Protection of Human Plasma Kallikrein from Inactivation by C1̄ Inhibitor and Other Protease Inhibitors. The Role of High Molecular Weight Kininogen†

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ABSTRACT: High M_r kininogen increases the activation rate of prekallikrein by activated factor XII on a surface. The resulting serine protease, plasma kallikrein, M_r 88 000, is inhibited in plasma by C1̄ inhibitor, M_r 105 000. Since prekallikrein circulates in plasma with high M_r kininogen as a complex and a kallikrein–high M_r kininogen complex can be formed in purified systems, we studied whether the inhibition of kallikrein by C1̄ inhibitor was influenced by high M_r kininogen. With C1̄ inhibitor in excess, the inactivation of kallikrein followed pseudo-first-order kinetics. The second-order rate constant for the reaction was 1.7×10^4 M^{-1} s^{-1}, and a kallikrein–C1̄ inhibitor complex, M_r 190 000, was identified on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Kallikrein and C1̄ inhibitor formed an irreversible complex without measurable prior equilibrium. The rate of this reaction was decreased by 50% in the presence of high M_r kininogen (1 unit/mL or 0.73 μM). Kinetic analysis indicated that this protection was the result of the formation of a reversible complex between kallikrein and high M_r kininogen, which had a dissociation constant of 0.75 μM. However, low M_r kininogen did not protect kallikrein from inactivation by C1̄ inhibitor. High M_r kininogen also protected kallikrein from inactivation by diisopropyl fluorophosphate. These findings suggest that the kallikrein–high M_r kininogen complex was formed by noncovalent interactions between the light chains of both kallikrein and high M_r kininogen.

Human plasma kallikrein is a serine protease that liberates bradykinin from high M_r kininogen (Thompson et al., 1978; Kerbiriou & Griffin, 1979). Plasma kallikrein also converts factor XII, plasminogen, and prorenin into their respective biologically active forms (Meier et al., 1977; Cochrane et al., 1973; Colman, 1969; Sealey et al., 1979). Kallikrein is inactivated in plasma by C1̄ inhibitor, an α_2-glycoprotein, which also inactivates plasmin, factor XIIa, factor XII fragments, C1̄s, and C1̄r (Ratnoff et al., 1969). The inactivation of kallikrein by C1̄ inhibitor follows second-order kinetics and results in the parallel loss of both kallikrein and C1̄-inhibitor activities (Gigli et al., 1970). Prekallikrein, the precursor of kallikrein, circulates in plasma associated with high M_r kininogen in a noncovalent bimolecular complex (Mandle et al., 1976). This complexed state influences the functional and immunological expression of prekallikrein (Scott & Colman, 1980; Silverberg et al., 1980). Plasma kallikrein also forms a complex with high M_r kininogen. This finding raised the possibility that this complexed state may protect kallikrein from its inhibitors (Scott & Colman, 1980).

This study investigates the interactions between plasma kallikrein, high M_r kininogen, and C1̄ inhibitor in the liquid phase. Kinetic evidence is presented that C1̄ inhibitor inactivates plasma kallikrein without formation of a detectable reversible complex. However, the formation of a reversible complex between kallikrein and high M_r kininogen decreases the rate of inactivation of kallikrein by C1̄ inhibitor and other irreversible inhibitors of kallikrein.

Materials and Methods

Materials. DEAE-cellulose (DE52) (Whatman Ltd., Maidstone, Kent, England), Sephadex G-100 and QAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ), antiserum to C1̄ inhibitor, α_1-antitrypsin, antithrombin III, α_2-macroglobulin, and ceruloplasmin (Behring Diagnostics, Somerville, NJ), urokinase (Abbott, Chicago, IL), H-D-Pro-Phe-Arg-*p*-nitroanilide (S-2302) and H-D-Val-Leu-Lys-*p*-nitroanilide (S-2251) (Ortho Diagnostics Inc., Raritan, NJ), normal pooled plasma (George King Bio-Medical Inc., Overland Park, KS), and dextran sulfate, ellagic acid, soybean trypsin inhibitor, human serum albumin, and diisopropyl fluorophosphate (DFP)[1] (Sigma Chemical Co., St. Louis, MO) were purchased from the designated supplier.

Fresh frozen plasma for protein purification was generously supplied by the American Red Cross, Philadelphia, PA. Kininogen-deficient plasma was donated by M. Williams (Colman et al., 1975).

Purification of Plasma Proteins. Prekallikrein was prepared by the method of Scott et al., (1979) and was a single band of M_r 88 000 on $NaDodSO_4$ gel electrophoresis (Weber & Osborn, 1969). Kallikrein was prepared by activation of prekallikrein with factor XII fragments as described (Scott et al., 1979). The specific activity was 14.5 μmol min^{-1} mg^{-1} with H-D-Pro-Phe-Arg-*p*-nitroanilide as substrate, and the preparation showed two chains of M_r 55 000 and 33 000 on reduced $NaDodSO_4$ gel electrophoresis.

In some experiments, kallikrein was derived from prekallikrein containing IgG as the only contaminant (Scott et al., 1979). No differences were noted between the two preparations regarding kinetic analysis.

Factor XII fragments were prepared by activation of 200 mL of acid-treated plasma (Colman et al., 1969) at 25 °C with 50 mg of dextran sulfate at 25 °C for 45 min. The activated plasma was then chromatographed on a 4 × 60 cm column containing QAE Sephadex A-50 in 10 mM sodium phosphate buffer, pH 8.0, containing 0.25 M NaCl. After being washed with the starting buffer, the factor XII fragments were eluted with a gradient of 2000 mL to a limit of 0.6 M NaCl in the same buffer. The protein was concentrated in an Amicon ultrafiltration unit by using a PM10 membrane and then gel

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[1] Abbreviations used: DFP, diisopropyl fluorophosphate; $NaDodSO_4$, sodium dodecyl sulfate.

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filtered on a 2.5 × 90 cm column of Sephadex G-100. The activity was determined by its ability to activate plasma prekallikrein. On reduced NaDodSO$_4$ gel electrophoresis, a single broad band of M_r 28 000 was found.

C1̄ inhibitor was prepared from a partially purified preparation (Wickerhauser et al., 1978) kindly provided by Dr. M. Wickerhauser (American Red Cross Blood Research Laboratory, Bethesda, MD). The procedure involved cryoprecipitation, kaolin precipitation, poly(ethylene glycol) precipitation, DEAE-Sephadex batch chromatography, CM-Sephadex batch chromatography, and hydroxylapatite chromatography (Gadek et al., 1980). The resultant preparation was 97% active as determined by the method of Levy & Lepow (1959), compared to its antigenic reactivity, assessed by electroimmunodiffusion (Laurell, 1966). C1̄ inhibitor was further purified by ion-exchange chromatography on DEAE-cellulose, as described by Reboul et al. (1977), using counterimmunoelectrophoresis (Howe et al., 1967) to monitor the presence of the protein. No contamination by α_1-antitrypsin, α_2-macroglobulin, antithrombin III, or ceruloplasmin was found by using double immunodiffusion (Ouchterlony & Nilsson, 1978). C1̄ inhibitor was quantitated by radial immunodiffusion (Mancini et al., 1964; Rosen et al., 1965) and by absorbance at 280 nm by using $A^{1\%}_{1cm}$ = 4.5 (Harpel, 1976).

High M_r kininogen was prepared according to Kerbiriou & Griffin (1979) and was a single band of M_r 110 000 on NaDodSO$_4$ gel electrophoresis. High M_r kininogen was assayed by a modification of the partial thromboplastin time (Colman et al., 1975) by using kininogen-deficient plasma. One unit was defined as that amount which is present in 1 mL of normal pooled plasma. The specific activity was 12.5 units/mg. One unit per milliliter corresponded to a 0.73 μM solution of the purified high M_r kininogen. Low M_r kininogen was prepared according to Colman et al. (1975).

Plasminogen (a gift of Dr. S. Olexa, this institution) was purified according to Deutsch & Mertz (1970); it was activated to plasmin by urokinase at a ratio of 100 units of urokinase/mg of plasminogen. The specific activity of plasmin was 18 CTA units/mg.

Kinetic Studies. Kallikrein was incubated with inhibitors and other proteins in freshly silicone-coated glass vessels. Kallikrein activity was quantitated by its amidolytic activity on the chromogenic substrate H-D-Pro-Phe-Arg-*p*-nitroanilide (Kluft, 1978). A 1 mM solution of the substrate was prepared in 0.1 M sodium phosphate buffer, pH 7.6, containing 0.15 M NaCl. Ten microliters of the solution to be tested was added to 330 μL of substrate at 37 °C, and the absorbance change at 405 nm was continuously recorded by using a Cary 210 double-beam spectrophotometer (Varian, Palo Alto, CA). Under these conditions, the hydrolysis rate of the substrate by a 1:20 dilution of normal plasma activated by ellagic acid (final concentration 10 μM) was 1.36 nmol/min. H-D-Val-Leu-Lys-*p*-nitroanilide, at a final concentration of 1 mM and in the above mentioned buffer, was used as a substrate for plasmin (Goldsmith et al., 1978).

Results

Formation of a Complex between Kallikrein and C1̄ Inhibitor. C1̄ inhibitor was a single component on NaDodSO$_4$ gel electrophoresis (M_r 105 000) as shown in Figure 1A. When kallikrein (final concentration 2.3 μM) was incubated with an excess of C1̄ inhibitor (final concentration 4.5 μM) for 1 h at 37 °C, a decrease in the C1̄ inhibitor band was noted (Figure 1B), with the formation of a new high M_r band (M_r 190 000). This coincided with a 1:1 stoichiometric complex between C1̄ inhibitor (M_r 105 000) and kallikrein (M_r 88 000).

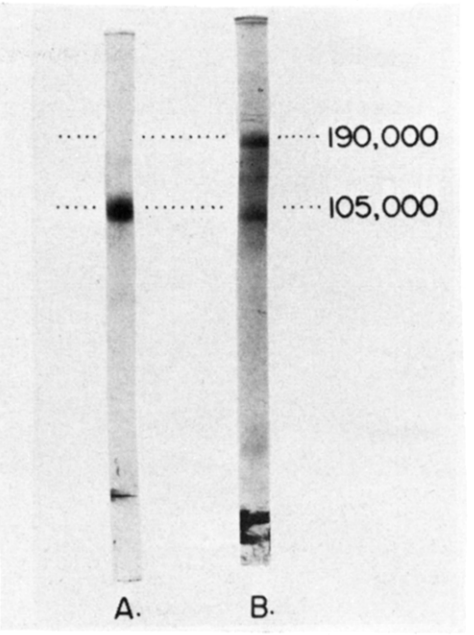

FIGURE 1: Formation of C1̄ inhibitor–kallikrein complex. When NaDodSO$_4$–polyacrylamide gels were used, gel A contained 15 μg of purified C1̄ inhibitor and gel B contained 23 μg of the protein mixture generated after a 1-h incubation at 37 °C of 15 μg of purified C1̄ inhibitor and 8 μg of purified kallikrein. The exact procedure was that of Weber & Osborn (1969) without reduction.

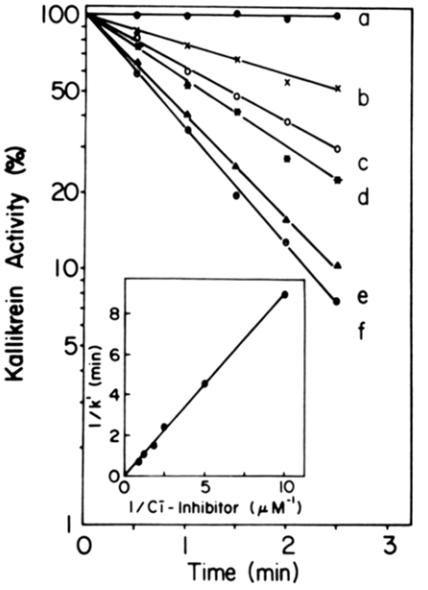

FIGURE 2: Kinetics of inactivation of kallikrein amidolytic activity by C1̄ inhibitor. Kallikrein (final concentration 50 nM) was incubated with various concentrations of C1̄ inhibitor and then assayed at various times for residual amidolytic activity. C1̄ inhibitor final concentration: (a) 0, (b) 0.1 μM, (c) 0.2 μM, (d) 0.4 μM, (e) 0.6 μM, and (f) 0.8 μM. The insert shows a double-reciprocal plot of the pseudo-first-order rate constant and the concentration of C1̄ inhibitor. The line drawn in a least-squares fit of the experimental points (r = 0.99). The equation of the line is $y = 0.96x - 0.04$.

Two minor bands, which have intermediate mobility between the inhibitor and the enzyme–inhibitor complex, were also seen.

Inactivation of Kallikrein by C1̄ Inhibitor. The kinetics of inactivation of kallikrein amidolytic activity by various concentrations of C1̄ inhibitor are shown in Figure 2. The inactivation of kallikrein followed pseudo-first-order kinetics when C1̄ inhibitor was in a 10- to 100-fold molar excess. The second-order rate constant, k'', for the inactivation of kallikrein by C1̄ inhibitor was calculated and has a value of 1.7×10^4 M^{-1} s^{-1}.

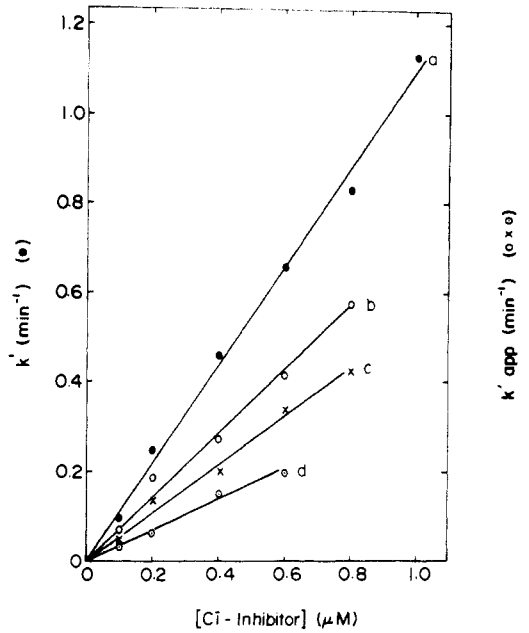

FIGURE 3: Effect of the Cī inhibitor concentration on the Cī inhibitor–kallikrein reaction rate in the absence and presence of high M_r kininogen. Kallikrein (final concentration 50 nM) was incubated with Cī inhibitor in the presence of high M_r kininogen at final concentrations: (a) 0, (b) 0.36 μM, (c) 0.73 μM, and (d) 1.46 μM. Each line drawn is a least-squares fit of the experimental points ($r > 0.97$).

The general mechanism of enzyme inactivation by an irreversible inhibitor is

$$E + I \rightleftharpoons EI \rightarrow EI^* \quad (1)$$

where the enzyme is E, the inhibitor is I, the dissociable complex is EI, and the stabilized complex is EI*. A double-reciprocal plot of the pseudo-first-order rate constant k' vs. the Cī inhibitor concentration was linear (Figure 2, insert), and regression analysis indicated that the line went almost through the origin. This observation indicates that the inactivation of kallikrein by Cī inhibitor does not follow Michaelis–Menten kinetics, and, therefore, the inactivation of kallikrein by Cī inhibitor occurs without formation of a detectable reversible enzyme–inactivator complex. This result indicates, therefore, that the general mechanism of enzyme inactivation can be simplified to

$$E + I \xrightarrow{k''} EI^* \quad (2)$$

when kallikrein is inactivated by Cī inhibitor.

Influence of High M_r Kininogen on the Inactivation of Kallikrein by Cī Inhibitor. The influence of high M_r kininogen on the rate constant for the inactivation of kallikrein by Cī inhibitor is shown in Figure 3. The inactivation rates in the presence of high M_r kininogen, at various inhibitor concentrations, were reduced as compared to the rates observed when kallikrein was inactivated by Cī inhibitor in the absence of high M_r kininogen. For example, the apparent inactivation rate of kallikrein k'_{app} in the presence of high M_r kininogen (0.73 μM) (Figure 3c) was diminished to half of the inactivation rate, k', observed in the absence of high M_r kininogen (Figure 3a). Assuming the model of eq 2 and 3 for the interrelationship between kallikrein (E), Cī inhibitor (I), and high M_r kininogen (C) (where k'' is the second-order rate constant for the reaction between E and I, and k_{+1}/k_{-1} is the dissociation constant K_d of the EC complex), the inactivation of kallikrein by Cī inhibitor (Figure 4a) is described by

$$E + I \xrightarrow{k''} EI^* \quad (2)$$

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \quad (3)$$

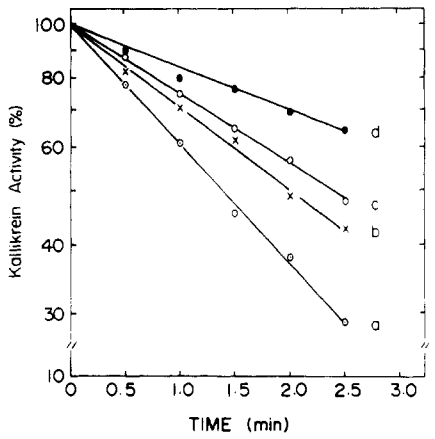

FIGURE 4: Kinetics of inactivation of kallikrein amidolytic activity by DFP in the absence and presence of high M_r kininogen. Kallikrein (final concentration 0.1 μM) was incubated with DFP (final concentration 1.25 mM) and then assayed at various times for residual amidolytic activity. High M_r kininogen final concentration: (a) 0, (b) 0.36 μM, (c) 0.73 μM, and (d) 1.10 μM.

$$k' = k''[I] \quad (4)$$

where k' is the pseudo-first-order reaction rate constant. When high M_r kininogen is present during the reaction (Figure 4b–d), the inactivation of kallikrein by Cī inhibitor is modified to

$$k'_{app} = k'/[1 + ([C]/K_d)] \quad (5)$$

where k'_{app} is the apparent pseudo-first-order reaction rate constant. By use of eq 5, a dissociation constant K_d was calculated for the kallikrein–high M_r kininogen complex and found to be 0.75 μM ± 0.04 (mean ± SD).

Influence of High M_r Kininogen on the Inactivation of Kallikrein by Soybean Trypsin Inhibitor or DFP. For assessment of whether the complex formation between kallikrein and high M_r kininogen could protect kallikrein from its inactivation by other protease inhibitors, additional experiments were performed by using soybean trypsin inhibitor or DFP as kallikrein inactivators. When kallikrein (final concentration 45 nM) was incubated with soybean trypsin inhibitor (final concentration 1.7 μM), the residual amidolytic activity at 1 min was 55 ± 1.6% (mean ± SD) in the absence of high M_r kininogen as compared to 68 ± 1.6% in the presence of high M_r kininogen (0.73 μM). This difference was significant ($p < 0.02$) by paired Student's t test.

The second-order rate constant for the inactivation of kallikrein by DFP was calculated to be 6.7 M^{-1} s^{-1}. High M_r kininogen was also found to protect kallikrein from inactivation by DFP (Figure 4). For example, the presence of high M_r kininogen (1.10 μM) decreased the inactivation rate of kallikrein to 34% of the rate observed in the absence of high M_r kininogen. Furthermore, a K_d of 0.82 ± 0.19 (mean ± SD) was calculated for the kallikrein–high M_r kininogen complex, using the data reported in Figure 4 and eq 5. Thus, the formation of a noncovalent molecular complex between kallikrein and high M_r kininogen provides protection against inactivation of kallikrein by soybean trypsin inhibitor and DFP.

Influence of Human Serum Albumin or Low M_r Kininogen on the Inactivation of Kallikrein by Cī Inhibitor. For determination of whether the protection of kallikrein by high M_r kininogen against inactivation by Cī inhibitor was a specific property of high M_r kininogen, experiments were performed substituting human serum albumin or low M_r kininogen for

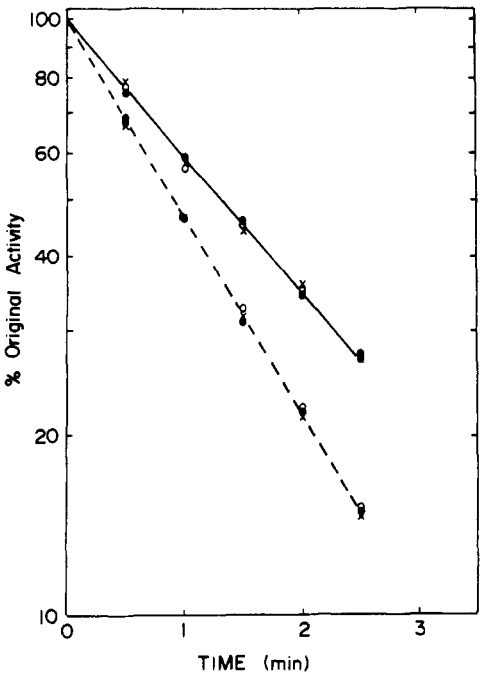

FIGURE 5: Kinetics of inactivation of kallikrein amidolytic activity by C1 inhibitor in the absence and presence of low M_r kininogen. Kallikrein (final concentration 50 nM) was incubated with various concentrations of C1 inhibitor and then assayed at various times for residual amidolytic activity. C1 inhibitor final concentration: (—) 0.4 μM and (---) 0.6 μM. Low M_r kininogen final concentration: (○) 0, (●) 1 μM, (×) 40 μM.

high M_r kininogen. Kallikrein (final concentration 23 nM) was incubated with C1 inhibitor (final concentration 0.4 μM) in the presence and in the absence of human serum albumin (final concentration 7.5 μM). The inactivation rate of kallikrein by C1 inhibitor in the presence of human serum albumin ($k' = 0.55$ min^{-1}) was similar to that in the absence of human serum albumin ($k' = 0.58$ min^{-1}).

When high M_r kininogen was substituted by low M_r kininogen, no protection of the kallikrein inactivation by C1 inhibitor was observed (Figure 5), even when the concentration of low M_r kininogen was 40 μM. These results indicate that the protection of kallikrein from inactivation by C1 inhibitor is a specific property of high M_r kininogen.

Influence of High M_r Kininogen on the Inactivation of Plasmin by C1 Inhibitor. To exclude the possibility that high M_r kininogen was inhibiting the action of C1 inhibitor and thereby preventing its action on kallikrein, we assessed the influence of high M_r kininogen on the inactivation of plasmin by C1 inhibitor. The second-order rate constant for the inactivation of plasmin by C1 inhibitor was calculated to be 3.6×10^4 M^{-1} s^{-1}. The inactivation rate of plasmin, at three different concentrations of C1 inhibitor, was unaffected by the presence of high M_r kininogen (0.73 μM) (Figure 6). These results demonstrate that high M_r kininogen does not inhibit the function of C1 inhibitor.

Discussion

High M_r kininogen is known to be a coagulation cofactor (Colman et al., 1975; Saito et al., 1975; Wuepper et al., 1975) as well as a substrate for plasma and tissue kallikreins (Jacobsen, 1966; Pierce & Guimaraes, 1976). High M_r kininogen, bound to a negatively charged surface, increases the rate of the reciprocal activation of factor XII by kallikrein and of prekallikrein by factor XII_a (Griffin & Cochrane, 1976; Meier et al., 1977) as well as the activation rate of factor XI by factor XII_a (Griffin & Cochrane, 1976; Saito, 1977; Wiggins et al., 1977). Moreover, high M_r kininogen forms bimolecular complexes with prekallikrein (Mandle et al., 1976) and factor XI (Thompson et al., 1977) when these factor XII substrates bind to a site which is located on the light chain derived from kinin-free high M_r kininogen (Thompson et al., 1979). We have also demonstrated that high M_r kininogen forms a molecular complex with plasma kallikrein (Scott & Colman, 1980), a finding which has been confirmed by others (Kerbiriou et al., 1980). The results of this study lead to further insight into the function of high M_r kininogen since they demonstrate that this cofactor protects plasma kallikrein from inactivation by C1 inhibitor as well as by soybean trypsin inhibitor and DFP.

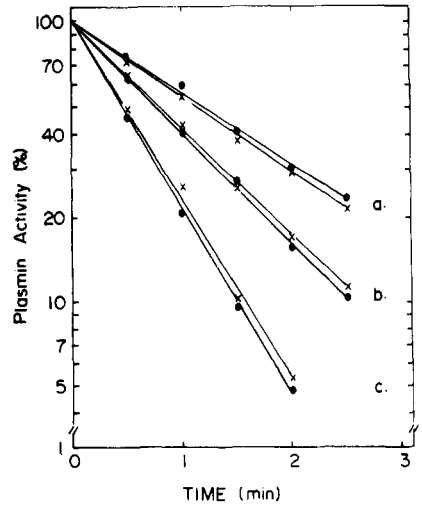

FIGURE 6: Kinetics of inactivation of plasmin amidolytic activity by C1 inhibitor in the absence and presence of high M_r kininogen. Plasmin (final concentration 0.1 μM) was incubated with various concentrations of C1 inhibitor in the absence (○) or presence (×) of high M_r kininogen (final concentration 0.73 μM) and then assayed at various times for residual amidolytic activity. C1 inhibitor final concentration: (a) 0.2 μM, (b) 0.4 μM, and (c) 0.8 μM.

Plasma kallikrein may be inactivated by several proteinase inhibitors such as α_2-macroglobulin (Harpel, 1970), antithrombin III (Lahiri et al., 1976), or α_1-antitrypsin (Fritz et al., 1972). However, the principal inhibitor of kallikrein in plasma was found to be C1 inhibitor. This was concluded from comparison of the different inhibition profiles of kallikrein obtained in normal human plasma and C1-inhibitor-deficient plasma (Gigli et al., 1970; McConnell, 1972). Our results confirm that C1 inhibitor interacts with kallikrein to form an inactive stoichiometric complex (Gigli et al., 1970; Harpel et al., 1975), exhibiting a M_r of 190000 upon $NaDodSO_4$ gel electrophoresis (Figure 1). As in the parallel case of the reaction between C1 inhibitor and plasmin (Harpel & Cooper, 1975), minor protein bands larger than C1 inhibitor but smaller than the C1 inhibitor–enzyme complex were visualized. By analogy, these may represent proteolysis of the complex by kallikrein.

The reaction between kallikrein and C1 inhibitor is not distinguishable from a simple bimolecular mechanism since C1 inhibitor inactivates kallikrein without the formation of a detectable reversible enzyme–inhibitor complex (Figure 2). This mechanism contrasts with the usual mechanism of enzyme inactivation, whereby Michaelis–Menten kinetics are observed (Hartley, 1960; Laskowski & Sealock, 1971). However, a similar mechanism of enzyme inactivation has been described wheh methanesulfonyl fluoride was used as an acetylcholinesterase inactivator (Kitz & Wilson, 1962).

The inactivation rate of kallikrein by C1 inhibitor was decreased by the presence of high M_r kininogen. Kinetic analysis

(Figure 3) suggested that high M_r kininogen decreased the rate of inactivation of kallikrein by forming a reversible complex with this enzyme. This conclusion was supported by experiments showing that high M_r kininogen also decreased the rate of inactivation of kallikrein by other inhibitors, such as soybean trypsin inhibitor or DFP (Figure 4). Moreover, the fact that high M_r kininogen protected kallikrein from inactivation by a small molecule such as DFP (M_r 184) indicates that the binding site for high M_r kininogen on kallikrein must be very closely linked to the DFP binding site. Alternatively, formation of the complex may alter the conformation of kallikrein in such a way that the catalytic site becomes unavailable for DFP. It is therefore likely that the binding site for high M_r kininogen is located on the light chain of the kallikrein molecule, as is the DFP site (Mandle & Kaplan, 1977).

When human serum albumin or low M_r kininogen was substituted for high M_r kininogen, no protection of the inactivation of kallikrein by C$\bar{1}$ inhibitor was noticed (Figure 5). This finding further indicates that the formation of a complex between kallikrein and high M_r kininogen is a prerequisite for the protection of kallikrein inactivation. The failure of low M_r kininogen to protect kallikrein from inactivation suggests that the protection observed with high M_r kininogen is mediated by the binding of kallikrein to the light chain of high M_r kininogen. This light chain is not part of low M_r kininogen, in contrast to the heavy chain of high M_r kininogen, which shares functional activity and antigenic determinants with low M_r kininogen (Pierce & Guimaraes, 1976; Thompson et al., 1978). Moreover, immunochemical studies indicate that the light chain of high M_r kininogen binds to kallikrein (Kerbiriou et al., 1980).

Both high and low M_r kininogens are substrates for kallikrein and yield bradykinin upon proteolysis. High M_r kininogen is, however, a better substrate for plasma kallikrein than low M_r kininogen (Habal et al., 1974). The K_M value for the cleavage of high M_r kininogen is 30-fold less than the K_M value for the cleavage of low M_r kininogen (Pierce & Guimaraes, 1976). However, low M_r kininogen failed to protect kallikrein from inactivation, even when the concentration of low M_r kininogen was 100 times greater than a concentration of high M_r kininogen known to provide protection (Figure 5). These experiments, therefore, argue against substrate protection as a likely mechanism for the protection of kallikrein inactivation by high M_r kininogen.

Finally, the influence of high M_r kininogen upon the function of C$\bar{1}$ inhibitor was tested. The inactivation of plasmin by C$\bar{1}$ inhibitor proceeded at a similar rate whether or not the cofactor was present (Figure 6), indicating that high M_r kininogen did not alter the function of C$\bar{1}$ inhibitor. Thus, our results provide good evidence that high M_r kininogen protects kallikrein from its inactivation by inhibitors, including C$\bar{1}$ inhibitor, the major inhibitor of kallikrein in plasma. This protection is dependent upon the formation of a reversible complex between the enzyme and the cofactor. The dissociation constant of this complex was found to be 0.75 μM. This value differs from the dissociation constant for the prekallikrein–high M_r kininogen complex which has been reported to be 0.029 μM (Thompson et al., 1979). This difference may indicate that the zymogen prekallikrein has a greater affinity for the substrate-cofactor high M_r kininogen than the enzyme kallikrein. Alternatively, it may reflect differences in experimental techniques since our results were obtained from kinetic analysis of liquid phase reactions, while Thompson et al. (1979) determined the binding of prekallikrein to high M_r kininogen which was adsorbed to a surface. Since the dissociation constant for the kallikrein–high M_r kininogen complex is almost identical with the concentration of high M_r kininogen in plasma, this contact phase cofactor may be important for regulating the action of kallikrein in vivo.

Acknowledgments

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Medium Effects in Enzyme-Catalyzed Decarboxylations†

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ABSTRACT: Carbon isotope effects and steady-state kinetic parameters have been measured for the decarboxylation of arginine and homoarginine by the pyridoxal 5′-phosphate dependent arginine decarboxylase from *Escherichia coli*. In water at pH 5.25, 5 °C, homoarginine shows an isotope effect $k^{12}/k^{13} = 1.061$, indicating that the decarboxylation step is entirely rate determining. In the presence of 16 mol % ethylene glycol under otherwise identical conditions, the decarboxylation rate is increased 3-fold, and the carbon isotope effect is 1.044, indicating that the rate of the decarboxylation step is increased by the presence of the less polar solvent. The decarboxylation of arginine under the same conditions shows a similar trend: in water, the isotope effect is 1.027, decreasing to 1.003 in 16% ethylene glycol, with little change in the steady-state rate. Again, the rate of the decarboxylation step is substantially increased by the presence of the nonpolar solvent. Thus, pyridoxal phosphate dependent enzymatic decarboxylations show a medium effect similar to that observed in a number of nonenzymatic decarboxylations. This suggests that these enzymes may accelerate the decarboxylation step by providing a nonpolar environment. Evidence is also presented that desolvation of the substrate carboxyl group may contribute to catalysis.

Most amino acid decarboxylases require pyridoxal 5′-phosphate (pyridoxal-P)[1] for activity. The decarboxylation mechanism (Scheme I) was suggested nearly simultaneously by Metzler et al. (1954) and by Westheimer (Mandeles et al., 1954). Subsequent studies of enzymes and models have confirmed most features of this mechanism (Boeker & Snell, 1972).

Considerable progress has been made in understanding the underlying mechanisms of catalysis of the various reaction steps. Initial substrate binding involves particularly the distal functional groups of the substrate (Boeker & Snell, 1972). In model reactions, the Schiff base interchange step is catalyzed by the 3-hydroxyl group of the coenzyme (Bruice & Benkovic, 1966), and similar catalysis probably also occurs in enzymatic decarboxylations. Catalysis of the decarboxylation step is still somewhat enigmatic (O'Leary, 1977). At a first level the coenzyme functions as an "electron sink", obviating the necessity for forming a highly unstable carbanion. This function has been demonstrated in numerous model studies with pyridoxal analogues (Bruice & Benkovic, 1966; Kalyankar & Snell, 1962). However, the rates of enzymatic decarboxylations exceed those of model reactions by many orders of magnitude, and other factors must also contribute to the catalysis. It is clear, following the suggestion of Dunathan (1966), that stereochemical factors also play an important role. Decarboxylases must control the conformation of the enzyme-bound pyridoxal-P–amino acid Schiff base in order to prevent the kinetically more favorable transamination. The

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[1] Abbreviation used: pyridoxal-P, pyridoxal 5′-phosphate.

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