by  $\alpha_2$ -macroglobulin, antithrombin III, and  $\alpha_1$ -antitrypsin.

### Materials and Methods

**Purification and Characterization of Plasma Proteins.** Kallikrein was prepared by activation of purified prekallikrein by factor XII fragments (Scott et al., 1979). The specific activity was  $14.5 \mu\text{mol min}^{-1} \text{ M}^{-1}$  with H-D-Pro-Phe-Arg-p-nitroanilide (0.09 mM) as substrate, and the resultant kallikrein contained two chains of  $M_r$  55 000 and 33 000 on reduced NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis<sup>1</sup> (Weber & Osborn, 1969). In some experiments, kallikrein contained IgG as the only contaminant. No differences were noted between the two preparations with regard to kinetic analysis.

$\alpha_2$ -Macroglobulin was prepared by the method of Song et al. (1975), using plasma supplemented with soybean trypsin inhibitor (50  $\mu\text{g/mL}$ ) as starting material (Harpel, 1976). Trypsin binding activity of the preparation was determined with the Remazol brilliant blue-hide assay (Rinderknecht et al., 1970), using active-site titrated trypsin (Chase & Shaw,

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<sup>1</sup> Abbreviations: NaDodSO<sub>4</sub>, sodium dodecyl sulfate; IgG, immunoglobulin G.

1970). One milligram of  $\alpha_2$ -macroglobulin inactivated 78  $\mu$ g of trypsin. The preparation migrated as a single band of  $M_r$  185 000 on reduced NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis.  $\alpha_2$ -Macroglobulin had no detectable amidolytic activity on H-D-Pro-Phe-Arg-*p*-nitroanilide. The molarity of  $\alpha_2$ -macroglobulin was calculated by assuming a 1:1 stoichiometry for the reaction of  $\alpha_2$ -macroglobulin with kallikrein ( $M_r$  88 000), by analogy to the stoichiometry for the reaction between  $\alpha_2$ -macroglobulin and plasmin ( $M_r$  85 000) (Jacquot-Armand & Guinand, 1976; Pochon et al., 1978; Gyzander & Teger-Nilsson, 1980).

$\alpha_1$ -Antitrypsin was prepared by the method of Cohen & James (1978). One milligram of this preparation completely inhibited 0.44 mg of trypsin.  $\alpha_1$ -Antitrypsin was a single band,  $M_r$  54 000, on reduced polyacrylamide gel electrophoresis in the presence of NaDodSO<sub>4</sub>.

Antithrombin III was purchased from Kabi Group, Greenwich, CT. The preparation was 78% active, as assessed by its reactivity with thrombin (active site titrated thrombin was a gift of John W. Fenton II). Antithrombin III was a single band,  $M_r$  62 000, on reduced polyacrylamide gel electrophoresis in the presence of NaDodSO<sub>4</sub>.

CI inhibitor was prepared as described by Schapira et al. (1981), using a partially purified preparation (Wickerhauser et al., 1978) as starting material. The resultant preparation,  $M_r$  105 000 on NaDodSO<sub>4</sub> reduced polyacrylamide gel electrophoresis, was 97% active as determined by the method of Levy & Lepow (1959) compared to its antigenic activity, assessed by electroimmunodiffusion (Laurell, 1966).

High molecular weight kininogen was prepared according to Kerbirou & Griffin (1979). The preparation was a single band of  $M_r$  110 000 on reduced NaDodSO<sub>4</sub> gel electrophoresis. High molecular weight kininogen was assayed by a modification of the partial thromboplastin time, using plasma from a kininogen-deficient individual as a substrate (Colman et al., 1975). One unit was defined as the amount present in 1 mL of normal pooled plasma and corresponded to a 0.73  $\mu$ M solution of the purified high molecular weight kininogen. The specific activity was 12.5 units/mg. The light chain derived from kinin-free high molecular weight kininogen was prepared according to Kerbirou & Griffin (1979). The preparation was a single band of  $M_r$  45 000 on reduced NaDodSO<sub>4</sub> gel electrophoresis. It was assayed as described for the parent molecule. The specific activity was 30.5 units/mg. Plasmin was obtained from Kabi Group, Greenwich, CT. Its specific activity was 18 CTA units/mg.

**Kinetic Studies.** Kallikrein was incubated with inhibitors and other proteins in freshly silicone coated glass vessels at 23 °C. Kallikrein activity was measured by its amidolytic activity on the chromogenic substrate H-D-Pro-Phe-Arg-*p*-nitroanilide (S-2302) (Ortho Diagnostics Inc., Raritan, NJ). A 0.6 mM solution of the substrate was prepared in 85 mM sodium phosphate buffer, pH 7.6, containing 127 mM NaCl. Ten microliters of the solution to be tested was added to 330  $\mu$ L of substrate at 37 °C, and the absorbance change at 405 nm was continuously recorded with a Cary 210 double-beam spectrophotometer. Under these conditions, the hydrolysis rate of the substrate by 1 nM kallikrein was 1.84 nmol/min. The presence of high molecular weight kininogen or of its light chain (final concentration  $\leq 43$  nM) did not cause any detectable changes in the hydrolysis rate of the substrate. H-D-Val-Leu-Lys-*p*-nitroanilide (S-2251) (Ortho Diagnostics Inc., Raritan, NJ), at a final concentration of 0.45 mM and in the above-mentioned buffer, was used as a substrate for plasmin.

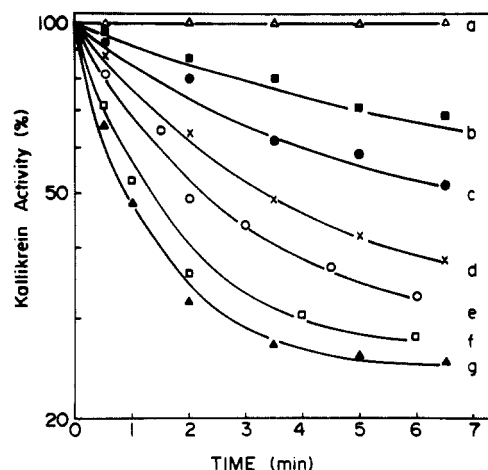


FIGURE 1: Kinetics of inactivation of kallikrein amidolytic activity by  $\alpha_2$ -macroglobulin. Kallikrein (final concentration 50 nM) was incubated with various concentrations of  $\alpha_2$ -macroglobulin and then assayed at various times for residual amidolytic activity.  $\alpha_2$ -macroglobulin final concentration: (a) 0, (b) 0.125, (c) 0.25, (d) 0.5, (e) 1.0, (f) 2.0, and (g) 3.0  $\mu$ M.

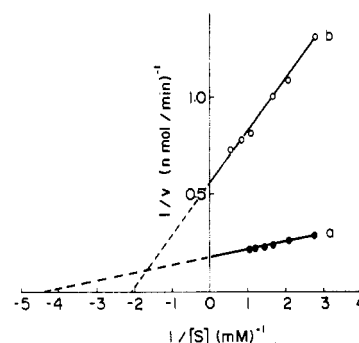


FIGURE 2: Lineweaver-Burk plots of the activity of kallikrein (a) and  $\alpha_2$ -macroglobulin-bound kallikrein (b) on H-D-Pro-Phe-Arg-*p*-nitroanilide. Kallikrein and  $\alpha_2$ -macroglobulin-bound kallikrein final concentration was 2.2 nM. The lines drawn are least-squares fits of the experimental points. The equations of the lines are (a)  $y = 0.04x + 0.18$ ,  $r = 0.98$ , and (b)  $y = 0.27x + 0.55$ ,  $r = 0.98$ .

## Results

**Inactivation of Kallikrein by  $\alpha_2$ -Macroglobulin.** The kinetics of inactivation of kallikrein amidolytic activity by various concentrations of  $\alpha_2$ -macroglobulin is shown in Figure 1. The inactivation of kallikrein did not follow pseudo-first-order kinetics, even when  $\alpha_2$ -macroglobulin was in a 60-fold molar excess (Figure 1, curve g). On the contrary, a curvilinear pattern of inhibition was observed when the logarithm of residual enzyme activity was examined as a function of time, suggesting that the kallikrein- $\alpha_2$ -macroglobulin complex might exhibit catalytic activity on H-D-Pro-Phe-Arg-*p*-nitroanilide. To assess the amidolytic activity of this complex, we compared the activity of free and  $\alpha_2$ -macroglobulin-bound kallikrein.  $\alpha_2$ -Macroglobulin-bound kallikrein was prepared by incubating kallikrein with a 30-fold molar excess of  $\alpha_2$ -macroglobulin for 2 h at 37 °C. No residual free kallikrein was present, since the addition of soybean trypsin inhibitor did not decrease the amidolytic activity of the mixture. Intersecting noncompetitive inhibition was observed (Figure 2). The  $K_m$  and  $K_{m_{app}}$  with free and  $\alpha_2$ -macroglobulin-bound kallikrein were respectively 0.22 and 0.45 mM. The  $V_{max}$  and  $V_{max_{app}}$  were respectively 5.6 and 1.8 nmol/min at free or  $\alpha_2$ -macroglobulin-bound kallikrein concentrations of 2.2 nM. At a concentration of H-D-Pro-Phe-Arg-*p*-nitroanilide of 0.6 mM, the kallikrein- $\alpha_2$ -macroglobulin complex retained 24% of the activity of the free enzyme. Appropriate corrections were thus made to the

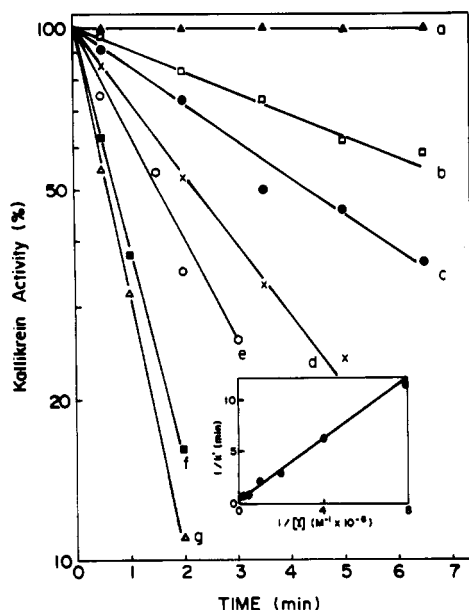


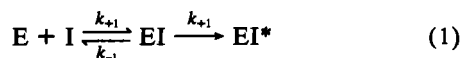
FIGURE 3: Kinetics of inactivation of kallikrein amidolytic activity by  $\alpha_2$ -macroglobulin. The actual points were replotted from Figure 1, after appropriate corrections for the residual amidolytic activity of  $\alpha_2$ -macroglobulin-bound kallikrein.  $\alpha_2$ -Macroglobulin final concentrations: (a) 0, (b) 0.125, (c) 0.25, (d) 0.5, (e) 1.0, (f) 2.0, and (g) 3.0  $\mu$ M. The inset shows a double-reciprocal plot of the pseudo-first-order rate constant and the concentration of  $\alpha_2$ -macroglobulin. The line drawn is a least-squares fit of the experimental points ( $r = 0.99$ ). The equation of the line is  $y = 1.45x + 0.48$ .

Table I: Comparison of Kinetic Constants for the Inactivation of Plasma Kallikrein by  $\alpha_2$ -Macroglobulin, Antithrombin III,  $\alpha_1$ -Antitrypsin, C $\bar$ I Inhibitor and  $\alpha_2$ -Plasmin Inhibitor<sup>a</sup>

| inhibitor                     | $K_i$<br>( $\mu$ M) | $k_{+2}$<br>( $\text{min}^{-1}$ ) | $k_{+2}/K_i$<br>( $\text{M}^{-1} \text{min}^{-1} \times 10^{-4}$ ) |
|-------------------------------|---------------------|-----------------------------------|--|
| $\alpha_2$ -macroglobulin     | 3.0                 | 2.08                              | 69   |
| antithrombin III              | 34                  | 0.61                              | 1.8  |
| $\alpha_1$ -antitrypsin       | 62                  | 0.015                             | 0.025  |
| C $\bar$ I inhibitor          | 62                  | 0.015                             | 102 <sup>b</sup>   |
| $\alpha_2$ -plasmin inhibitor |                     |                                   | $\leq 3.5^c$   |

<sup>a</sup> The reversible dissociation constants of the enzyme-inhibitor complex,  $K_i$ , and the first-order constants of the irreversible step of the reaction,  $k_{+2}$ , were calculated according to Kitz & Wilson (1962). <sup>b</sup> This value was calculated by using the data of Schapira et al. (1981), assuming that the second-order rate constant for the irreversible inactivation of kallikrein by C $\bar$ I inhibitor was equal to  $k_{+2}/K_i$ . <sup>c</sup> This value was calculated by using the data reported by Saito et al. (1979). This value represents a maximum, which may only be obtained if no saturation of the inactivation rate is observed with increasing concentrations of  $\alpha_2$ -plasmin inhibitor.

data shown in Figure 1 by subtracting the calculated residual activity of the complex at each time point. After correction, the inactivation of kallikrein by  $\alpha_2$ -macroglobulin followed pseudo-first-order kinetics (Figure 3). A double-reciprocal plot of the pseudo-first-order rate constant  $k'$  vs. the  $\alpha_2$ -macroglobulin concentration (Figure 3, inset) indicated that the inactivation of the enzyme (E) by the inhibitor (I) was described by



where the dissociable enzyme-inhibitor complex is EI, the stabilized complex is EI\*, the second-order rate constant for the formation of EI is  $k_{+1}$ , the first-order rate constant for the dissociation of EI is  $k_{-1}$ , the dissociation constant of EI is  $K_i = k_{-1}/k_{+1}$ , and the first-order rate constant for the formation of EI\* is  $k_{+2}$  (Kitz & Wilson, 1962). The kinetic constants

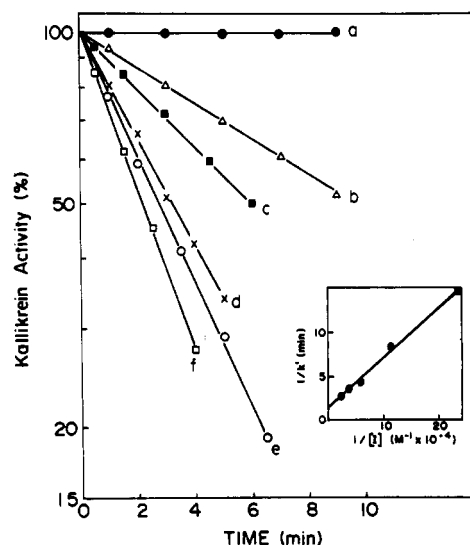


FIGURE 4: Kinetics of inactivation of kallikrein amidolytic activity by antithrombin III. Kallikrein (final concentration 75 nM) was incubated with various concentrations of antithrombin III and then assayed at various times for residual amidolytic activity. Antithrombin III final concentrations: (a) 0, (b) 4.3, (c) 8.6, (d) 17, (e) 25, and (f) 42  $\mu$ M. The inset shows a double-reciprocal plot of the pseudo-first-order rate constant and the concentration of antithrombin III. The line drawn is at least-squares fit of the experimental points ( $r = 0.99$ ). The equation of the line is  $y = 0.55x + 1.63$ .

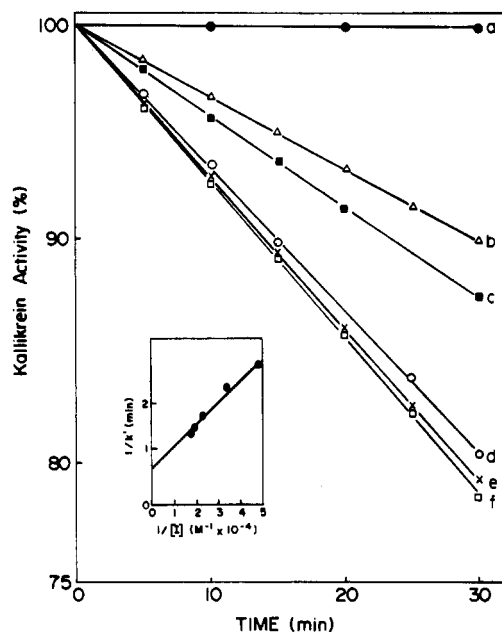


FIGURE 5: Kinetics of inactivation of kallikrein amidolytic activity by  $\alpha_1$ -antitrypsin. Kallikrein (final concentration 75 nM) was incubated with various concentrations of  $\alpha_1$ -antitrypsin and then assayed at various times for residual amidolytic activity.  $\alpha_1$ -antitrypsin final concentrations: (a) 0, (b) 21, (c) 31, (d) 41, (e) 52, and (f) 55  $\mu$ M. The inset shows a double-reciprocal plot of the pseudo-first-order rate constant and the concentration of  $\alpha_1$ -antitrypsin. The line drawn is at least-squares fit of the experimental points ( $r = 0.97$ ). The equation of the line is  $y = 0.40x + 0.65$ .

for the inactivation of kallikrein by  $\alpha_2$ -macroglobulin were derived from the double-reciprocal plot (Figure 3, inset) and are indicated in Table I.

**Inactivation of Kallikrein by Antithrombin III and  $\alpha_1$ -Antitrypsin.** The kinetics of inactivation of kallikrein amidolytic activity by various concentrations of antithrombin III (Figure 4) or  $\alpha_1$ -antitrypsin (Figure 5) are shown. Antithrombin III was a more efficient inhibitor of kallikrein than  $\alpha_1$ -antitrypsin. For example, 10% of kallikrein activity was inhibited by antithrombin III (4.3  $\mu$ M) in 1.5 min (Figure 4,

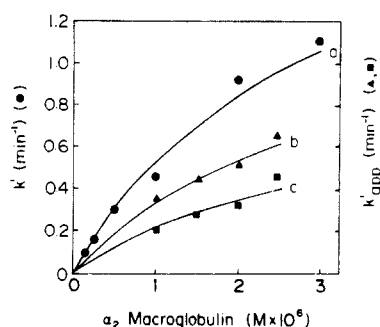


FIGURE 6: Effect of the  $\alpha_2$ -macroglobulin concentration on the pseudo-first-order rate constant for the inactivation of kallikrein in the absence and presence of high molecular weight kininogen. Kallikrein (final concentration 50 nM) was incubated with  $\alpha_2$ -macroglobulin in the presence of molecular weight kininogen at final concentrations of (a) 0, (b) 0.50, and (c) 1.04  $\mu$ M. Curve a was drawn by using eq 2. Curves b and c were drawn by using eq 3.

curve b), while the same amount of enzyme was inhibited in 13.5 min when  $\alpha_1$ -antitrypsin was 55  $\mu$ M (Figure 5, curve f). The kinetic constants for kallikrein inactivation were calculated from double-reciprocal plots (Figure 4, inset; Figure 5, inset) and are presented in Table I.

The second-order rate constants for irreversible kallikrein inactivation  $k_{+2}/k_i$  indicate; however, that both antithrombin III and  $\alpha_1$ -antitrypsin are very ineffective kallikrein inhibitors when compared to  $\alpha_2$ -macroglobulin (Table I). Moreover, after inclusion (Table I) of the data of Saito et al. (1979) and Schapira et al. (1981) reporting the second-order rate constants for the inactivation of kallikrein by  $\alpha_2$ -plasmin inhibitor and C $\bar{I}$  inhibitor, it appeared that  $\alpha_2$ -macroglobulin and C $\bar{I}$  inhibitor were respectively 13 and 19 times more effective in the inactivation of kallikrein than antithrombin III,  $\alpha_1$ -antitrypsin, and  $\alpha_2$ -plasmin inhibitor combined. Moreover, the pseudo-first-order inactivation rate constants, determined at normal plasma inhibitor concentrations, indicate that C $\bar{I}$  inhibitor and  $\alpha_2$ -macroglobulin would account for 58 and 38% of kallikrein inhibition, respectively, while antithrombin III,  $\alpha_1$ -antitrypsin, and  $\alpha_2$ -plasmin inhibitor combined would account for only 4%.

**Influence of High Molecular Weight Kininogen on the Inactivation of Kallikrein by  $\alpha_2$ -Macroglobulin, Antithrombin III, and  $\alpha_1$ -Antitrypsin.** The influence of high molecular weight kininogen on the inactivation rate of kallikrein by  $\alpha_2$ -macroglobulin and antithrombin III is shown in Figures 6 and 7. High molecular weight kininogen decreased the inactivation rate of kallikrein by both inhibitors. For example, the apparent rate constant of inactivation,  $k'_{app}$ , of kallikrein by  $\alpha_2$ -macroglobulin (2.0  $\mu$ M) in the presence of high molecular weight kininogen (1.04  $\mu$ M) (Figure 6, curve c) was diminished to 35% of the inactivation rate constant  $k'$ , observed in the absence of high molecular weight kininogen (Figure 6, curve a). Similarly,  $k'_{app}$  of kallikrein (75 nM) by antithrombin III (25  $\mu$ M) in the presence of high molecular weight kininogen (1.46  $\mu$ M) (Figure 7, curve c) was diminished to 28% of  $k'$  observed in the absence of high molecular weight kininogen (Figure 7, curve a). Moreover, when kallikrein (75 nM) was inactivated by  $\alpha_1$ -antitrypsin (55  $\mu$ M),  $k'_{app}$ , in the presence of high molecular weight kininogen (0.73  $\mu$ M), was  $3.8 \times 10^{-3} \text{ min}^{-1}$ , while  $k'$ , in its absence, was  $7.7 \times 10^{-3} \text{ min}^{-1}$ . Theoretical curves describing  $k'$  as a function of the concentration of  $\alpha_2$ -macroglobulin (Figure 6, curve a) and of antithrombin III (Figure 7, curve a) were computed by using the data presented in Table I. The equation for these curves was

$$k' = k_{+2}[I]/([I] + k_i) \quad (2)$$

When the high molecular weight complex was present during

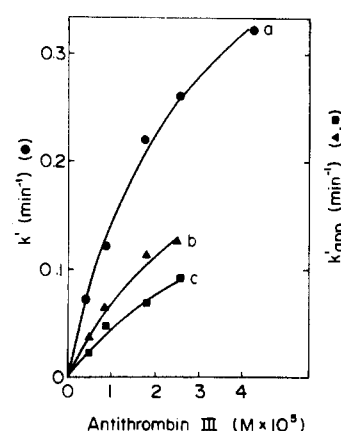


FIGURE 7: Effect of the antithrombin III concentration on the pseudo-first-order rate constant for the inactivation of kallikrein in the absence and presence of high molecular weight kininogen. Kallikrein (final concentration 75 nM) was incubated with antithrombin III in the presence of high molecular weight kininogen at final concentrations of (a) 0, (b) 0.73, and (c) 1.46  $\mu$ M. Curve a was drawn by using eq 2. Curves b and c were drawn by using eq 3.

the inactivation of kallikrein by  $\alpha_2$ -macroglobulin or by antithrombin III, eq 2 was modified to

$$k'_{app} = k_{+2}[I]/([I] + k_i)(1 + [C]/K_d^{\text{Kal-C}}) \quad (3)$$

where C is high molecular weight kininogen and  $K_d^{\text{Kal-C}}$  is the dissociation constant for the kallikrein-high molecular weight kininogen complex. Equation 3 implies that the inhibitors cannot compete with high molecular weight kininogen and, therefore, that high molecular weight kininogen does not bind to the active site of kallikrein. The theoretical curves shown (Figure 6, curves b and c; Figure 7, curves b and c) were calculated by using  $K_d^{\text{Kal-C}} = 0.75 \mu\text{M}$  (Schapira et al., 1981). There is an excellent agreement between the experimental points and the theoretical curves.

**Influence of High Molecular Weight Kininogen on the Inactivation of Plasmin by  $\alpha_2$ -Macroglobulin.** To exclude the possibility that high molecular weight kininogen was inhibiting the action of  $\alpha_2$ -macroglobulin, we studied the influence of the cofactor on the inactivation of plasmin by  $\alpha_2$ -macroglobulin. The pseudo-first-order rate constant for the inhibition of plasmin by  $\alpha_2$ -macroglobulin (0.25  $\mu$ M) was  $0.07 \text{ min}^{-1}$ , in the absence and in the presence of high molecular weight kininogen (1.46  $\mu$ M).

**Influence of the Light Chain Derived from Kinin-free High Molecular Weight Kininogen on the Inactivation of Kallikrein by C $\bar{I}$  Inhibitor and  $\alpha_2$ -Macroglobulin.** The inactivation rates of kallikrein by C $\bar{I}$  inhibitor (Figure 8A) and by  $\alpha_2$ -macroglobulin (Figure 8B) were reduced in the presence of the light chain, as compared to the rates observed when kallikrein was inactivated in its absence. For example, the presence of the light chain (1.46  $\mu$ M) reduced the rate of inactivation of kallikrein by C $\bar{I}$  inhibitor (0.38  $\mu$ M) (Figure 8A, curve f) by 90% when compared to the inactivation rate observed in its absence (Figure 8A, curve a). The dissociation constant  $K_d^{\text{Kal-C(L)}}$  for the complex between kallikrein and the light chain derived from kinin-free high molecular weight kininogen was calculated with the equation:

$$K_d^{\text{Kal-C(L)}} = [L]/(k'/k'_{app} - 1) \quad (4)$$

where L is the light chain derived from kinin-free high molecular weight kininogen. Using the data reported in Figure 8A,B, we found  $K_d^{\text{Kal-C(L)}}$  to be  $0.14 \pm 0.03 \mu\text{M}$  (mean  $\pm$  SD).

**Influence of the Light Chain of High Molecular Weight Kininogen on the Inactivation of Plasmin by C $\bar{I}$  Inhibitor and  $\alpha_2$ -Macroglobulin.** To exclude that the light chain was altering

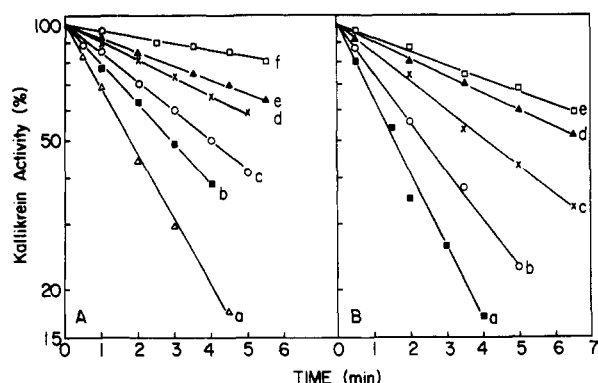


FIGURE 8: Kinetics of inactivation of kallikrein amidolytic activity by C1 inhibitor (A) and  $\alpha_2$ -macroglobulin (B) in the absence and presence of the light chain derived from kinin-free high molecular weight kininogen. Kallikrein (final concentration 50 nM) was incubated with C1 inhibitor at a final concentration of 0.38  $\mu$ M (A) or with  $\alpha_2$ -macroglobulin at a final concentration of 0.25  $\mu$ M (B) and then assayed at various times for residual amidolytic activity. Appropriate corrections were made to correct the raw data for the amidolytic activity of  $\alpha_2$ -macroglobulin-bound kallikrein. Light chain concentration: (A), (a) 0, (b) 0.10, (c) 0.21, (d) 0.42, (e) 0.73, and (f) 1.46  $\mu$ M; (B), (a) 0, (b) 0.10, (c) 0.21, (d) 0.31, and (e) 0.42  $\mu$ M.

the reactivity of either C1 inhibitor or  $\alpha_2$ -macroglobulin, we assessed the effect of the light chain on the inactivation of plasmin by these inhibitors. The pseudo-first-order rate constant for the inactivation of plasmin by C1 inhibitor (0.1  $\mu$ M) was 0.19  $\text{min}^{-1}$ . This value was not modified by the presence of the light chain of high molecular weight kininogen (0.18  $\mu$ M). When plasmin was inactivated by  $\alpha_2$ -macroglobulin (0.25  $\mu$ M) in the absence or in the presence of the light chain (0.18  $\mu$ M), identical pseudo-first-order rate constants were obtained (0.07  $\text{min}^{-1}$ ). The results demonstrate that the light chain derived from kinin-free high molecular weight kininogen was not inhibiting the function of C1 inhibitor or  $\alpha_2$ -macroglobulin.

### Discussion

The results obtained in this study, together with the data from Saito et al. (1979) and Schapira et al. (1981), allow a comparison between the different kallikrein inhibitors (Table I), since the kinetic constants determined were obtained by using the same substrate and similar conditions. It appears, when comparing the second-order rate constants  $k_{+2}/K_i$  that C1 inhibitor and  $\alpha_2$ -macroglobulin are the only efficient plasma inhibitors of kallikrein, since they are respectively 17 and 13 times more effective than antithrombin III,  $\alpha_1$ -antitrypsin, and  $\alpha_2$ -plasmin inhibitor combined.

The inactivation of plasma kallikrein by C1 inhibitor results in the formation of a bimolecular complex that is catalytically inactive (Gigli et al., 1970; Schapira et al., 1981). In contrast, the complex formed when kallikrein was inactivated by  $\alpha_2$ -macroglobulin retained part of its activity on H-D-Pro-Phe-Arg-p-nitroanilide,  $M_r$  612 (Figures 1 and 2). Similar observations were made by Harpel (1970), using  $N^\alpha$ -(p-toluenesulfonyl)-L-arginine methyl ester,  $M_r$  379, as substrate for  $\alpha_2$ -macroglobulin-bound kallikrein. Other investigators have shown that  $\alpha_2$ -macroglobulin-bound proteolytic enzymes usually retain part of their catalytic activity toward low molecular weight substrates (Haverback et al., 1962; Lanchantin et al., 1966; Barrett & Starkey, 1973; Harpel & Mosesson, 1973). When the kinetics of free and  $\alpha_2$ -macroglobulin-bound kallikrein were analyzed (Figure 2), it appeared that  $\alpha_2$ -macroglobulin acted as a mixed type or intersecting noncompetitive inhibitor. This behavior indicates that  $\alpha_2$ -macroglobulin interfered both with the catalytic properties of kal-

likrein and with its binding to the substrate.

High molecular weight kininogen decreased the inactivation rate of kallikrein by  $\alpha_2$ -macroglobulin, antithrombin III, and  $\alpha_1$ -antitrypsin. This observation amplifies the conclusion of our previous report (Schapira et al., 1981) that high molecular weight kininogen decreased the inactivation rate of kallikrein by C1 inhibitor by forming a reversible molecular complex with the enzyme. The dissociation constant of the kallikrein-high molecular weight kininogen complex was found to be 0.75  $\mu$ M (Schapira et al., 1981). Our present results show an excellent fit to curves calculated by using 0.75  $\mu$ M as an estimate of the dissociation constant for the complex (Figures 6 and 7) as well as indicate that high molecular weight kininogen does not bind kallikrein at its active site. Therefore, substrate protection is not the mechanism involved in the decrease in kallikrein activation rate. High molecular weight kininogen is probably an important factor in the regulation of kallikrein activation in vivo, since the dissociation constant for the kallikrein-high molecular weight kininogen complex is almost identical with the concentration of this contact phase cofactor in normal human plasma.

High molecular weight kininogen, a single-chain molecule, is also a substrate for plasma kallikrein (Thompson et al., 1978; Kerbiriou & Griffin 1979). Concomitant with bradykinin liberation, kallikrein cleaves high molecular weight kininogen, producing two chains that remain linked by disulfide bridges (Thompson et al., 1978; Kerbiriou & Griffin, 1979). The heavy chain ( $M_r$  65 000) shares antigenic determinants with low molecular weight kininogen and does not exhibit coagulant activity (Thompson et al., 1978; Kerbiriou & Griffin, 1979; Schiffman et al., 1980). The light chain ( $M_r$  45 000) possesses the procoagulant activity of the single-chain parent molecule (Thompson et al., 1978; Kerbiriou & Griffin, 1979; Schiffman et al., 1980) and a single binding site for prekallikrein and kallikrein (Kerbiriou et al., 1980) as well as a binding site for factor XI (Thompson et al., 1979). Since the light chain protected kallikrein from inactivation by C1 inhibitor and  $\alpha_2$ -macroglobulin (Figure 8), our results demonstrate kinetically that kallikrein complexes with the light chain of high molecular weight kininogen, complementing the immunochemical data of Kerbiriou et al. (1980). The dissociation constant for the kallikrein-light chain complex was found to be 0.14  $\mu$ M. The kallikrein-light chain complex is thus 5 times tighter than the kallikrein-high molecular weight kininogen complex, indicating steric differences between the free light chain and the light chain incorporated in the parent molecule. Protection against inhibitors by the light chain, which is not proteolyzed by kallikrein, further eliminates any possibility of substrate protection accounting for the decrease in inactivation rate. Further studies will be needed to establish the precise molecular details of complex formation between kallikrein and high molecular weight kininogen.

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