Contribution of Plasma Protease Inhibitors to the Inactivation of Kallikrein in Plasma

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ABSTRACT Although CĪ-inhibitor (CĪ-INH) and α_2-macroglobulin (α_2M) have been reported as the major inhibitors of plasma kallikrein in normal plasma, there is little quantitative support for this conclusion. Thus, we studied the inactivation of purified kallikrein in normal plasma, as well as in plasma congenitally deficient in CĪ-INH, or artificially depleted of α_2M by chemical modification of the inhibitor with methylamine. Under pseudo-first-order conditions, the inactivation rate constant of kallikrein in normal plasma was 0.60 min^{-1}. This rate constant was reduced to 0.35, 0.30, and 0.06 min^{-1}, in plasma deficient respectively in CĪ-INH, α_2M, or both inhibitors. Thus CĪ-INH (42%) and α_2M (50%) were found to be the major inhibitors of kallikrein in normal plasma. Moreover all the other protease inhibitors present in normal plasma contributed only for 8% to the inactivation of the enzyme. To confirm these kinetic results, ^{125}I-kallikrein (M_r 85,000) was completely inactivated by various plasma samples, and the resulting mixtures were analyzed by gel filtration on Sepharose 6B CL for the appearance of ^{125}I-kallikrein-inhibitor complexes. After inactivation by normal plasma, 52% of the active enzyme were found to form a complex (M_r 370,000) with CĪ-INH, while 48% formed a complex (M_r 850,000) with α_2M. After inactivation by CĪ-INH-deficient plasma, >90% of the active ^{125}I-kallikrein was associated with α_2M. A similar proportion of the label was associated with CĪ-INH in plasma deficient in α_2M. After inactivation by plasma deficient in both CĪ-INH and α_2M, ^{125}I-kallikrein was found to form a complex of M_r 185,000. This latter complex, which may involve antithrombin III, α_1-protease inhibitor, and/or α_1-plasmin inhibitor, was not detectable in appreciable concentrations in the presence of either CĪ-INH or α_2M, even after the addition of heparin (2 U/ml). These observations demonstrate that CĪ-INH and α_2M are the only significant inhibitors of kallikrein in normal plasma confirming previous predictions based on experiments in purified systems. Moreover, in the absence of either CĪ-INH or α_2M, the inactivation of kallikrein becomes almost entirely dependent on the other major inhibitor.

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INTRODUCTION

Human plasma kallikrein, a serine protease, has a central role in the contact phase activation of blood coagulation, fibrinolysis, and kinin formation (1, 2). Studies using plasma fractions or purified proteins have indicated that kallikrein can be inactivated by several plasma protease inhibitors, including CĪ-inhibitor (CĪ-INH)[1], α_2 macroglobulin (α_2M), antithrombin III, α_2-protease inhibitor (3-11). Using purified kallikrein and inhibitors, we have recently examined the kinetics of the inactivation of kallikrein by CĪ-INH, α_2M, antithrombin III, and α_1-protease inhibitor (12, 13). Our results, together with the study of α_2-plasmin inhibitor by Saito et al. (11), indicate that CĪ-INH and α_2M are respectively 19 and 13 times more efficient, on a molar basis, than antithrombin III, α_1-protease inhibitor, and α_2-plasmin inhibitor combined (13).

Less information is available on the inactivation of kallikrein in plasma, which however is a more relevant issue for the understanding of the regulation of the plasma kinin-forming system. Two reports suggest that CĪ-INH is the most important inhibitor of kallikrein in plasma, because the plasma of patients with hereditary angioedema possesses little inhibitory activity toward the esterolytic activity of the enzyme (5, 14). A third paper indicates that α_2M is a major inhibitor of kallikrein in plasma, because the residual arginine-esterase activity present in normal plasma (37%), 5

[1] *Abbreviations used in this paper:* CĪ-INH, CĪ-inhibitor; α_2M, α_2-macroglobulin; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

min after plasma activation by kaolin, is not inhibitable by soybean trypsin inhibitor (6). This same investigator shows that kallikrein, when bound to α_2M, retains 48% of the esterolytic activity of the free enzyme (6). These data suggest that α_2M might account for 79% of the inactivation of kallikrein in normal plasma, and CĪ-INH for ≤21%. Thus, although these studies clearly establish that CĪ-INH and α_2M are both significant inhibitors of kallikrein in plasma, the respective contribution of each inhibitor was not determined, nor was the relative contribution of the other inhibitors of kallikrein. One problem in determining the relative contribution of these inhibitors has been the lack of a plasma selectively deficient in α_2M.

Our study is designed to assess the respective contribution of the different plasma protease inhibitors to the inactivation of kallikrein in plasma. The use of a new technique that selectively inactivates α_2M in plasma facilitates the comparison of the kinetics of kallikrein inactivation. Furthermore, we quantify the concentration of each kallikrein-inhibitor complex, following the complete inactivation of the enzyme in normal plasma, as well as in the plasma cogenitally deficient or depleted of one or more protease inhibitors.

METHODS

Antiserum to CĪ-INH (Behring Diagnostic, Inc., Woodbury, N. Y.); C4-Quiplate kit (Helena Laboratories, Beaumont, Tex.); H-D-proline-phenylalanine-arginine-*p*-nitroanilide (H-D-Pro-Phe-Arg-*p*-nitroanilide) (S 2302, Ortho Pharmaceutical, Raritan, N. J.); methylamine (Fisher Scientific Co., Pittsburgh, Pa.); ^{125}I 3-(4-hydroxyphenyl) propionic acid *N*-hydroxysuccinimide ester (Bolton-Hunter reagent, New England Nuclear, Boston, Mass.); Sepharose CL 6B (Pharmacia Fine Chemicals, Piscataway, N. J.); heparin (Riker Laboratories, Inc., Northridge, Calif.); and trypsin (Sigma Chemical Co., St. Louis, Mo.) were purchased from the designed supplier. All other reagents used were reagent grade.

Normal pooled plasma was obtained from George King Bio-Medical Inc., Overland Park, Kans. CĪ-INH-deficient plasma was generously donated by a patient presenting the classical form of hereditary angioedema. CĪ-INH and C4 were assessed by radial immunodiffusion (15) in the plasma of this patient. CĪ-INH was 17% and C4 18% of the level observed in normal pooled plasma.

Preparation of plasma proteins. Kallikrein was prepared by activating purified prekallikrein with factor XII fragments (16). The specific activity was 15.4 μmol/min per mg with H-D-Pro-Phe-Arg-*p*-nitroanilide as a substrate. The factor XII fragments were not removed but contributed <0.01% of the amidolytic activity. Kallikrein migrated as a single band of M_r 88,000 on unreduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and as two bands, M_r 55,000 and 33,000, on reduced SDS-PAGE (17). Kallikrein was radioiodinated with the Bolton-Hunter reagent (18). Its specific radioactivity was 29 μCi/mg. The resultant enzyme was unchanged in specific enzymatic activity after radiolabeling. Radioactivity was measured with a CG 4000 Intertechnique gamma counter.

CĪ-INH was prepared as described elsewhere (12). The resultant preparation, M_r of 105,000 on reduced and unreduced SDS-PAGE, was 97% active as determined by the method of Levy and Lepow (19), compared with its antigenic reactivity, assessed by electroimmunodiffusion (20).

α_2M was purified by a modification (21) of the method described by Song et al. (22). The preparation migrated as a single band of M_r = 185,000 on reduced SDS-PAGE. 1 mg of α_2M inactivated 78 μg of trypsin, as assessed by the Remazol brilliant Blue-hide assay (23), indicating that it was fully active. The concentration of trypsin was measured by active site titration (24). α_2M had no detectable amidolytic activity on H-D-Pro-Phe-Arg-*p*-nitroanilide indicating that it did not contain measurable amounts of bound kallikrein or plasmin.

Kinetic studies. Kallikrein was incubated with inhibitors or plasma 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 (S2302). A 0.6-mM solution of the substrate was prepared in 85 mM sodium phosphate buffer, pH 7.6, containing 127 mM NaCl. 10 μl of the solution to be tested were added to 330 μl of substrate at 37°C, and the absorbance change at 405 nm was continuously recorded using a Cary 210 double-beam spectrophotometer. Under these conditions, the hydrolysis rate of the substrate by 1 nM kallikrein was 1.84 nmol/min.

RESULTS

Influence of methylamine on purified α_2M and CĪ-INH. The kinetics of the inactivation of kallikrein amidolytic activity by a 60-molar excess of purified α_2M yielded a curvilinear pattern of inhibition (Fig. 1a), because the kallikrein-α_2M complex retained 24%

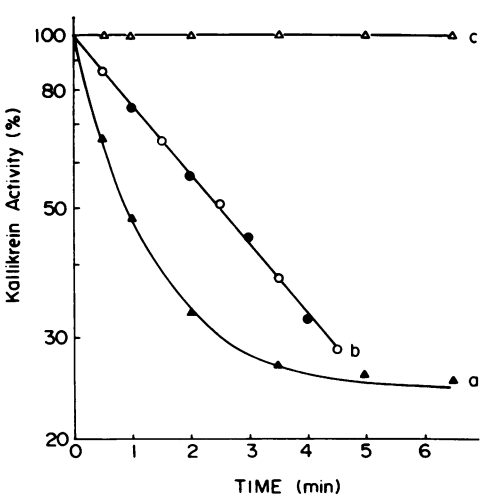

FIGURE 1 Effect of methylamine treatment on purfied α_2M or CĪ-INH. Kallikrein (final concentration 50 nM) was incubated with α_2M or with CĪ-INH, in 0.1 M sodium phosphate, pH 7.6 containing 0.15 M NaCl, and then assayed at various times for residual amidolytic activity. Alpha-2-macroglobulin final concentration was 3 μM in (a) and (c). CĪ-INH final concentration was 0.27 μM in (b). Inhibitors were preincubated for 2 h at 23°C, either with buffer (closed symbols), or with methylamine at a final concentration of 40 mM (open symbols).

of the activity of the free enzyme, when assayed on H-D-Pro-Phe-Arg-*p*-nitroanilide (13). After α_2M was incubated for 2 h at 23°C with methylamine (40 mM), its reactivity toward kallikrein was abolished (Fig. 1c). The inactivation of kallikrein amidolytic activity by purified CĪ-INH followed pseudo-first-order kinetics (Fig. 1b, closed circles), and methylamine treatment did not affect the function of CĪ-INH (Fig. 1b, open circles). Thus, methylamine appeared to be a suitable agent for selectively inactiviting α_2M while sparing CĪ-INH function.

Inactivation of kallikrein by normal plasma or by plasma deficient in protease inhibitors: kinetic studies. The kinetics of the inactivation of kallikrein amidolytic activity by normal plasma (Fig. 2a) is contrasted with the kinetics observed with plasma deficient in α_2M (Fig. 2b), CĪ-INH (Fig. 2c), or both inhibitors (Fig. 2d). CĪ-INH-deficient plasma was obtained from an individual with hereditary angioedema. α_2M-deficient plasma was generated by incubating normal plasma with methylamine at a final concentration of 40 mM for 2 h at 23°C. Plasma deficient in both inhibitors was obtained by methylamine-treatment of the plasma from an individual with hereditary angioendema. The inactivation of kallikrein by α_2M-deficient plasma (Fig. 2b) and by plasma deficient in both inhibitors (Fig. 2d) followed first-order kinetics. In contrast, curvilinear patterns of inhibition were seen, when functional α_2M was present, as in normal plasma (Fig. 2a) or in CĪ-INH-deficient plasma (Fig. 2c). The pseudo-first-order rate constants for the inactivation of kallikrein by normal plasma, CĪ-INH-deficient plasma, α_2M-deficient plasma, or plasma deficient in both inhibitors were 0.60, 0.35, 0.30, and 0.06 min^{-1}, respectively. The pseudo-first-order rate constants k'_{obs}, for the interaction of the enzyme with each inhibitor were derived from these results, and are listed in Table I. The k'_{obs}, are in excellent agreement with the theoretical pseudo-first-order rate constants, k'_{theor}, which were calculated using the kinetic constants obtained by studies in purified systems (11, 12, 13) and the concentrations of inhibitors used in Fig. 2. For example, CĪ-INH accounted for 42–48% of kallikrein inactivation in plasma, when assessed by k'_{obs}, while it represented 43% of kallikrein inhibition when determined by k'_{theor}. The pseudo-first-order rate constants for the inactivation of kallikrein in undiluted plasma, k'_{plasma}, were calculated for normal plasma concentrations of inhibitors. CĪ-INH (1.7 μM) accounted for 58% of the inhibition of kallikrein, α_2M (3.5 μM) for 38%, and the combined action of α_1-protease inhibitor (45.5 μM), antithrombin III (4.7 μM) and α_2-plasmin inhibitor (1.1 μM) for 4%. The differences between k'_{theor} and k'_{plasma} are a result of differences in inactivation kinetics: the inactivation rate of kallikrein by CĪ-INH is a linear function of the concentration of the inhibitor (12), whereas the inactivation rate of kallikrein by α_2M is hyperbolic function of the inhibitor concentration (13). Therefore, at low concentrations of plasma, the contribution of α_2M will predom-

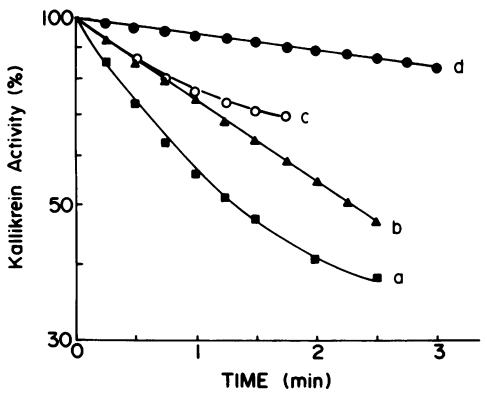

FIGURE 2 Kinetics of inactivation of kallikrein amidolytic activity. Kallikrein (final concentration 50 nM) was incubated with various plasma diluted in 0.1 M sodium phosphate, pH 7.6 containing 0.15 M NaCl (final plasma dilution 1:8.75), and then assayed, at various times, for residual amidolytic activity. (a) normal plasma, (b) α_2M-deficient plasma, (c) CĪ-INH-deficient plasma, and (d) plasma deficient in both α_2M and CĪ-INH.

TABLE I
Contribution of CĪ-INH, α_2M, and Other Protease Inhibitors to the Inactivation of Kallikrein

INHIBITOR	k'_{obs},* min^{-1}	k'_{theor}, min^{-1}	k'_{plasma},‡ min^{-1}
C1-INH	0.25–0.29 (42–48%)	0.20 (43%)	1.73 (58%)
α_2M	0.30 (50%)	0.24 (52%)	1.12 (38%)
Others	0.01–0.06§ (2–10%)	0.02‖ (4%)	0.12¶ (4%)

* The observed pseudo-first order rate constants k'_{obs}, were derived using the data presented in Fig. 2. The theoretical pseudo-first-order rate constants, k'_{theor}, were calculated using kinetic constants described elsewhere (11, 12)[2] and inhibitor concentrations as in the experimental conditions of Fig. 2.

‡ The plasma pseudo-first-order rate constants k'_{plasma}, were calculated using kinetic constants described elsewhere (11, 12)[2] and normal plasma concentrations of inhibitors.

§ This value represents k'_{obs} for all the kallikrein inhibitors present in normal plasma, excluding CĪ-INH and α_2M.

‖ This value represents k'_{theor} for antithrombin III, α_2-plasmin inhibitor, and α_1-protease inhibitor combined.

¶ This value represents k'_{plasma} for antithrombin III, α_2-plasmin inhibitor, and α_1-protease inhibitor combined.

inate, whereas at higher concentrations CĪ-INH will provide the majority of the inhibitory activity.

Inactivation of kallikrein by normal plasma and by plasma deficient in protease inhibitors: gel filtration studies. To confirm the preponderant importance of CĪ-INH and α_2M as kallikrein inhibitors in normal plasma, additional experiments were designed where ^{125}I-kallikrein was completely inactivated in various plasma. The resulting mixtures were then analyzed by gel filtration for the appearance of ^{125}I-kallikrein-inhibitor complexes. 1–1.4 μg ^{125}I-kallikrein was used for each experiment. Purified ^{125}I-kallikrein behaved as a single symmetric component (Fig. 3a) of M_r = 85,000. When ^{125}I-kallikrein was preincubated with normal plasma until the inhibition reached a maximum, and then gel filtered, 32% of the radioactivity was associated with a peak of apparent M_r = 850,000, 35% was associated with a peak of apparent M_r = 370,000, while 32% remained at a M_r of 85,000 (Fig. 3B). The M_r in this experiment as well as those in Figs. 4–6 were determined by a calibration curve constructed with normal plasma. Three peaks were identified by absorbance at 280 nm and used as internal standards: IgM (M_r = 900,000), IgG (M_r = 160,000), and albumin (M_r = 68,000). The log M_r was plotted versus fraction number and unknown M_r interpolated.

To characterize the radioactive peaks formed as a result of the inactivation of ^{125}I-kallikrein in normal plasma, the gel filtration behavior of complexes of purified components was determined. Preformed ^{125}I-kallikrein-CĪ-INH and ^{125}I-kallikrein-α_2M complexes were gel filtered (Fig. 4A and 4B); the ^{125}I-kallikrein-CĪ-INH complex had an apparent M_r of 370,000, and the ^{125}I-kallikrein-α_2M complex an apparent M_r of 850,000. Moreover, in all the gel filtration experiments, 31–33% of the radiolabeled enzyme remained in an uncomplexed form. This percentage was constant in all chromatograms and appeared to represent inactive ^{125}I-kallikrein. No enzymatic activity was detectable in the mixture of kallikrein and CĪ-INH, but amidolytic activity was detected in the mixture of kallikrein and α_2M (13). However, the active enzyme was entirely complexed to the inhibitor because this amidolotic activity was not inhibitable by soybean trypsin inhibitor. Therefore, it appeared that the inactivation of kallikrein in normal plasma (Fig. 3B) resulted almost exclusively in the formation of complexes with molecular weights characteristic of kallikrein-CĪ-INH and kallikrein-α_2M complexes. Moreover, a similar amount of enzyme was complexed to CĪ-INH and to α_2M (Fig. 3B).

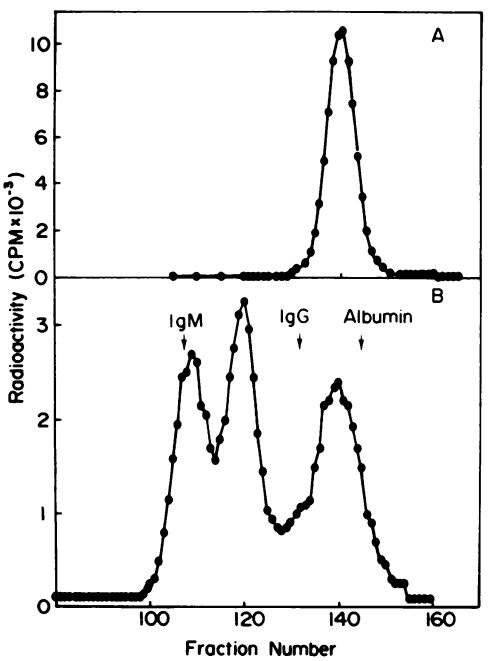

FIGURE 3 Gel filtration of ^{125}I-kallikrein alone or with normal plasma. 2 ml of the mixture generated after a 2-h incubation at 37°C of (A) ^{125}I-kallikrein (0.2 ml) and 1.8 buffer, or (B) ^{125}I-kallikrein (0.2 ml) and normal human plasma (1.8 ml) were separated by gel filtration on Sepharose CL 6B at 23°C in 0.1 M sodium phosphate, pH 7.6, containing 0.15 M NaCl, 1% bovine serum albumin, and 0.02% sodium azide. 1-ml fractions were collected. Column: 1.6 × 95 cm. Flow rate: 8 ml/min.

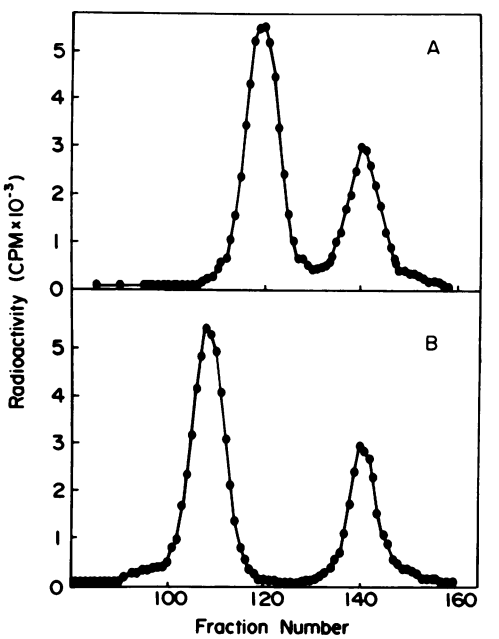

FIGURE 4 Gel filtration of ^{125}I-kallikrein after interaction with purified CĪ-INH or α_2M. 2 ml of the mixture generated after a 2-h incubation at 37°C of (A) ^{125}I-kallikrein and CĪ-INH and (B) ^{125}I-kallikrein and α_2M were gel filtered as described in Fig. 3. CĪ-INH final concentration: 6.8 μM. Alpha-2-macroglobulin final concentration: 3.5 μM.

To further confirm these results, ^{125}I-kallikrein was incubated with α_2M-deficient or CĪ-INH-deficient plasma. Analysis of the resultant mixtures revealed that the CĪ-INH-kallikrein complex was the only detectable enzyme-inhibitor complex in α_2M-deficient plasma (Fig. 5A), whereas the α_2M-kallikrein complex was the only observed complex involving kallikrein in CĪ-INH-deficient plasma (Fig. 5B). Indeed, the pattern observed when kallikrein was incubated with α_2M-deficient plasma (Fig. 5A) closely resembled the complex of purified CĪ-INH with kallikrein (Fig. 4A). Similarly, the pattern observed when kallikrein was incubated with CĪ-INH-deficient plasma (Fig. 5B) closely resembled the complex of purified α_2M with kallikrein (Fig. 4B).

To demonstrate the existence of minor inhibitor(s) of kallikrein in plasma, ^{125}I-kallikrein was then completely inhibited in the methylamine-treated plasma of an individual with hereditary angioedema, i.e., in plasma deficient in both CĪ-INH and α_2M. The resultant mixture was then gel filtered and analyzed for radioactivity (Fig. 6). 67% of the label was found to be associated to a peak of apparent M_r = 185,000. Therefore kallikrein can form complex(es) in plasma with other protease inhibitors than α_2M or CĪ-INH. These complex(es) probably account for the minor discrepancies observed between the chromatograms presented in Figs. 5A and B and those presented on Figs. 4A and B. To assess if heparin could increase significantly the inactivation rate of kallikrein by these inhibitor(s) and therefore increase the amount of enzyme complexed to these inhibitor(s), ^{125}I-kallikrein was inactivated in CĪ-INH deficient, or in α_2M deficient plasma, respectively, after these plasmas were adjusted to contain 2 U/ml with heparin. The resultant chromatograms (not shown) were identical to those illustrated in Fig. 5A and B.

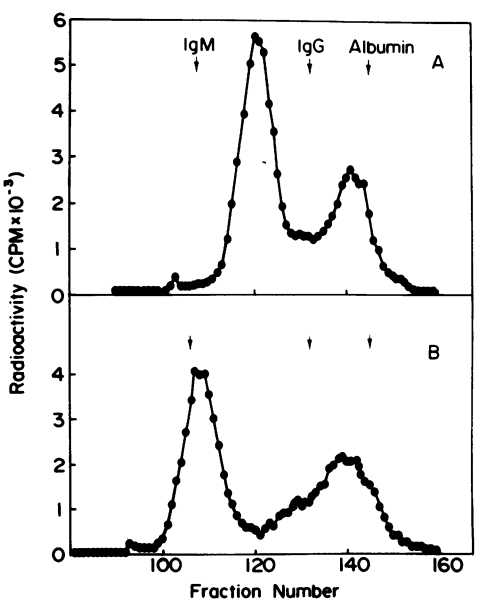

FIGURE 5 Gel filtration of ^{125}I-kallikrein with plasma deficient at α_2M or CĪ-INH. 2 ml of the mixture generated after a 2-h incubation at 37°C of (A) ^{125}I-kallikrein and α_2M-deficient plasma and (B) ^{125}I-kallikrein and CĪ-INH deficient plasma were gel filtered as described in Fig. 3.

DISCUSSION

The results obtained in this study indicate that CĪ-INH and α_2M are the major inhibitors of kallikrein in normal human plasma. This conclusion is supported by (*a*) the analysis of the kinetics of kallikrein inactivation in normal and protease inhibitor-deficient plasma, and (*b*) by the quantitation upon gel filtration of the kallikrein-inhibitor complexes formed in plasma as the result of the inactivation of purified radiolabeled enzyme.

Because no complete deficiency of α_2M has been documented in human plasma, quantitative assessment of the role of this inhibitor in plasma has been difficult. The discovery that methylamine can inactivate the purified inhibitor, most likely by scission of a thiolester reactive site (25, 26), led us to assess the effect of this nucleophilic agent in plasma. Kinetic observations, including the elimination of the characteristic curvilinear inhibition profile, indicate that methylamine is just as effective in plasma as in purified systems. Moreover, although it can modify other plasma proteins, notably C3 and C4 (27, 28), it does not inactivate CĪ-INH (Fig. 1).

Kinetic studies revealed that the rate constant of kallikrein inactivation by plasma deficient either in CĪ-INH, or in α_2M, was reduced to 50% of the rate constant observed when kallikrein was inactivated by normal plasma (Fig. 2). Thus, CĪ-INH and α_2M each contributed equally to the inactivation rate constant of the enzyme in normal human plasma (Fig. 2). Moreover, the inactivation rate constant of kallikrein by normal plasma was accurately predicted by adding the theoretical contributions of CĪ-INH and α_2M, assessed by studies in purified systems (Table I). This observation indicates that these inhibitors act independently in the inactivation of the enzyme. This finding is not surprising because CĪ-INH is a competitive inhibitor of kallikrein and α_2M is a noncompetitive one (13). When kallikrein was inactivated by plasma deficient in both CĪ-INH and α_2M, its inactivation rate constant was only 10% of the rate constant observed in normal plasma. This observation indicated that all the other plasma protease inhibitors contributed only marginally to the inactivation of kallikrein in normal plasma.

To independently assess the role of CĪ-INH and α_2M in the inactivation of kallikrein, ^{125}I-kallikrein was

incubated with normal and deficient plasma until complete inactivation, and the resulting mixtures were analysed by gel filtration for the formation of enzyme-inhibitor complexes. The radiolabeled kallikrein used in these experiments was 67–69% active, as assessed by its ability to form complexes with purified α_2M or CĪ-INH. This value is similar to that obtained for other proteolytic enzymes such as trypsin (29) by active site titration. In the mixture resulting from the incubation of kallikrein with normal plasma, 52% of the active enzyme was associated with CĪ-INH and 48% with α_2M (Fig. 3B). The difference between these results and those of Harpel (6) could result from the fact that in that study kallikrein was activated endogenously by adding kaolin to plasma whereas in this study preformed kallikrein was added to plasma. Such a difference has been recently reported for the distribution of plasmin between its plasma inhibitors (30). Additional experiments, where kallikrein was incubated with plasma deficient in protease inhibitors, revealed that >90% of the active enzyme was associated with CĪ-INH in plasma deficient in α_2M (Fig. 5A), while a similar proportion of active kallikrein was associated to α_2M in CĪ-INH-deficient plasma (Fig. 5B). These experiments confirmed the preponderent role of CĪ-INH and α_2M for the inactivation of kallikrein in plasma.

In the absence of either CĪ-INH or α_2M, almost all the active enzyme was complexed to the other major inhibitor. The apparent M_r for the α_2M-kallikrein complex was 850,000 daltons close to the predicted value of 813,000 (725,000 + 88,000). However, the apparent M_r for the kallikrein CĪ-INH complex was 370,000 daltons, exceeding the predicted value of 193,000 (105,000 + 88,000). This finding suggested that the kallikrein-C Ī-INH complex might have a high frictional ratio with a more assymetric shape than either of its components.

Finally, minor inhibitors of kallikrein were observed, using the gel filtration technique but only after the enzyme had been inactivated in plasma deficient both in CĪ-INH and α_2M (Fig. 6). The M_r of the kallikrein-inhibitor complex(es) was 185,000, indicating that antithrombin III, α_2M-plasmin inhibitor, and/or α_1-antitrypsin may be part of these complexes (31). Because these complex(es) were clearly identifiable only in the absence of both major inhibitors, this experiment confirmed that the reaction of kallikrein with its other plasma protease inhibitors was significantly slower than its reaction with either CĪ-INH or α_2M.

The plasma used in the gel filtration experiments were almost undiluted, in contrast to the plasma used for kinetic experiments, which was diluted 1:8.75. However, the respective contributions of CĪ-INH and α_2M to the inactivation of kallikrein was adequately predicted (k'_{plasma}, Table I) by the kinetic constants

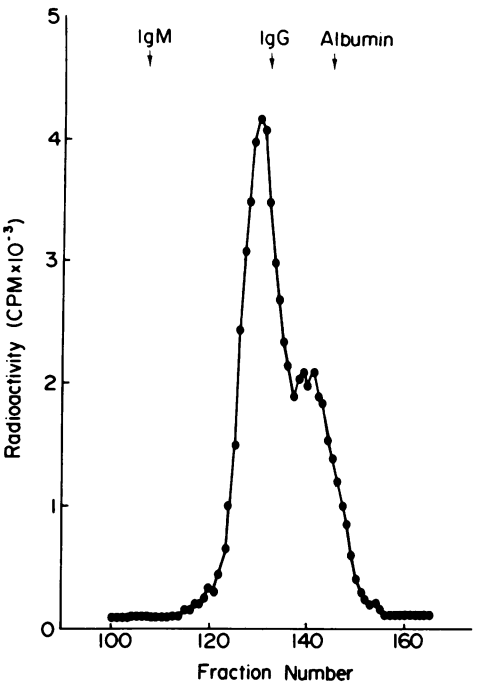

FIGURE 6 Gel filtration of ^{125}I-kallikrein with plasma deficient in both α_2M and CĪ-INH. 2 ml of the mixture generated after a 2-h incubation at 37°C of ^{125}I-kallikrein and plasma deficient in both CĪ-INH and α_2M were gel filtered as described in Fig. 3.

derived from studies in purified systems (13–15). This observation validates the use of these kinetic constants for the determination of the fluid phase kallikrein inactivation rate in systems containing the concentration of protease inhibitors present in normal plasma.

We have recently reported that the rate of kallikrein inactivation was reduced by the presence of the contact phase cofactor high molecular weight kininogen (12). This reduction in inactivation rate was the result of the formation of a reversible complex, K_d = 0.75 μM, between kallikrein and high molecular weight kininogen. The protective role of high molecular weight kininogen was ignored in the kinetic experiments described in the present report, because the concentration of this cofactor in the preincubation mixture was only 0.08 μM. At this concentration, high molecular weight kininogen is expected to decrease the inactivation rate of kallikrein by only 10% (12). Moreover, the influence of high molecular weight kininogen was not detectable in the gel filtration experiments, which were performed after complete inactivation of the enzyme because the cofactor decreases the rate but not the extent of kallikrein inactivation (12, 32). For analysis of the rate of kallikrein inactivation in vivo, it is however clear that the protective effect of high molecular weight kininogen should be considered.

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