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Interaction of Human Plasma Kallikrein and Its Light Chain with $C\bar{1}$ Inhibitor†

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ABSTRACT: The light chain of human plasma kallikrein contains the enzymatic active site. The inactivation of kallikrein and of its isolated light chain by $C\bar{1}$ inhibitor was investigated to assess the functional contributions of the heavy-chain region of kallikrein and of high molecular weight kininogen to this reaction. The second-order rate constants for the inactivation of kallikrein or its light chain were respectively 2.7×10^6 and 4.0×10^6 M^{-1} min^{-1}. High molecular weight kininogen did not influence the rate of kallikrein inactivation. The nature of the complexes formed between kallikrein or its light chain and $C\bar{1}$ inhibitor was studied by using sodium dodecyl sulfate (SDS) gradient polyacrylamide slab gel electrophoresis. Kallikrein as well as its light chain combined with $C\bar{1}$ inhibitor to form stable stoichiometric complexes that were not dissociated by SDS and that exhibited apparent molecular weights (M_r's) of 185 000 and 135 000, respectively, on nonreduced SDS gels. Reduction of the kallikrein–$C\bar{1}$ inhibitor complex gave a band at M_r 135 000 that comigrated with the complex seen for the light chain–$C\bar{1}$ inhibitor complex. During the inactivation of both kallikrein and its light chain, a M_r 94 000 fragment of $C\bar{1}$ inhibitor was formed which was unable to inactivate or bind kallikrein or its light chain. Kallikrein inactivated by diisopropyl phosphofluoridate did not form SDS-stable complexes with $C\bar{1}$ inhibitor. These results demonstrate that the functional binding site for $C\bar{1}$ inhibitor is localized in the light chain of kallikrein. In addition, the mechanism of interaction between kallikrein or its isolated light chain with $C\bar{1}$ inhibitor appears identical, and the rate of inactivation of both forms of the enzyme by $C\bar{1}$ inhibitor is very similar. Neither the heavy-chain region of kallikrein nor high molecular weight kininogen is significantly involved in the inactivation of kallikrein by $C\bar{1}$ inhibitor.

Plasma kallikrein, which circulates in its inactive zymogen form prekallikrein, participates in the contact activation reactions of plasma. Activation of this system occurs upon exposure of blood to negatively charged surfaces and initiates the intrinsic pathway of blood coagulation (Ratnoff, 1966), the kinin-forming pathway (Margolis, 1958), and the fibrinolytic system (Niewiarowski & Prou-Wartelle, 1959). The mechanism of this surface-dependent activation probably involves the binding of blood coagulation factor XII to the surface followed by the reciprocal activation of factor XII and prekallikrein (Nagasawa et al., 1968; Kaplan & Austen, 1970; Cochrane et al., 1973; Revak et al., 1977; Griffin & Cochrane, 1979; Dunn et al., 1982). For optimal generation of factor XII_a and kallikrein activity, a third plasma protein, high molecular weight kininogen, is necessary. High molecular weight kininogen circulates in plasma in a noncovalent complex with prekallikrein (Mandle et al., 1976; Donaldson et al., 1977) and functions as a nonenzymatic cofactor in these reactions by linking prekallikrein to the surface, thereby bringing it adjacent to surface-bound Factor XII (Griffin & Cochrane, 1976b; Meier et al., 1977; Wiggins et al., 1977; Silverberg et al., 1980). Plasma kallikrein also cleaves high molecular weight kininogen to liberate the vasoactive peptide bradykinin (Nagasawa & Nakayasu, 1973; Habal et al., 1974).

Several studies have identified $C\bar{1}$ inhibitor as an important inhibitor of plasma kallikrein (Gigli et al., 1970; McConnell, 1972; Fritz et al., 1972; Trumpi-Kalshoven & Kluft, 1978; Gallimore et al., 1979; Schapira et al., 1981). Recently, a major role for $C\bar{1}$ inhibitor in the inactivation of kallikrein in plasma was demonstrated in a quantitative manner (Schapira et al., 1982; van der Graaf et al., 1983). $C\bar{1}$ inhibitor is a plasma protease inhibitor which operates as an important regulator of the complement and the contact activation system of plasma. It is an α_2-globulin with a high content of amino sugars (Pensky & Schwick, 1969) and is the only plasma proteinase inhibitor capable of inactivating $C\bar{1}$, the activated first component of complement (Donaldson, 1979; Ziccardi,

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1981). The broad spectrum of inhibitory functions of C1 inhibitor became evident when C1 inhibitor was found to block the activities of plasma kallikrein (Kagan & Becker, 1963; Ratnoff et al., 1969), factor XII_a (Forbes et al., 1970; Schreiber et al., 1973), factor XI_a (Forbes et al., 1970; Harpel, 1971), and plasmin (Ratnoff et al., 1969; Schreiber et al., 1973). Gigli et al. (1970) found that an apparent stoichiometric reaction, which followed second-order kinetics, took place between kallikrein and C1 inhibitor. Harpel & Cooper (1975) demonstrated that the interaction of plasmin with C1 inhibitor is quite distinct from the interaction of C1s with C1 inhibitor.

Recently we isolated the alkylated heavy and light chains of human plasma kallikrein in order to study the functional roles of these chains (van der Graaf et al., 1982b). In this paper, the kinetics of inactivation of kallikrein and its light chain that contains the active site were studied and compared. Furthermore, the nature of the molecular complexes formed between kallikrein or its light chain and C1 inhibitor was investigated.

Materials and Methods

All chemicals obtained from commercial sources were the best grade available.

Purification of Proteins. High molecular weight kininogen and prekallikrein were isolated from human plasma as described elsewhere (Kerbiriou & Griffin, 1979; Kerbiriou et al., 1980). C1 inhibitor was purified from human citrated fresh plasma. The plasma was obtained as previously described (Bouma et al., 1980). Throughout the purification procedure, C1 inhibitor was detected by rocket immunoelectrophoresis according to the method of Laurell (1966) using 0.9% agarose (Seakem) containing 2% (v/v) anti-C1 inhibitor antiserum (Behringwerke A. G. Marburg, West Germany). One liter of plasma was dialyzed at 22 °C against the starting buffer for chromatography on DEAE-Sephadex A-50 (Pharmacia) as described before for the purification of factor XII (Griffin & Cochrane, 1976a). C1 inhibitor eluted at a buffer conductivity of 18–21 $m\Omega^{-1}$ at pH 7.7. All further steps were performed at 4 °C. The fractions containing C1 inhibitor were pooled (350 mL) and dialyzed against 0.04 M sodium acetate, pH 5.3, containing 1 mM ethylenediaminetetraacetic acid (EDTA),[1] 1 mM benzamidine, and 0.02% NaN_3 with a conductivity of 3.8 $m\Omega^{-1}$. After dialysis, a precipitate was removed by centrifugation (5 min, 1500*g*, 22 °C), and the dialyzed material was applied to a column (5 × 26 cm) of SP-Sephadex C-50 (Pharmacia) equilibrated in the same buffer at a flow rate of 90 mL/h. The column was washed with 2 L of starting buffer. C1 inhibitor was subsequently eluted by using a linear gradient formed by 1.3 L of starting buffer in the proximal chamber and 1.3 L of starting buffer containing an additional 0.3 M NaCl, with a conductivity 30 $m\Omega^{-1}$ in the distal chamber. Fractions containing C1 inhibitor were pooled and dialyzed against the starting buffer for a second chromatography on DEAE-Sephadex A-50. The column (2.5 × 30 cm) containing 6 g of DEAE-Sephadex A-50 was equilibrated in the starting buffer, 0.04 M sodium acetate, 0.15 M NaCl, 1 mM EDTA, 1 mM benzamidine, and 0.02% NaN_3, pH 5.3 (conductivity 18 $m\Omega^{-1}$). The dialyzed C1 inhibitor pool was loaded on this column at a flow rate of 35 mL/h. After the column was washed with 600 mL of starting buffer, elution of C1 inhibitor was effected with 375 mL of starting buffer in the stirred proximal chamber and 375 mL of starting buffer containing 0.5 M NaCl in the distal chamber. The last step employed affinity chromatography on concanavalin A–Sepharose (Pharmacia). After chromatography on the second DEAE-Sephadex column, the fractions containing C1 inhibitor were pooled, dialyzed against 0.01 M sodium phosphate, 0.4 M NaCl, 1 mM benzamidine, and 0.02% NaN_3, pH 7.4, and applied on a column (2.5 × 2 cm) containing concanavalin A–Sepharose equilibrated in the same buffer at a flow rate of 10 mL/h. The column was washed with 50 mL of starting buffer, and C1 inhibitor was subsequently eluted in a single step by using 0.5 M methyl α-D-glucoside in the starting buffer. Fractions containing C1 inhibitor were combined, dialyzed against storage buffer (5 mM sodium acetate, 0.15 M NaCl, 0.5 mM EDTA, and 0.02% NaN_3, pH 5.3), and stored at −70 °C. The average yield of C1 inhibitor by this method was in the range of 4–6%. On the basis of the observed specific antigen concentration of 5–6 units/mg, the C1 inhibitor concentration in normal human plasma is inferred to be 165–200 μg/mL. Purified C1 inhibitor analyzed on unreduced SDS 3–25% gradient PAA gels demonstrated two bands, a major band designated band I with a M_r = 110 000 and a minor band designated band II with a M_r = 94 000. After reduction of C1 inhibitor, only one band with M_r = 94 000 could be observed.

C1 inhibitor functional activity was assessed by its ability to inhibit the esterolytic activity of a partially purified preparation of activated C1 (Donaldson, 1968) by using *N*-acetyl-L-tyrosine ethyl ester (ATEe) as a substrate. Acid liberated by activated C1 was determined by addition of NaOH using the pH-state technique as described by Berrens & Guikers (1972). The functional activity of purified C1 inhibitor based on its ability to inhibit the esterolytic activity of activated C1 was compared to the measurement of C1 inhibitor antigen obtained with a quantitative immunological assay. All C1 inhibitor preparations appeared 100% active because a good correlation was obtained between the functional inhibitory activity and the antigen level.

The protein concentration of kallikrein and its light chain was calculated from data obtained from amino acid analysis following a 24-h hydrolysis. Cys and Trp were not determined and were not considered in calculating the protein concentration of kallikrein and its light chain. Concentrations of other proteins were determined by the method of Lowry et al. (1951) with bovine serum albumin as a reference.

Preparation of Kallikrein and Its Light Chain. Kallikrein was generated from prekallikrein by using β-factor XIIa (Hageman factor fragments) as previously described (van der Graaf et al., 1982a). Kallikrein obtained in this way gave three fragments on reduced gels in the presence of SDS, a heavy chain with a M_r = 43 000 and two light chains of M_r 36 000 and 33 000. These fragments are held together by disulfide bonds, because on nonreduced SDS gels two bands were observed with an approximate M_r = 80 000. Some preparations of kallikrein contained two minor additional bands with a slightly lower apparent molecular weight than the two kallikrein bands (Figure 4a,b). These bands might result from further proteolytic cleavage of kallikrein by β-factor XIIa during its activation or from autodigestion. However, the specific amidolytic activity of the kallikrein preparation was not changed when these two additional bands were present. The light chain of kallikrein was prepared from kallikrein by mild reduction and alkylation as described before (van der Graaf et al., 1982b) and gave two bands of M_r 36 000 and

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAA, polyacrylamide; M_r, molecular weight; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; pNA, *p*-nitroanilide.

33 000 on SDS–polyacrylamide gels in the presence or absence of reducing agents. The specific amidolytic activity was 4100 μmol of pNA min^{-1} μmol^{-1} for kallikrein as well as 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–1 mg/mL BSA, pH 8.3 (at 37 °C). Molecular weight values were assumed to be 80 000 and 35 000 for the native kallikrein and the light chain, respectively.

Radiolabeling of Kallikrein. Radiolabeling of kallikrein was performed with ^{125}I by using the Bolton–Hunter reagent (Bolton & Hunter, 1973). Kallikrein was radiolabeled in the presence of 1 mM benzamidine. After the radiolabeling procedure, benzamidine was removed by extensive dialysis. The radiolabeled kallikrein retained at least 60% of its specific amidolytic activity and contained 0.6 μCi/μg.

Kinetic Studies of the Inactivation of Kallikrein or Its Light Chain by C1 Inhibitor. Kallikrein or its light chain was incubated in the presence or absence of high molecular weight kininogen in 0.05 M Tris, pH 7.4, and 1 mg/mL BSA, at 37 °C in plastic tubes. After 5 min, C1 inhibitor was added, and the inactivation of kallikrein amidolytic activity was followed by adding a 20-μL sample from the incubation mixture to a plastic cuvette containing 980 μL of 0.2 mM H-D-Pro-Phe-Arg-*p*-nitroanilide in 0.1 M Tris, 0.05 M NaCl, pH 8.0, and 1 mg/mL BSA at various times. The change in absorbance at 405 nm was followed continuously by using a Cary 210 double-beam spectrophotometer or a Beckman Model 3600 double-beam spectrophotometer. The observed ΔA per minute was converted to the percent of maximum activity by comparison with the ΔA per minute of the sample which did not contain C1 inhibitor.

Studies on the Interaction of Kallikrein or Its Light Chain Using SDS Gradient PAA Slab Gel Electrophoresis. For the study reported in Figures 4 and 5, a mixture of kallikrein and ^{125}I-kallikrein was incubated with C1 inhibitor in 0.05 M Tris, pH 7.4, in a final volume of 450 μL at 37 °C. At various times, a 6.5-μL aliquot was withdrawn and tested for kallikrein amidolytic activity. At the same times, a 32-μL samples was also withdrawn and added to 32 μL of a 3% SDS solution, whereas another 32-μL sample was added to 32 μL of a 3% SDS solution containing 0.1 M dithiothreitol. Both samples were incubated for 5 min at 100 °C and used for analysis by SDS 3–25% gradient PAA slab gel electrophoresis. For the study reported in Figure 6, various amounts of the light chain of kallikrein were incubated with C1 inhibitor in 0.05 M Tris, pH 7.4, for 30 min at 37 °C. Sixty-five-microliter samples were then added to a SDS solution (final concentration 1% SDS) and analyzed by SDS 3–18% gradient PAA slab gel electrophoresis. SDS gradient PAA slab gel electrophoresis was carried out on gradient slab gels (280 × 140 × 0.75 mm) essentially according to the method of Laemmli (1970). The gels were run at 150 V, 25 mA, for 18 h using running tap water for cooling. After electrophoresis, the gels were stained for protein with Coomassie blue R-250 and dried on Whatman 3MM paper. Autoradiography of the dried gels was performed by using Sakura X-ray film A (Konishiraku Photo Inc. Co. Ltd, Tokyo). The following standard proteins were included as references on each slab gel: myosin (M_r 200 000), β-galactosidase (M_r 116 500), phosphorylase (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), and carbonic anhydrase (M_r 30 000) (Bio-Rad, Richmond, CA).

Results

Kinetics of Inactivation of Kallikrein or Its Light Chain by C1 Inhibitor. Kallikrein or its light chain was incubated with different concentrations of C1 inhibitor. At various times, samples were withdrawn and analyzed for remaining kallikrein or kallikrein light chain amidolytic activity. Irreversible inhibition, progressive with time, was observed for both kallikrein and its light chain (Figure 1). The values of the apparent pseudo-first-order reaction rate constant (k_{app}) were calculated from this figure and plotted as a double-reciprocal plot of k_{app} vs. the C1 inhibitor concentration (Figure 2). For the inactivation of kallikrein as well as its light chain by C1 inhibitor, a straight line passing through the origin was obtained (Figure 2), which indicated that the mechanism of the inactivation of kallikrein or its light chain by C1 inhibitor can be regarded as a reaction in which no reversible enzyme–inhibitor complex

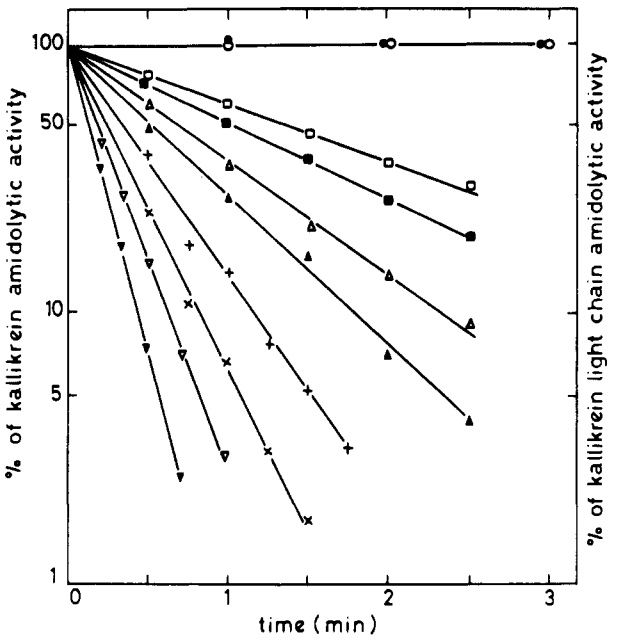

FIGURE 1: Kinetics of inactivation of kallikrein or kallikrein light chain amidolytic activity by C1 inhibitor. Kallikrein or its light chain was incubated at 37 °C with different concentrations of C1 inhibitor in 0.05 M Tris, pH 7.4, containing 1 mg/mL BSA. At various times, samples were withdrawn and assayed for remaining kallikrein or light chain amidolytic activity. The inactivation of kallikrein was assayed at 85 nM in the presence of C1 inhibitor at 0 (○), 0.2 (□), 0.4 (△), 0.8 (+), and 1.2 μM (▽). The inactivation of the light chain of kallikrein was assayed at 85 nM in the presence of C1 inhibitor at 0 (●), 0.2 (■), 0.4 (▲), 0.8 (×), and 1.2 μM (▼).

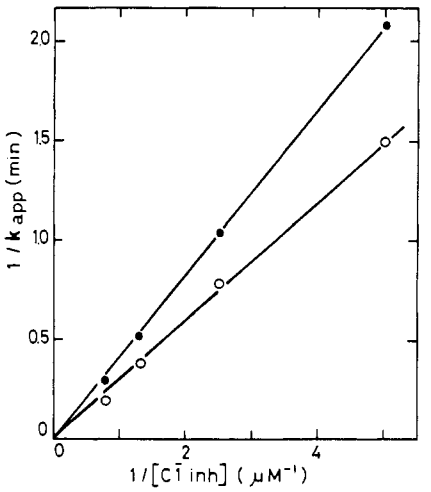

FIGURE 2: Double-reciprocal plot of the pseudo-first-order rate constant of the inactivation of kallikrein (●) or its light chain (○) vs. the concentration of C1 inhibitor. The pseudo-first-order rate constants were calculated from the data from Figure 1. The lines are a least-squares fit of the points.

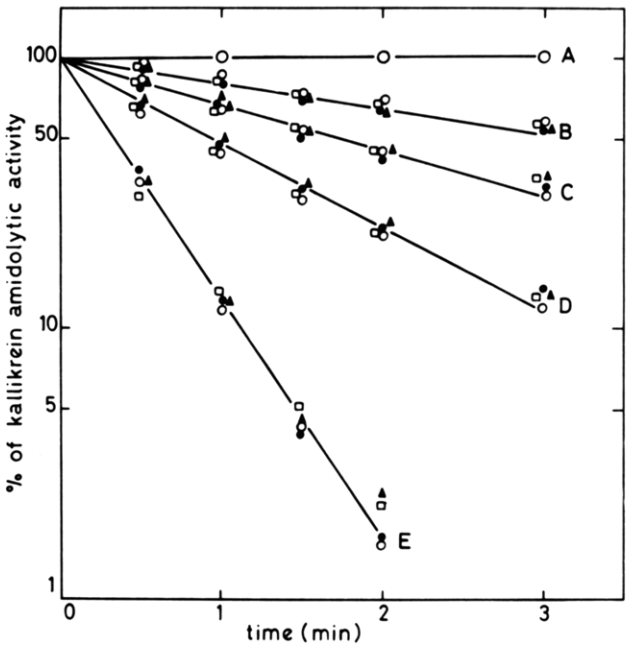

FIGURE 3: Effect of high molecular weight kininogen on the inactivation rate of kallikrein by C1̄ inhibitor. Kallikrein at 85 nM was preincubated with different concentrations of high molecular weight kininogen for 5 min in 0.05 M Tris, pH 7.4, and 1 mg/mL BSA. C1̄ inhibitor was then added at a final concentration of 0 (A), 0.11 (B), 0.21 (C), 0.42 (D), and 1.0 μM (E), and kallikrein amidolytic activity was determined at various times. High molecular weight kininogen was present at 0 (○), 0.42 (●), 1.05 (□), and 2.10 μM (▲).

is formed (Kitz & Wilson, 1962). The second-order rate constant (k'') for the reaction between an enzyme (E) and an irreversible inhibitor (I)

$$E + I \xrightarrow{k''} E\text{–}I$$

can be calculated from the equation

$$k_{app} = k''[I]$$

For the reaction between kallikrein and C1̄ inhibitor, the second-order rate constant has a value of 2.7×10^6 M^{-1} min^{-1}, while that for the reaction between the light chain of kallikrein and C1̄ inhibitor was calculated to be 4.0×10^6 M^{-1} min^{-1}.

Influence of High Molecular Weight Kininogen on the Reaction between Kallikrein and C1̄ Inhibitor. High molecular weight kininogen forms a 1 to 1 stoichiometric complex with kallikrein (Kerbiriou et al., 1980; Scott & Colman, 1980). Therefore, it was of interest to investigate whether the rate of kallikrein inactivation by C1̄ inhibitor is influenced by the high-affinity binding between kallikrein and high molecular weight kininogen. Since high molecular weight kininogen is also a substrate for kallikrein, high concentrations of high molecular weight kininogen relative to kallikrein present during the inactivation of kallikrein might decrease the rate of kallikrein inactivation due to substrate protection due to secondary binding. Therefore, kallikrein was preincubated with various concentrations of high molecular weight kininogen for 5 min at 37 °C before different amounts of C1̄ inhibitor were added to the mixture. Then at various times, a sample was withdrawn and added to a cuvette containing 0.2 mM H-D-Pro-Phe-Arg-*p*-nitroanilide to measure kallikrein amidolytic activity. Laurell rocket immunoelectrophoresis using anti-prekallikrein antibodies as described by Kerbiriou et al. (1980) was used to establish if kallikrein had indeed formed a complex with high molecular weight kininogen under the conditions used in this experiment. No effect of high molecular weight kininogen could be observed on the rate of kallikrein inactivation by C1̄ inhibitor (Figure 3) at any of the concentrations of high molecular weight kininogen or C1̄ inhibitor examined.

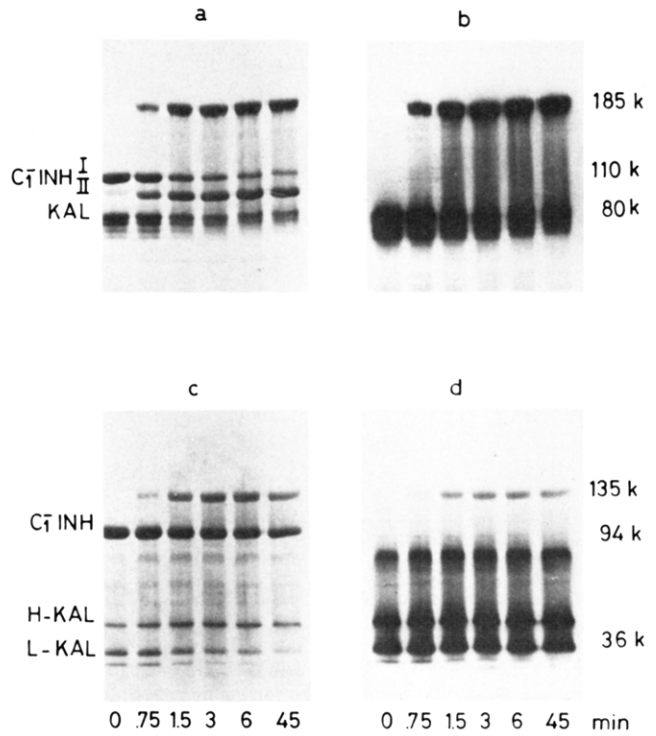

FIGURE 4: SDS 3–25% gradient PAA slab gel electrophoretic analysis of the reaction between C1̄ inhibitor and kallikrein. Kallikrein (1.0 μM), ^{125}I-kallikrein (0.05 μM), and C1̄ inhibitor (1.5 μM) were incubated at 37 °C. Samples were withdrawn at various times, incubated in a SDS solution either with or without dithiothreitol for 5 min at 100 °C, and subsequently applied to the gel. (a) Unreduced stained gel; (b) autoradiogram of gel (a); (c) reduced stained gel; (d) autoradiogram of gel (c). The different incubation times as well as C1̄ inhibitor bands I and II (C1̄ INH I and II), kallikrein (KAL), and the heavy and light chains of kallikrein (H-KAL and L-KAL, respectively) are indicated.

Studies on the Interaction between Kallikrein and C1̄ Inhibitor. Kallikrein, ^{125}I-kallikrein, and C1̄ inhibitor were incubated in a molar ratio of 1.5 of C1̄ inhibitor relative to kallikrein at 37 °C. Samples were withdrawn at various times and analyzed by SDS gradient PAA slab gel electrophoresis and for kallikrein amidolytic activity. In the absence of reducing agent, a new slower moving band, with increasing intensity in time, was observed (Figure 4a). This band was also present on the autoradiogram of this gel (Figure 4b), indicating that it contained kallikrein. The molecular weight of this band was 185000, which is the approximate molecular weight expected for stoichiometric complex formation between kallikrein and C1̄ inhibitor. In concert with this, a parallel decrease of C1̄ inhibitor band I was observed, while the simultaneous formation of a new band at the C1̄ inhibitor band II position was apparent. The density of the kalikrein bands on the stained gel as well as on the autoradiogram decreased during the incubation. The amount of ^{125}I-kallikrein still present after 45-min inactivation (Figure 4b) is probably inactive ^{125}I-kallikrein caused by the radiolabeling procedure. These findings suggested that kallikrein had produced a lower molecular weight derivative of C1̄ inhibitor band I, indicated here as C1̄ inhibitor band II. Addition of kallikrein to the reaction mixture after 45 min at 37 °C had no effect on the C1̄ inhibitor band II, nor was there an increase in the M_r 185000 band as compared to the 45-min samples. Furthermore, no decrease in kallikrein amidolytic activity could be demonstrated (data not shown). Therefore, the lower mo-

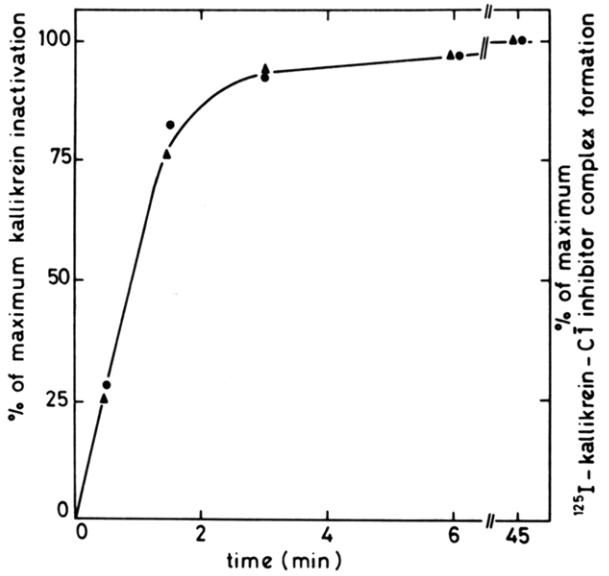

FIGURE 5: Kinetics of inactivation of kallikrein by C1 inhibitor (●) and the formation of ^{125}I-kallikrein–C1 inhibitor complex (▲), Kallikrein (1.0 μM), ^{125}I-kallikrein (0.05 μM), and C1 inhibitor (1.5 μM) were incubated at 37 °C. At various times, two samples were withdrawn. One sample was analyzed for the presence of kallikrein amidolytic activity. The second sample was analyzed on an unreduced SDS 3–25% gradient PAA slab gel (Figure 4). After being dried, the gel was sliced into sections and counted for the presence of radioactivity. The formation of kallikrein–C1 inhibitor complex at M_r 185 000 is expressed as the percent of the maximum radioactivity incorporated into the complex after a 45-min incubation. The inactivation of kallikrein is expressed as the percent of maximum kallikrein inactivation at 45 min.

lecular weight derivative of C1 inhibitor generated by kallikrein failed to complex with kallikrein and was unable to inactivate kallikrein activity. Further studies showed that kallikrein inactivated by diisopropyl phosphofluoridate prior to its reaction with C1 inhibitor was unable to generate the M_r 185 000 band and the inactive M_r 94 000 C1 inhibitor derivative, indicating that the active site of kallikrein is necessary for these reactions to occur.

Analysis of the same samples under reducing conditions indicated the formation of a band with an approximate M_r = 135 000 which contained radioactivity as demonstrated by autoradiography. Parallel to the increase in the M_r 135 000 band, a decrease in the intensity of the light chains of kallikrein was apparent, whereas the density of the heavy chain remained unaltered during the incubation (Figure 4c). C1 inhibitor which appeared as a single protein band with a M_r = 94 000 under reducing conditions slowly decreased in intensity during time, but still after 45 min, a substantial amount of this band was present. These results suggest that the band with an apparent M_r = 130 000 represented a complex between C1 inhibitor and the light chain of kallikrein. They also indicate that no SDS-stable interactions are formed between C1 inhibitor and the heavy chain of kallikrein. On the autoradiogram of the reduced gel, an additional band of M_r 80 000 could be observed (Figure 4d). This band most likely represents trace amounts of prekallikrein, which was present in small amounts in the kallikrein preparation.

To assess the relation between the kinetics of kallikrein inactivation and the formation of the M_r 185 000 complex band, we cut the unreduced gel longitudinally and sliced it into sections and then counted the slices for radioactivity. The rate of formation of the kallikrein–C1 inhibitor complex band closely paralleled the rate of kallikrein inactivation (Figure 5), Which suggests that kallikrein is inactivated by C1 inhibitor due to formation of a complex that is stable in SDS.

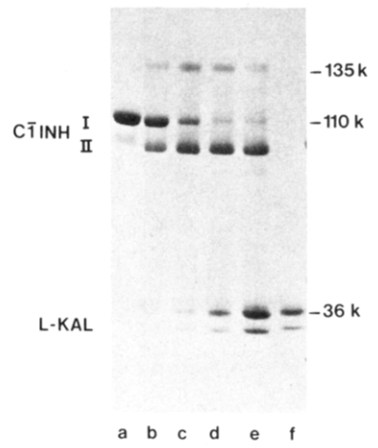

FIGURE 6: SDS 3–18% gradient PAA slab gel analysis of the reaction between the light chain of kallikrein and C1 inhibitor. C1 inhibitor (1.20 μM) was incubated with various concentrations of the light chain of kallikrein for 30 min at 37 °C and analyzed in the absence of reducing agents: (a) 0 μM, (b) 0.6 μM, (c) 1.2 μM, (d) 1.8 μM, (e) 3.6 μM light chain of kallikrein, and (f) 1.2 μM light chain of kallikrein a no C1 inhibitor present. C1 inhibitor bands I and II (C1 INH I and II) and the light chains of kallikrein (L-KAL) are indicated.

Studies on the Interaction between the Light Chain of Kallikrein and C1 Inhibitor. C1 inhibitor was incubated with different concentrations of the light chain of kallikrein for 30 min at 37 °C before the samples were subjected to SDS gradient PAA gel electrophoresis. Incubation of increasing molar ratios of the light chain of kallikrein (0.5, 1.0, 1.5, and 3) relative to C1 inhibitor produced a proportional increase in a band of M_r 135 000 and a band of M_r 94 000 which comigrated with the C1 inhibitor band II (Figure 6). In concert with this, a concomitant loss of C1 inhibitor band I was observed. The formation of the band with M_r = 135 000 indicated that C1 inhibitor formed a 1:1 molar complex with the light chain of kallikrein. When the light chain of kallikrein was present in a molar excess of 1.5 over C1 inhibitor, no band at the C1 inhibitor band I position was present, whereas a substantial amount of a band with M_r = 94 000 was apparent. Addition of more light chain of kallikrein to this mixture did not result in a decrease in the intensity of this band, and no decrease of kallikrein light chain amidolytic activity could be detected (data not shown). Thus, it can be inferred that in analogy with the interaction between kallikrein and C1 inhibitor, the light chain of kallikrein also produced an inactive C1 inhibitor form at M_r = 94 000. When the light chain of kallikrein was present in a molar ratio of 3 to 1 relative to C1 inhibitor, an additional minor band with M_r = 82 000 could be identified. This band was not observed when the same amount of the light chain of kallikrein was analyzed on the gel. Therefore, this band might be derived from the inactive M_r 94 000 C1 inhibitor band.

Discussion

Human plasma kallikrein contains heavy and light chains linked by disulfide bonds (Mandle & Kaplan, 1977; Bouma et al., 1980). For investigation of the functional roles of the heavy- and light-chain regions of human plasma kallikrein, kallikrein was reduced and alkylated, and the heavy and light chains were isolated (van der Graaf et al., 1982b). The isolated light chain of kallikrein contains the active site and exhibits full amidolytic activity against a synthetic substrate and full proteolytic activity against factor XII in solution. The heavy chain of kallikrein possesses the high-affinity binding site for high molecular weight kininogen and is required for potent

surface-dependent coagulant activity in plasma. In order to determine if the heavy chain of kallikrein plays a significant role in the inhibition of kallikrein, we studied the inactivation reactions between kallikrein or its light chain and the protease inhibitors, C$\bar{1}$ inhibitor and α_2-macroglobulin. In this report, the kinetics of inactivation of kallikrein and its light chain by C$\bar{1}$ inhibitor were investigated as well as the nature of the molecular complexes that form due to the inactivation reaction. Kinetic data indicate that the apparent pseudo-first-order rate constant for the reaction between kallikrein or its light chain increases linearly with the C$\bar{1}$ inhibitor concentration. Hence, it can be inferred that the inactivation reaction does not involve the formation of a reversible enzyme–inhibitor complex (Kitz & Wilson, 1962). The second-order rate constant for the inactivation of kallikrein by C$\bar{1}$ inhibitor is 2.7×10^6 M^{-1} min^{-1}, in agreement with the results of Schapira et al. (1981). The second-order rate constant for the inactivation of the light chain is 4.0×10^6 M^{-1} min^{-1}, which is slightly higher than that of kallikrein. This suggests that the heavy chain of kallikrein does not significantly affect the rate of kallikrein inactivation by C$\bar{1}$ inhibitor.

Since kallikrein possesses a high-affinity binding site for high molecular weight kininogen, it was of interest to study the effect of high molecular weight kininogen on the rate of inactivation of kallikrein by C$\bar{1}$ inhibitor. In contrast to previous reports (Schapira et al., 1981), we were unable to demonstrate any detectable effect of high molecular weight kininogen on the rate of inactivation of kallikrein by C$\bar{1}$ inhibitor (Figure 3). This complements the observation that the heavy chain of kallikrein, which provides the high-affinity binding site for high molecular weight kininogen (van der Graaf et al., 1982b), does not significantly affect the rate of kallikrein inactivation by C$\bar{1}$ inhibitor. Complex formation between kallikrein and high molecular weight kininogen in our studies was confirmed by using rocket immunoelectrophoresis as described by Kerbiriou et al. (1980). High molecular weight kininogen is also known to be a substrate for kallikrein (Nagasawa & Nakayasu, 1973; Habal et al., 1974). Competition between high molecular weight kininogen and C$\bar{1}$ inhibitor for the active site of kallikrein could influence the rate of kallikrein inactivation by C$\bar{1}$ inhibitor. Therefore, in our experiments, high molecular weight kininogen was preincubated with kallikrein before addition of C$\bar{1}$ inhibitor. Nonetheless, no influence of high molecular weight kininogen on the inactivation reaction was observed.

The nature of the reactions that occur between kallikrein or its light chain and C$\bar{1}$ inhibitor was studied by using SDS gradient PAA slab gel electrophoresis (Figure 4). Purified C$\bar{1}$ inhibitor demonstrated two bands on analysis by SDS gradient PAA slab gel electrophoresis, a major band designated band I with a M_r = 110000 and a minor band designated band II with a M_r = 94000. The concentration of band II relative to band I was approximately 10% or less. After reduction of C$\bar{1}$ inhibitor, only one band with a M_r = 94000 could be identified. Similar SDS band patterns of C$\bar{1}$ inhibitor on unreduced gels were observed by Harpel & Cooper (1975). However, in contrast to our results, they found that the apparent molecular weight of the C$\bar{1}$ inhibitor band I was not altered by reduction.

Kallikrein forms a complex with C$\bar{1}$ inhibitor which is not dissociated by SDS. The apparent M_r of 185000 reflects the sum of the molecular weights of both molecules, which is indicative of a stoichiometric complex. This finding is consistent with earlier reports (Gigli et al., 1970; Harpel et al., 1975; Schapira et al., 1981). Recently, we demonstrated that inactivation of kallikrein in plasma also results in the formation of a M_r 185000 complex with C$\bar{1}$ inhibitor (van der Graaf et al., 1983). Active kallikrein is necessary for complex formation because no complex was formed between C$\bar{1}$ inhibitor and kallikrein that was previously treated with diisopropyl phosphofluoridate. In association with the formation of the kallikrein–C$\bar{1}$ inhibitor complex, a second band, derived by kallikrein from C$\bar{1}$ inhibitor, was formed. This band is apparently generated from C$\bar{1}$ inhibitor band I by kallikrein and has a M_r = 94000. However, no putative fragment of approximate M_r 15000 associated with the production of this band could be observed on the slab gel. The absence of this fragment might be explained by its failure to be stained, or a small polypeptide containing a high amount of amino sugars might be released, resulting in a different apparent molecular weight. The C$\bar{1}$ inhibitor derivative band (M_r 94000) was not able to bind or inactivate kallikrein, since addition of kallikrein never resulted in a loss of intensity of this fragment or in a decrease of kallikrein amidolytic activity. The fact that the inactivation of kallikrein parallels the formation of the kallikrein–C$\bar{1}$ inhibitor complex (Figure 5) and the formation of the inactive C$\bar{1}$ inhibitor derivative suggests that these reactions are closely linked.

The results obtained after reduction of kallikrein–C$\bar{1}$ inhibitor mixtures indicated that the complex is formed through covalent interaction between the light chain of kallikrein and C$\bar{1}$ inhibitor. Conclusive evidence for this was provided by SDS–PAA gel electrophoretic analysis of mixtures of the isolated light chain of kallikrein and C$\bar{1}$ inhibitor. A complex of C$\bar{1}$ inhibitor–kallikrein light chain is seen on gels at M_r = 135000, which is the same molecular weight as that for the complex observed on the reduced gel of kallikrein–C$\bar{1}$ inhibitor mixtures. Interestingly, reduction of C$\bar{1}$ inhibitor alone and of the kallikrein–C$\bar{1}$ inhibitor mixtures results in indistinguishable mobilities on gels for the active C$\bar{1}$ inhibitor band I and for the inactive fragment derived from C$\bar{1}$ inhibitor. Possibly, reduction changes the apparent molecular weight of the active C$\bar{1}$ inhibitor band I to that of the nonreduced inactive C$\bar{1}$ inhibitor band, since no putative fragment with an approximate M_r of 15000 could be demonstrated on the gel.

Analogous studies of the interaction of plasmin with C$\bar{1}$ inhibitor were performed by Harpel & Cooper (1975). The interaction of plasmin with C$\bar{1}$ inhibitor shows some similarities and some differences when compared to kallikrein and C$\bar{1}$ inhibitor. In analogy to kallikrein, the light chain of plasmin containing the active site formed a stoichiometric complex with C$\bar{1}$ inhibitor. However, C$\bar{1}$ inhibitor band I as well as an active M_r 96000 derivative band of C$\bar{1}$ inhibitor formed a complex with plasmin. Plasmin also generated a M_r 96000 fragment from C$\bar{1}$ inhibitor which was inactive with regard to C$\bar{1}$s and plasmin binding. However, this fragment was formed only when plasmin was present in excess over C$\bar{1}$ inhibitor. The interaction between C$\bar{1}$s and C$\bar{1}$ inhibitor as studied by several investigators (Harpel & Cooper, 1975; Reboul et al., 1977) differs considerably from that between kallikrein or plasmin and C$\bar{1}$ inhibitor. Complex formation was shown between C$\bar{1}$s and C$\bar{1}$ inhibitor bands I and II. No inactive fragment was generated from C$\bar{1}$ inhibitor by C$\bar{1}$s.

The results of this study demonstrated that no SDS-stable interactions exist between the heavy chain of kallikrein and C$\bar{1}$ inhibitor. The mechanism of interaction between kallikrein or its light chain and C$\bar{1}$ inhibitor appeared to be identical. Inactivation of both forms of the enzyme by C$\bar{1}$ inhibitor leads to the formation of enzyme–C$\bar{1}$ inhibitor complexes that are stable in SDS, and both reactions can produce an inactive C$\bar{1}$

inhibitor derivative. Moreover, the rate constants for the inactivation of both kallikrein and its light chain by $C\bar{1}$ inhibitor are very similar. Hence, the heavy-chain region of kallikrein is not significantly involved in the inactivation of kallikrein by $C\bar{1}$ inhibitor, and no influence of high molecular weight kininogen on this reaction is evident.

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