# Interaction of Human Plasma Kallikrein and Its Light Chain with $\alpha_2$-Macroglobulin†

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ABSTRACT: Human plasma kallikrein participates in the contact activation system of plasma. The light chain of kallikrein contains the enzymatic active site; the heavy chain is required for binding to high molecular weight kininogen and for surface-dependent activation of coagulation. This study has examined the functional contributions of the heavy chain of kallikrein and of high molecular weight kininogen in the inactivation of kallikrein and of its isolated light chain by $\alpha_2$-macroglobulin ($\alpha_2$M). Irreversible inhibition was observed for both kallikrein and its light chain, with the initial formation of a reversible enzyme–inhibitor complex. The second-order rate constants for these reactions were $3.5 \times 10^5$ and $4.8 \times 10^5$ $M^{-1}$ $min^{-1}$ for kallikrein and its light chain, respectively. When present in excess, high molecular weight kininogen decreased the rate of kallikrein inactivation by $\alpha_2$M, whereas the rate of inactivation of the light chain was unaffected by high molecular weight kininogen. Although at a drastically reduced rate, high molecular weight kininogen was cleaved by $\alpha_2$M-bound kallikrein. Sodium dodecyl sulfate gradient polyacrylamide gel electrophoresis was used to study complex formation between $\alpha_2$M and kallikrein or its light chain. Under reducing conditions, four kallikrein–$\alpha_2$M complexes were observed. Three of these complexes consisted of $\alpha_2$M and the light chain of kallikrein ($M_r$ 123 000, 235 000, and 330 000). Two $\alpha_2$M–kallikrein light chain complexes incorporated [$^3$H]diisopropyl fluorophosphate ([$^3$H]DFP) whereas the $M_r$ 330 000 complex did not react with [$^3$H]DFP. These results indicate that high molecular weight kininogen decreases the rate of kallikrein inactivation by $\alpha_2$M by high-affinity interaction with the heavy chain of kallikrein. In the absence of high molecular weight kininogen, the heavy chain does not contribute significantly to the inactivation of kallikrein by $\alpha_2$M. Complex formation between kallikrein and $\alpha_2$M occurs by covalent bond formation between predominantly the light chain of kallikrein and $\alpha_2$M.

Initiation of the contact activation system by negatively charged surfaces results in the reciprocal activation of prekallikrein and factor XII (Kaplan & Austen, 1970; Cochrane et al., 1973; Revak et al., 1977). High molecular weight kininogen serves as a cofactor in these reactions (Griffin & Cochrane, 1976; Meier et al., 1977). Both prekallikrein and its activated form, kallikrein, form a complex with high molecular weight kininogen (Mandle et al., 1976; Kerbiriou et al., 1980). The two very similar forms of human plasma kallikrein consist of one heavy chain ($M_r$ 43 000) and one light chain ($M_r$ either 36 000 or 33 000) which are linked by disulfide bonds (Mandle & Kaplan, 1977; Bouma et al., 1980). Recent studies showed that the light chain of kallikrein possesses the enzymatic site (Mandle & Kaplan, 1977; van der Graaf et al., 1982b) and adequately accounts for the enzymatic properties of kallikrein in solution on the protein substrate factor XII and on oligopeptide substrates (van der Graaf et al., 1982b). The binding site for high molecular weight kininogen is located on the heavy chain region. Further, the heavy chain is required for potent surface-dependent procoagulant activity of kallikrein (van der Graaf et al., 1982b).

C1 inhibitor and $\alpha_2$-macroglobulin ($\alpha_2$M) are the major inhibitors of kallikrein in plasma (Schapira et al., 1982a; van der Graaf et al., 1983a). The demonstration of Harpel (1970) that the residual kallikrein esterolytic activity, present in normal plasma after activation with kaolin, was resistant to inhibition by soybean trypsin inhibitor (STI) was the first indication of kallikrein inactivation by $\alpha_2$M. Other reports confirmed the role of $\alpha_2$M in the inactivation of kallikrein in plasma (McConnell, 1972; Trumpi-Kalshoven & Kluft, 1978). Like trypsin, thrombin, and plasmin, kallikrein was shown to cleave the subunit chain of $\alpha_2$M, producing two $M_r$ 85 000 fragments (Harpel, 1973). Recently, Schapira et al. (1982b) demonstrated that high molecular weight kininogen decreased the rate of kallikrein inactivation by $\alpha_2$M and suggested that this was a result of the binding between high molecular weight kininogen and kallikrein.

Human $\alpha_2$-macroglobulin ($M_r$ 726 000) is a tetramer of four identical subunits ($M_r$ 185 000) formed by the noncovalent association of two disulfide-linked pairs of subunits (Harpel, 1973). $\alpha_2$M is capable of forming complexes with endopeptidases from all known classes of proteases (Barrett & Starkey, 1973). Only active proteases appear to be bound by $\alpha_2$M. It was argued that the process was initiated by the cleavage of a vulnerable region near the middle of the $\alpha_2$M subunit chain, followed by a conformational change resulting in steric entrapment of the enzyme (Barrett & Starkey, 1973). The active site of the bound protease is not involved in maintaining the complex, since activity toward small substrates is usually partly impaired or not impaired at all (Starkey, 1979). It is generally assumed that large protein inhibitors cannot react with a $\alpha_2$M-bound protease. Previous reports, however, showed that STI can react with $\alpha_2$M-bound trypsin (Hayes & Harpel, 1979; Bieth et al., 1981; Wang et al., 1981).

Recently, we investigated the inactivation reaction of kallikrein and its isolated light chain with C1 inhibitor (van der Graaf et al., 1983b). Here we report the kinetics of the inactivation of kallikrein and of its isolated light chain by $\alpha_2$M in the absence and presence of high molecular weight kininogen in order to assess the functional contributions of the heavy chain of kallikrein and of high molecular weight kininogen to this reaction. In addition, the nature of the molecular complexes formed by interactions between kallikrein or its light chain and $\alpha_2$M was examined.

## Materials and Methods

High molecular weight kininogen and prekallikrein were isolated from human plasma as described elsewhere (Kerbiriou

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& Griffin, 1979; Kerbiriou et al., 1980). $\alpha_2$M was isolated from citrated fresh human plasma. The plasma was obtained as previously described (Bouma et al., 1980). Throughout the purification procedure, $\alpha_2$M was detected by rocket immunoelectrophoresis according to the method of Laurell (1966) using 0.9% agarose (Seakem) containing 1% (v/v) anti-$\alpha_2$M antiserum (Behringwerke A. G., Marburg, West Germany). Two hundred milliliters of plasma was diluted with 2 volumes of 0.02 M sodium phosphate and 0.1 M NaCl, pH 7.4. This solution was ajusted to 4% (w/v) polyethylene glycol ($M_r$ 6000) by using a 50% (w/v) stock solution at 4 °C. After 30 min of mixing, the suspension was centrifuged and the supernatant adjusted to 12% polyethylene glycol. The precipitated protein was collected after 45 min, dissolved in 0.01 M phosphate buffer–5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6, and dialyzed against deionized water. After removal of a precipitate by centrifugation, the solution was dialyzed against 0.02 M sodium phosphate–0.8 M NaCl, pH 6.8, and applied at a flow rate of 15 mL/h to a column (2.5 × 3.7 cm) containing a $Zn^{2+}$ chelate coupled to cross-linked 4% agarose beads (Pierce Chemical Co., Rockford, IL), prepared as described by Kurecki et al. (1979) and equilibrated in 0.02 M sodium phosphate–0.8 M NaCl, pH 6.8. After the column was washed (25× bed volume), $\alpha_2$M was eluted by using a linear gradient formed by 45 mL of starting buffer in the stirred proximal chamber and 45 mL of 0.1 M sodium acetate, pH 5.1, in the distal chamber. Fractions (1 mL) containing $\alpha_2$M were collected and dialyzed against 0.01 M sodium phosphate–0.15 M NaCl, pH 7.4, and stored frozen at −70 °C. The average yield of $\alpha_2$M by this method was in the range of 26–34%. On the basis of the observed specific antigen concentration of 0.32–0.39 unit/mg of protein, the $\alpha_2$M concentration in normal plasma is inferred to be 2.6–3.1 mg/mL. Analysis of the $\alpha_2$M preparation under reducing conditions indicated that approximately 90% of the protein migrated as a single band with an apparent molecular weight of 170000 (Figure 5A). $\alpha_2$M was found to bind 1.7–2.0 mol of trypsin/mol of $\alpha_2$M by using trypsin assayed by active-site titration with 4-nitrophenyl-4-guanidinobenzoate hydrochloride (BDH Chemicals Ltd., England).

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a reference.

Kallikrein was generated from prekallikrein by using $\beta$-factor $XII_a$ (Hageman factor fragments) as previously described (van der Graaf et al., 1982a). The light chain of kallikrein was prepared from kallikrein by mild reduction and alkylation as described previously (van der Graaf et al., 1982b). The specific amidolytic activity was 4100 $\mu$mol of *p*-nitroanilide (*p*NA) $min^{-1}$ $\mu mol^{-1}$ for kallikrein as well as for its light chain when H-D-Pro-Phe-Arg-*p*-nitroanilide (S-2302, Kabi Diagnostica) was used at a concentration of 0.2 mM in 0.15 M tris(hydroxymethyl)aminomethane (Tris)–1 mg/mL bovine serum albumin (BSA), pH 8.3 (at 37 °C). Molecular weight values were assumed to be 80000 and 35000 for the native kallikrein and the light chain, respectively.

Radiolabeling of kallikrein was performed with $^{125}I$ by using the Bolton–Hunter reagent (Bolton & Hunter, 1973) essentially as described elsewhere (van der Graaf et al., 1983a). The radiolabeled kallikrein retained at least 60% of its specific amidolytic activity and contained 0.44 $\mu$Ci/$\mu$g. High molecular weight kininogen was radiolabeled with $^{125}I$ by using the insolubilized lactoperoxidase method (David & Reisfeld, 1974). The high molecular weight kininogen preparation retained its procoagulant activity after the radiolabeling procedure and contained 0.24 $\mu$Ci/$\mu$g.

For the kinetic studies of the inactivation of kallikrein or its light chain by $\alpha_2$M, a constant amount of kallikrein (85 nM) or kallikrein light chain (85 nM) was incubated at 37 °C with increasing amounts of $\alpha_2$M in 0.05 M Tris–0.15 M NaCl, pH 7.4, in a final volume of 400 $\mu$L. Twenty-microliter samples were withdrawn at various times and added to a 1-cm cuvette containing 980 $\mu$L of 2 mM H-D-Pro-Phe-Arg-*p*-nitroanilide in 0.1 M Tris, 0.05 M NaCl, and 1 mg/mL BSA, pH 8.0. The change in absorbance at 405 nm was followed continuously by using a Beckman Model 3600 double-beam spectrophotometer. The observed change in absorbance per minute was converted to the percent of maximum activity by comparison with the change in absorbance per minute of the sample which did not contain $\alpha_2$M. When high molecular weight kininogen was present, kallikrein or its light chain was preincubated with high molecular weight kininogen for 10 min at 37 °C before $\alpha_2$M was added. The kinetic constants for the hydrolysis of H-D-Pro-Phe-Arg-*p*-nitroanilide by kallikrein and its light chain and those for $\alpha_2$M-bound kallikrein and kallikrein light chain were determined by incubating kallikrein (0.2 $\mu$M) or its light chain (0.2 $\mu$M) with a 25-fold molar excess of $\alpha_2$M as described above. Samples of 9 and 10.2 $\mu$L for kallikrein mixtures and kallikrein light chain mixtures, respectively, were added to a 1-cm cuvette containing 990 $\mu$L of the substrate solution in 0.1 M Tris, 0.15 M NaCl, and 1 mg/mL BSA, pH 8.0.

Sodium dodecyl sulfate (SDS) gradient polyacrylamide (PAA) slab gel electrophoresis was performed on 3–25% and 3–18% gradient PAA slab gels essentially according to the method of Laemmli (1970) under conditions as described by van der Graaf et al. (1983a).

## Results

*Kinetics of Inactivation of Kallikrein or Its Light Chain by $\alpha_2$M.* Incubation of kallikrein or its light chain with a 20-fold molar excess of $\alpha_2$M at 37 °C resulted in a decrease of the amidolytic activity of kallikrein or its light chain, which reached a plateau at 35% and 30% of the original amidolytic activity of kallikrein and kallikrein light chain, respectively, when H-D-Pro-Phe-Arg-*p*-nitroanilide was used at a concentration of 0.2 mM. The remaining activity was resistant to inhibition by soybean trypsin inhibitor (STI), when STI was used at a thousandfold molar excess over $\alpha_2$M-bound kallikrein or light chain and during relatively short incubation times (1 min). However, at longer incubation times, this residual amidolytic activity decreased slowly. After 1-h incubation of STI and the $\alpha_2$M–enzyme complexes, 31% of the original kallikrein and 27.5% of the original kallikrein light chain amidolytic activity were present. Incubation of $\alpha_2$M-bound kallikrein (final concentration of kallikrein 0.1 $\mu$M) and diisopropyl fluorophosphate (final concentration 1 mM) for 1 h at 37 °C resulted in a complete elimination of $\alpha_2$M-bound kallikrein amidolytic activity.

To study the kinetics of inactivation of kallikrein and its light chain by $\alpha_2$M, kallikrein or its light chain was incubated with different concentrations of $\alpha_2$M. At various times, samples were withdrawn and analyzed for amidolytic activity. At each time point, the obtained values were corrected by subtraction of the calculated remaining $\alpha_2$M-bound enzyme amidolytic activity. The resulting free kallikrein or kallikrein light chain amidolytic activity was plotted against the incubation time (Figure 1). Irreversible inactivation of kallikrein and its light chain by $\alpha_2$M progressive with time was observed. From the data of Figure 1, the apparent pseudo-first-order rate constants, $k_{app}$s, were calculated and plotted as a double-reciprocal plot of $k_{app}$ and $\alpha_2$M concentration. For both kallikrein and its

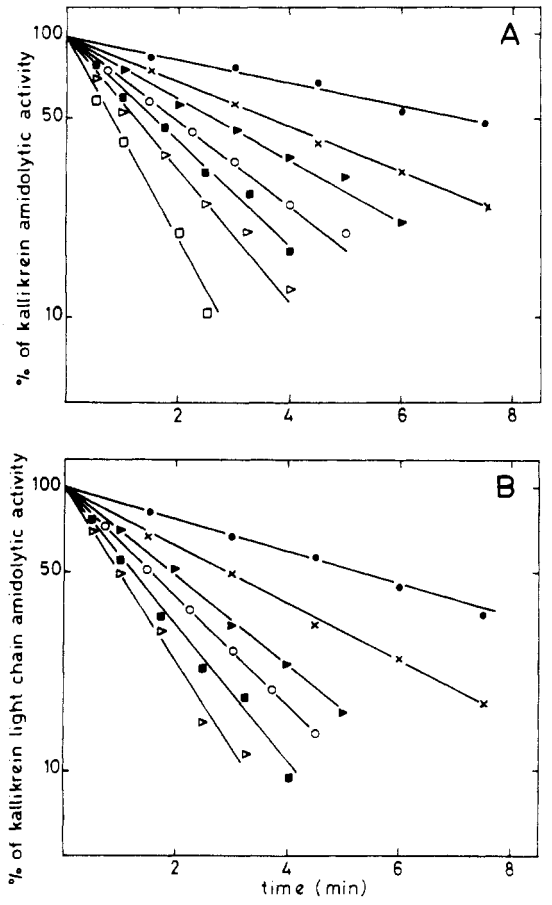


FIGURE 1: Kinetics of inactivation of the amidolytic activities of kallikrein and its light chain. Kallikrein (85 nM) or its light chain (85 nM) was incubated with different concentrations of $\alpha_2$M in 0.05 M Tris, 0.15 M NaCl, and 1 mg/mL BSA, pH 7.4 at 37 °C. The inactivation of kallikrein or kallikrein light chain amidolytic activity was followed by determination of the amidolytic activities of samples withdrawn at various times. The obtained activities were corrected by subtraction of the calculated $\alpha_2$M-bound enzyme amidolytic activities and plotted on a semilogarithmic scale as the percent of maximum kallikrein or light chain amidolytic activity. $\alpha_2$M was present at 0.30 (●), 0.60 (×), 0.90 (▶), 1.2 (○), 1.50 (■), 1.80 (▷), and 4.0 $\mu$M (□) during the inactivation of kallikrein (A) and at 0.30 (●), 0.60 (×), 0.90 (▶), 1.20 (○), 1.50 (■), and 1.80 $\mu$M (▷) during the inactivation of kallikrein light chain (B).

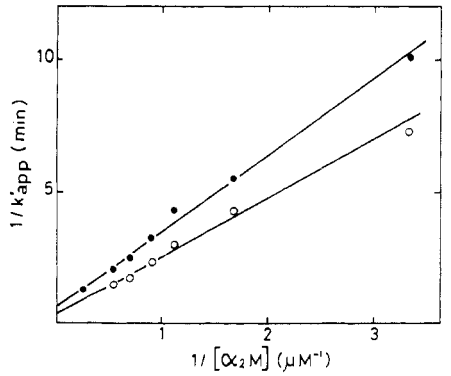


FIGURE 2: Double-reciprocal plot of the pseudo-first-order rate constants of the inactivation of kallikrein (●) or its light chain (○) vs. the concentration of $\alpha_2$M. The pseudo-first-order rate constants were calculated from the data of Figure 1. The lines are a least-squares fit of the points.

light chain, a straight line was obtained which intercepted the positive $y$ axis (Figure 2). Therefore, the inactivation of kallikrein and its light chain by $\alpha_2$M can be regarded as a process in which the enzyme (E) and the inhibitor (I) first form a reversible complex (EI) which then reacts to form an irreversible enzyme–inhibitor complex (E–I):

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} E\text{–}I$$

The dissociation constant for the initial reversible enzyme–inhibitor complex is $K_i$ which equals $k_{-1}/k_{+1}$. $k_{+2}$ is the first-order rate constant for the conversion of the reversible complex to the irreversible inhibited enzyme. $k_{+2}$ is also the first-order rate constant for the reaction at high inhibitor concentrations. At low inhibitor concentrations, the kinetics will correspond to a simple bimolecular mechanism with a second-order rate constant, $k_{+2}'$, which equals $k_{+2}/K_i$ (Kitz & Wilson, 1962). The kinetic constants for the reaction between kallikrein or its light chain and $\alpha_2$M can be determined from Figure 2. For the inactivation of kallikrein, the values for $K_i$, $k_{+2}$, and the second-order rate constant ($k_{+2}/K_i$) were 4.0 $\mu$M, 1.43 min$^{-1}$, and 36 × 10$^4$ M$^{-1}$ min$^{-1}$, respectively, and for the inactivation of the light chain of kallikrein, they were 4.4 $\mu$M, 2.2 min$^{-1}$, and 48 × 10$^4$ M$^{-1}$ min$^{-1}$, respectively.

Table I: Kinetic Constants for the Hydrolysis of H-D-Pro-Phe-Arg-*p*-nitroanilide by Kallikrein and Its Light Chain, $\alpha_2$M-Bound Kallikrein, and the $\alpha_2$M-Bound Light Chain of Kallikrein[a]

| | $K_m$ (mM) | $k_{cat}$ (min$^{-1}$) |
|---|---|---|
| kallikrein | 0.36 | 10.700 |
| $\alpha_2$M-kallikrein | 0.15 | 2.600 |
| light chain of kallikrein | 0.38 | 11.000 |
| $\alpha_2$M-bound light chain of kallikrein | 0.16 | 1.960 |

[a] $K_m$ and $k_{cat}$ values were obtained by using Lineweaver-Burk plots. Amidolytic activities were determined as described under Materials and Methods. The Lineweaver-Burk plot is a least-squares fit of the means of duplicate determinations. The correlation coefficients were 0.998 or higher. Molecular weight values were assumed to be 80 000 and 35 000 for native kallikrein and the light chain, respectively.

As noted above, binding of kallikrein or its light chain to $\alpha_2$M resulted in the formation of a complex which still possessed amidolytic activity. Therefore, it was of interest to determine whether binding of kallikrein or its light chain to $\alpha_2$M affected the kinetic parameters $K_m$ and $k_{cat}$ for the hydrolysis of H-D-Pro-Phe-Arg-*p*-nitroanilide by the two enzymes. $\alpha_2$M-bound kallikrein and $\alpha_2$M-bound kallikrein light chain were prepared by incubating kallikrein or its light chain with a 25-fold molar excess of $\alpha_2$M for 2 h at 37 °C. The results for the determination of the $K_m$ and $k_{cat}$ values are listed in Table I. The $K_m$ and $k_{cat}$ for this enzymatic reaction decreased approximately 2-fold and 5-fold, respectively, when kallikrein and its light chain had formed a complex with $\alpha_2$M.

*Influence of High Molecular Weight Kininogen on the Reaction between Kallikrein or Its Light Chain and $\alpha_2$M.* Kallikrein forms an equimolar complex with high molecular weight kininogen (Kerbiriou et al., 1980; Scott & Colman, 1980). To test whether the rate of kallikrein inactivation by $\alpha_2$M is influenced by the high-affinity interaction between these two proteins, kallikrein and various concentrations of high molecular weight kininogen were preincubated for 10 min at 37 °C. Laurell rocket immunoelectrophoresis using anti-prekallikrein antiserum as described by Kerbiriou et al. (1980) was used to establish if kallikrein had indeed formed a complex with high molecular weight kininogen under these conditions. Different amounts of $\alpha_2$M were then added to the mixture containing kallikrein and high molecular weight kininogen, and the inactivation of kallikrein was followed at 37 °C. Increasing concentrations of high molecular weight kininogen

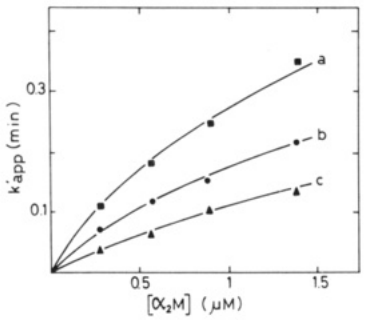


FIGURE 3: Effect of high molecular weight kininogen on the inactivation rate of kallikrein by $\alpha_2$M. Kallikrein (85 nM) was preincubated with high molecular weight kininogen at 0 (a), 1.1 (b), and 2.2 $\mu$M (c) in 0.05 M Tris, 0.15 M NaCl, and 1 mg/mL BSA, pH 7.4, for 10 min at 37 °C. Various amounts of $\alpha_2$M were then added, and the inactivation of kallikrein amidolytic activity was followed at 37 °C. Pseudo-first-order rate constants were determined from plots as shown in Figure 1.

caused a decrease of the apparent pseudo-first-order rate constant for the reaction between kallikrein and $\alpha_2$M (Figure 3). When high molecular weight kininogen was present at 1.1 $\mu$M, the apparent pseudo-first-order rate constant was reduced to 60% as compared to the rate constant determined in the absence of high molecular weight kininogen. However, high molecular weight kininogen did not affect the rate of inactivation of the light chain of kallikrein by $\alpha_2$M when tested at $\alpha_2$M concentrations of 0.6 and 1.2 $\mu$M, kallikrein light chain concentration of 85 nM, and high molecular weight kininogen concentrations as indicated in Figure 3.

*Interaction between Kallikrein and $\alpha_2$M: Analysis by SDS-Gradient PAA Slab Gel Electrophoresis.* Mixtures of $\alpha_2$M and increasing amounts of kallikrein and $^{125}$I-labeled kallikrein were incubated at 37 °C for 60 min and subjected to electrophoresis on SDS-gradient PAA slab gels. In the absence of reducing agent, the formation of several new bands with approximate molecular weights of 400 000–1 000 000 could be observed on the stained gel (Figure 4A). These bands were also present on the autoradiogram of the gel (Figure 4B), indicating that all bands contained kallikrein. In another experiment, a mixture of kallikrein and $\alpha_2$M preincubated for 1 h at 37 °C was incubated with [$^3$H]DFP for 30 min and then analyzed on a SDS-gradient PAA slab gel. Autoradiography of this gel demonstrated the formation of the same high molecular weight complex bands (Figure 4C). Kallikrein that had been inactivated by DFP, prior to its reaction with $\alpha_2$M, was not able to form the high molecular weight complex bands.

Analysis of the samples after reduction showed the appearance of a number of new bands (Figure 5A). The bands with $M_r$ 330 000, 235 000, 135 000, and 123 000 seen on the stained gel were also apparent on the autoradiogram (Figure 5B), suggesting that these bands represented complexes between kallikrein and $\alpha_2$M. Addition of increasing amounts of kallikrein to $\alpha_2$M led to the formation of increasing quantities of a $M_r$ 85 000 fragment. This fragment did not contain kallikrein, suggesting that it is the result of a proteolytic cleavage in the subunit chain of $\alpha_2$M by kallikrein. Furthermore, in association with the formation of these bands, a decrease in the intensity of the band with $M_r$ 170 000, representing the $\alpha_2$M subunit chain, was observed. However, even when kallikrein was present in a molar ratio of 14 relative to $\alpha_2$M, approximately 50% of $\alpha_2$M remained at $M_r$ 170 000. Thus, only part of $\alpha_2$M is proteolytically cleaved during its reaction with kallikrein. On the representative gel shown (Figure 5A), the generation of an additional fragment with a molecular weight of 88 000 was observed. Since this fragment did not contain kallikrein, it is likely that this band was derived from $\alpha_2$M. However, in other experiments, the formation of this band was not observed. Reaction between $\alpha_2$M and kallikrein resulted in a decrease in the intensity of both heavy and light chains of kallikrein (Figure 5A, lane e) as compared to unreacted kallikrein (Figure 5A, lane b), which suggests that the heavy chain as well as the light chains of kallikrein form SDS-stable complexes with $\alpha_2$M. Analysis under reducing conditions of the kallikrein–$\alpha_2$M complexes that were labeled with [$^3$H]DFP revealed two bands which comigrated with the $\alpha_2$M–kallikrein complex bands of $M_r$ 123 000 and 235 (Figure 5C). This observation demonstrates that these bands represent kallikrein–$\alpha_2$M complexes which contain the light chain of kallikrein.

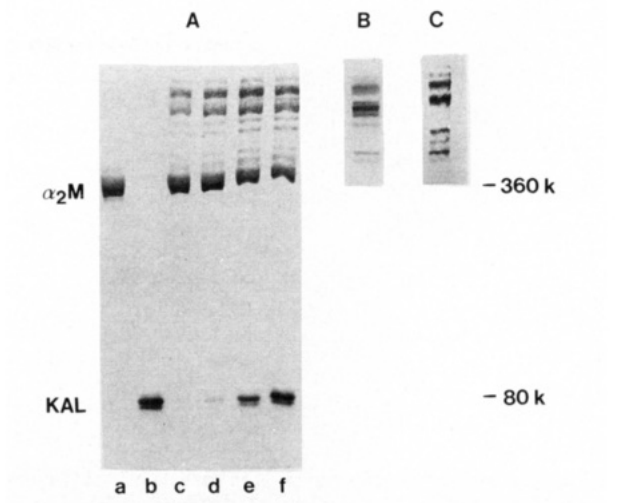


FIGURE 4: SDS 3–25% gradient PAA slab gel electrophoretic analysis of the reaction between kallikrein and $\alpha_2$M. Different amounts of kallikrein and $^{125}$I-labeled kallikrein were incubated with $\alpha_2$M (0.25 $\mu$M) in 0.05 M Tris–0.15 M NaCl, pH 7.4 at 37 °C. Twenty-five-microliter samples were incubated in a SDS solution for 45 min at 37 °C and analyzed on the gel. (A) Stained gel; in the incubation mixtures, kallikrein was present at 0 (a), 1.8 (no $\alpha_2$M present) (b), 0.23 (c), 0.46 (d), 1.8 (e), and 3.6 $\mu$M (f). (B) Autoradiogram of gel A. In another experiment, kallikrein was incubated with $\alpha_2$M (final concentrations 0.4 and 0.2 $\mu$M, respectively) for 2 h at 37 °C. [$^3$H]DFP (0.1 mM) was subsequently added and incubated for another 30 min at 37 °C. A 100-$\mu$L sample was analyzed in the presence of SDS on a 3–25% gradient PAA slab gel. (C) Autoradiogram of this gel. $\alpha_2$M and kallikrein (KAL) are indicated.

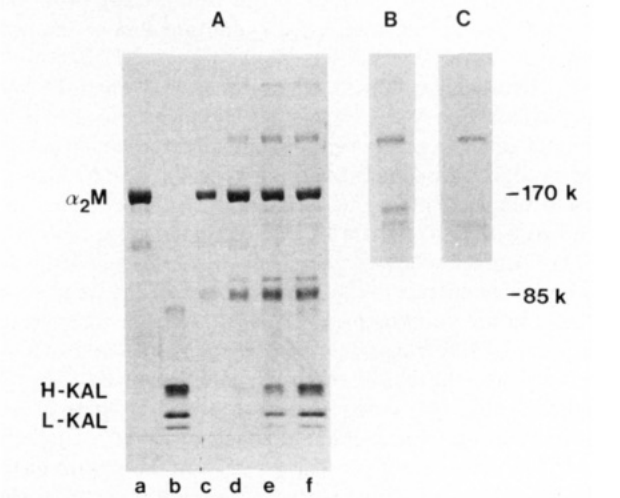


FIGURE 5: SDS 3–25% gradient PAA slab gel electrophoretic analysis of the reaction between kallikrein and $\alpha_2$M. The incubation mixtures described in the legend to Figure 4 were reduced prior to analysis on the gel. $\alpha_2$M and the heavy chain (H-KAL) and light chains (L-KAL) of kallikrein are indicated.

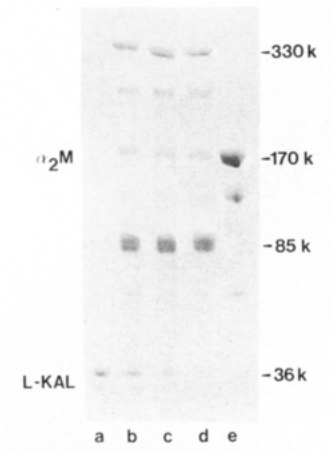


FIGURE 6: Analysis of the reaction between the light chain of kallikrein and $\alpha_2$M on a SDS 3–18% gradient PAA slab gel. $\alpha_2$M (0.55 $\mu$M) was incubated with various concentrations of the light chain of kallikrein in 0.05 M Tris–0.15 M NaCl, pH 7.4, for 1 h at 37 °C. The mixtures (30 $\mu$L) were then incubated in the presence of SDS and $\beta$-mercaptoethanol for another 45 min at 37 °C before analysis on the gel. The light chain of kallikrein (L-KAL) was present at 1.50 (no $\alpha_2$M present) (a), 2.5 (b), 1.5 (c), 0.5 (d), and 0 $\mu$M (e).

*Studies on the Interaction between the Light Chain of Kallikrein and $\alpha_2$M.* The interaction between the light chain of kallikrein and $\alpha_2$M was examined by incubation of $\alpha_2$M with various amounts of the light chain of kallikrein. The mixture was subsequently analyzed by SDS-gradient PAA slab gel electrophoresis under reducing conditions (Figure 6). In analogy with the results obtained with kallikrein, a $M_r$ 85 000 band was generated, whereas concomitant loss of the $\alpha_2$M band at $M_r$ 170 000 was observed. Approximately 20% of the $\alpha_2$M subunit chains appeared to be unaltered when the light chain of kallikrein was present in a 5-fold molar excess to $\alpha_2$M. Complex formation between the light chain of kallikrein and $\alpha_2$M produced three new bands (Figure 6). The bands with apparent molecular weights of 235 000 and 330 000 appeared identical with two bands with apparently the same molecular weight formed by the interaction between kallikrein and $\alpha_2$M. However, in contrast to the predominance of the band at $M_r$ 235 000 in the kallikrein–$\alpha_2$M mixture (Figure 5), with the light chain of kallikrein the $M_r$ 330 000 band was predominant. Furthermore, the kallikrein–$\alpha_2$M complex of $M_r$ 123 000 (Figure 5) that was shown to contain the light chain of kallikrein by using [$^3$H]DFP could not be observed. The band at $M_r$ 330 000 was not present on the autoradiogram of the gel containing kallikrein–$\alpha_2$M complexes that were labeled with [$^3$H]DFP (Figure 5C), suggesting that the light chain of kallikrein present in this complex is not able to react with [$^3$H]DFP. In addition to the complexes at $M_r$ 235 000 and 330 000, an additional complex band of $M_r$ 395 000 was present. As compared to the other two bands, its intensity was relatively weak. Furthermore, this band could not be identified on the gel containing reduced samples of kallikrein–$\alpha_2$M mixtures (Figure 5).

*Comparison of the Cleavage of $^{125}$I-Labeled High Molecular Weight Kininogen by Kallikrein and $\alpha_2$M-Bound Kallikrein.* To examine the possibility that in addition to its amidolytic activity $\alpha_2$M-bound kallikrein displays proteolytic activity, kallikrein was preincubated with a 25-fold molar excess of $\alpha_2$M. The proteolytic activity of this kallikrein–$\alpha_2$M preparation was then determined by incubation with $^{125}$I-labeled high molecular weight kininogen and compared to the proteolytic activity of the same amount of free kallikrein. Within 20 min, the single chain $^{125}$I-labeled high molecular weight kininogen was converted completely into a two-chain form by kallikrein. Cleavage of high molecular weight kininogen also occurred when kallikrein was complexed to $\alpha_2$M, although at a drastically reduced rate (Table II). Comparison of the rate of cleavage of high molecular weight kininogen indicated that the proteolytic activity of the $\alpha_2$M-band kallikrein had been reduced to less than 0.15% of that of an equivalent amount of free kallikrein.

Table II: Comparison of the Proteolytic Activities of Kallikrein and $\alpha_2$M-Bound Kallikrein against $^{125}$I-Labeled High Molecular Weight Kininogen[a]

| time of incubation (h) | % of max cleavage of $^{125}$I-labeled high mol wt kininogen | | |
|---|---|---|---|
| | kallikrein | kallikrein–$\alpha_2$M | buffer |
| 0.05 | 45 | 0 | 0 |
| 0.1 | 78 | 0 | |
| 0.3 | 100 | 0 | |
| 1.5 | | 2 | 0 |
| 19 | | 21 | |
| 27 | | 33 | |
| 43 | | 56 | 3 |

[a] Kallikrein (13.5 nM) or an equivalent amount of kallikrein preincubated with a 25-fold molar excess of $\alpha_2$M was incubated with high molecular weight kininogen (2.6 $\mu$M) and $^{125}$I-labeled high molecular weight kininogen in 0.02 M Tris, pH 8.0 at 37 °C. At the times indicated, samples were withdrawn and subjected to SDS gel electrophoresis in the presence of reducing agents. Cleavage of radiolabeled high molecular weight kininogen was assessed by analyzing the radioactivity of the sliced SDS gels.

## Discussion

The molecular mechanism of the interaction of $\alpha_2$M with different proteases, such as trypsin and plasmin, involves the formation of an $\alpha_2$M–protease complex that still exhibits considerable reactivity toward low molecular weight substrates (Starkey, 1979). When complexed to $\alpha_2$M, both kallikrein and its alkylated light chain retain part of their amidolytic activity on H-D-Pro-Phe-Arg-*p*-nitroanilide. Previous work demonstrated that $\alpha_2$M-bound kallikrein possessed esterolytic (Harpel, 1970) and amidolytic activity (Schapira et al., 1981; van der Graaf et al, 1981). $\alpha_2$M-bound kallikrein also retains proteolytic activity against high molecular weight kininogen. However, the rate of cleavage of high molecular weight kininogen by $\alpha_2$M-bound kallikrein is reduced to less than 0.15% of that of free kallikrein. Therefore, the possibility cannot be excluded that the observed cleavage of high molecular weight kininogen by the $\alpha_2$M–kallikrein mixture is caused by traces of free kallikrein or partially degraded $\alpha_2$M-bound kallikrein. Harpel & Mosesson (1973) and Harpel & Rosenberg (1976) showed that $\alpha_2$M-bound plasmin exhibited low but detectable proteolytic activity toward fibrinogen. When STI was incubated in a 50-fold molar excess over $\alpha_2$M-bound kallikrein or $\alpha_2$M-bound kallikrein light chain for 5 min, we failed to observe inhibition (data not shown). However, when present in a thousandfold molar excess and during longer incubation times, STI gave a minor but significant decrease of the amidolytic activity of the $\alpha_2$M–kallikrein and $\alpha_2$M–kallikrein light chain complexes. This is in agreement with the recent observation that STI can react with $\alpha_2$M-bound trypsin, resulting in a ternary complex (Bieth et al., 1981; Wang et al., 1981). The reaction of $\alpha_2$M-bound kallikrein with STI reinforces the notion that high molecular weight substrates have access to the active site of $\alpha_2$M-associated kallikrein. It is generally assumed that the reduced enzymatic activity of the $\alpha_2$M–protease complex is a reflection of sterically hindered access to the active site. In addition to steric factors, changes in the enzymatic properties may contribute to the changes in enzy-

matic activity observed for $\alpha_2$M-associated enzymes. Our data demonstrate that binding of kallikrein and its light chain to $\alpha_2$M is associated with a decrease in the apparent $K_m$ and $k_{cat}$ for the hydrolysis of the low molecular weight substrate, H-D-Pro-Phe-Arg-*p*-nitroanilide. In analogy, Rinderknecht et al. (1975) found a lower apparent $K_m$ for the hydrolysis of Z-Gly-Gly-Arg-2-naphthylamide hydrochloride by $\alpha_2$M-bound trypsin as compared to the free enzyme. Van Leuven et al. (1981) also demonstrated differences, dependent on the substrate, for the $K_m$ and $V_{max}$ of native trypsin and of the $\alpha_2$M–trypsin complex. The kinetic effects of entrapment of an enzyme within $\alpha_2$M are likely to be complex and probably depend to a large degree on the altered microenvironment created by the "trap".

The inhibition process of kallikrein or its light chain with $\alpha_2$M involves the initial formation of a reversible enzyme–inhibitor complex which then reacts to form an irreversible complex. The second-order rate constants, $k_{+2}/K_i$, are $3.5 \times 10^5$ and $4.8 \times 10^5$ $M^{-1}$ $min^{-1}$ for kallikrein and its light chain, respectively. Similar results for kallikrein were reported recently (Schapira et al., 1982b). The inactivation of kallikrein by $C\bar{1}$ inhibitor occurs with a second-order rate constant of $2.7 \times 10^6$ $M^{-1}$ $min^{-1}$ (van der Graaf et al., 1983b). Consequently, the $C\bar{1}$ inhibitor is approximately 7–8 times more effective than $\alpha_2$M in the inhibition of kallikrein. $C\bar{1}$ inhibitor and $\alpha_2$M were shown to contribute 55% and 35%, respectively, to the inactivation of kallikrein in plasma (Schapira et al., 1982a; van der Graaf et al., 1983a). These values are in close agreement with values calculated by using the kinetic constants noted above and assuming normal plasma concentrations of 1.7 $\mu$M for $C\bar{1}$ inhibitor and 3.8 $\mu$M for $\alpha_2$M.

In analogy to results obtained with $C\bar{1}$ inhibitor (van der Graaf et al., 1983b), the rate of reaction of $\alpha_2$M with the light chain of kallikrein is only slightly higher than that with kallikrein, which suggests that the heavy chain region of kallikrein does not affect the rate of kallikrein inactivation by $\alpha_2$M. The presence of high molecular weight kininogen decreases the inactivation rate of kallikrein by $\alpha_2$M, which confirms results obtained by Schapira et al. (1982b). In contrast, high molecular weight kininogen fails to protect the light chain of kallikrein from inactivation by $\alpha_2$M. These observations, together with the fact that the heavy chain of kallikrein provides the high-affinity binding site for high molecular weight kininogen, allow the conclusion that high molecular weight kininogen influences the rate of reaction between kallikrein and $\alpha_2$M due to its high-affinity binding to the heavy chain of kallikrein. Recent studies, however, demonstrated that high molecular weight kininogen, present at normal plasma concentrations, had no effect on the rate of kallikrein inactivation in plasma (van der Graff et al., 1983a). This can be explained by the observation that high molecular weight kininogen does not affect the rate of kallikrein inactivation by $C\bar{1}$ inhibitor (van der Graaf et al., 1983b), which is the major inhibitor of kallikrein in plasma. In addition, in the representative experiment (Figure 3), high molecular weight kininogen was present in a 12- or 24-fold molar excess over kallikrein, whereas high molecular weight kininogen and kallikrein are present in normal plasma in equimolar amounts. Therefore, it is likely that the effect of high molecular weight kininogen on the rate of kallikrein inactivation by $\alpha_2$M in plasma is much less. Nonetheless, at low $C\bar{1}$ inhibitor concentrations in plasma, high molecular weight kininogen may show some protective effect.

As studied by SDS-gradient PAA gel electrophoresis, the interaction between kallikrein and $\alpha_2$M results in the formation of a number of complexes with approximate molecular weights of 400 000–1 000 000. The same high molecular weight complexes were observed when $^{125}$I-labeled kallikrein was inactivated in plasma (van der Graaf et al., 1983a). Although the nature of these complexes seems rather complicated and at present unclear, the molecular weights of the complexes might suggest that one or more kallikrein molecules are linked to one or more $\alpha_2$M dimers. The fact that the interactions are stable in SDS implies that the formed bonds possess a stability normally characteristic of covalent bonds. The reaction of kallikrein with $\alpha_2$M leads to a cleavage in some of the $\alpha_2$M subunits, producing a $M_r$ 85 000 fragment. As shown previously (Harpel, 1973), reduction of $\alpha_2$M–kallikrein mixtures is necessary for the detection of this derivative band. Cleavage in the central region, generating two $M_r$ 85 000 fragments linked by disulfide bonds, appear to be a constant feature of a protease–$\alpha_2$M reaction (Harpel, 1973; Starkey, 1979). Further analysis of the reduced samples on SDS-gradient PAA slab gels revealed the presence of four kallikrein–$\alpha_2$M complexes. The $M_r$ 235 000 and the $M_r$ 330 000 complexes consist of the light chain of kallikrein and $\alpha_2$M, since apparently identical complexes are formed between the isolated alkylated light chain of kallikrein and $\alpha_2$M. In addition to the $M_r$ 235 000 and 330 000 complexes, the $M_r$ 123 000 complex also contains the light chain as was evidenced by the uptake of [$^3$H]DFP. In spite of the fact that it contains the light chain, the $M_r$ 330 000 complex reacts not at all or very slowly with [$^3$H]DFP which indicates either that the light chain in this complex is inactive or that its active site is no longer accessible for even small substrates. Additional support for this conclusion is given by the observation that all remaining amidolytic activity of the $\alpha_2$M-bound kallikrein could be eliminated by DFP. Although there are many similarities, there are also some differences in the complex formation between kallikrein and its light chain wht $\alpha_2$M. Thus, the presence of the heavy chain of kallikrein may influence to some degree complex formation with $\alpha_2$M. Moreover, the fact that the interaction of kallikrein with $\alpha_2$M is also associated with a loss in intensity of the heavy chain may suggest the formation of complexes that contain the heavy chain as well. Taken together, these findings may indicate that both heavy and light chains of kallikrein form a 1:1 stoichiometric complex with the $M_r$ 85 000 fragment of $\alpha_2$M ($M_r$ 135 000 and 123 000, respectively). The $M_r$ 235 000 complex may be formed by the binding of one kallikrein light chain to either one $M_r$ 185 000 subunit chain or two $M_r$ 85 000 fragments of $\alpha_2$M. Furthermore, to interpret the $M_r$ 330 000 and 395 000 complexes, one kallikrein light chain might become attached to even three and four $M_r$ 85 000 fragments or, alternatively, to $M_r$ 185 000 subunits instead of two $M_r$ 85 000 fragments of $\alpha_2$M. Nonetheless, the results of this study do not definitely distinguish between the possibilities that exist for the composition of the $M_r$ 235 000, 330 000, and 395 000 complexes. Previous studies on the interaction between plasmin and $\alpha_2$M suggested that the light chain of plasmin forms a 1:1 stoichiometric complex with both the $M_r$ 85 000 fragment and the subunit chain of $\alpha_2$M (Harpel, 1979). Other studies, however, indicated that no binding to intact subunits of $\alpha_2$M occurs with plasmin.

The $\alpha_2$M molecule contains an alkylamine reactive site which was recently identified as a Glx residue bound as a $\gamma$-glutamyl thiol ester to the SH group of a Cys residue (Swenson & Howard, 1979; Sottrup-Jensen et al., 1980; Howard, 1981). After the reaction with trypsin or elastase, a free thiol group was detectable in each subunit of $\alpha_2$M (Sottrup-Jensen et al., 1980; Salvesen et al., 1981). Prelim-

inary results indicated the appearance of free SH groups as a result of the inactivation of kallikrein by $\alpha_2$M. In addition, $\alpha_2$M preincubated with methylamine was unable to inactivate kallikrein or to form SDS-resistant complexes with kallikrein. Further studies are in progress to determine the number of free SH groups released in the reaction of $\alpha_2$M with kallikrein and its light chain.

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