Functional Sites of Bovine High Molecular Weight Kininogen as a Cofactor in Kaolin-Mediated Activation of Factor XII (Hageman Factor)†

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ABSTRACT: Bovine high molecular weight (HMW) kininogen is a single chain glycoprotein with a molecular weight of 76 000. It contains four domains including a heavy chain, a kinin moiety, fragment 1.2, and a light chain, which were designated from proteolytic fragmentation of HMW kininogen by plasma kallikrein. The heavy chain and light chain are linked by a single disulfide bond. In this paper, the accelerating effect of HMW kininogen and various portions of this molecule on the activation of factor XII and prekallikrein was determined in a system containing factor XII, plasma prekallikrein, and kaolin. In this reaction, the kallikrein that was generated was measured by a fluorometric assay using carbobenzoxyphenylalanylarginine-4-methylcoumaryl-7-amide as substrate. The generation of kallikrein activity under certain conditions was increased 180-fold by the addition of 1.3–5.2 pmol of HMW kininogen. This effect was not observed, however, in the presence of more than 10 pmol of HMW kininogen. Kinin-free protein and a large fragment consisting of fragment 1.2 and light chain also accelerated the reaction. The accelerating effect of HMW kininogen was inhibited by fragment 1.2. Kinin and fragment 1.2-free protein, heavy chain, light chain, or low molecular weight kininogen neither accelerated nor inhibited the kaolin-mediated activation of factor XII and prekallikrein. These results indicate that both fragment 1.2 and the light chain region in HMW kininogen are essential for the kaolin-mediated activation of factor XII and prekallikrein.

Factor XII (Hageman factor) is a glycoptorein present in plasma as a precursor of a serine protease called activated factor XII or factor XIIa. The latter protein activates factor XI or prekallikrein and triggers the intrinsic pathway of blood coagulation, kinin formation, and fibrinolysis (Davie & Fujikawa, 1975; Kaplan et al., 1976; Kaplan, 1978). It has been known for some time that factor XII is activated on contact with negatively charged surfaces such as kaolin and glass (Nossel, 1972) and that high molecular weight (HMW)[1] kininogen accelerates this reaction (Griffin & Cochrane, 1976; Meier et al., 1977; Liu et al., 1977; Wiggins et al., 1977; Saito, 1977). The mechanism of the role of HMW kininogen in the contact-mediated activation of factor XII, however, remains to be clarified.

HMW kininogen, a precursor protein of bradykinin, has been purified from bovine (Komiya et al., 1974a), human (Habal et al., 1974; Pierce & Guimaraes, 1974; Nakayasu & Nagasawa, 1979), and horse (Sugo et al., 1979a) plasmas. The chemical properties of bovine HMW kininogen have been extensively studied in our laboratory. Bovine HMW kininogen is a single chain glycoprotein with a molecular weight of 76 000. By the proteolytic fragmentation of HMW kininogen by plasma kallikrein, it has been found that HMW kininogen consists of four domains, including a heavy chain (MW 48 500), a kinin moiety (MW 1000), fragment 1.2 (MW 14 500), and light chain (MW 16 000) (Kato et al., 1976). The amino acid sequences of fragment 1.2 (histidine-rich peptide) and the light chain (Han et al., 1975, 1976, 1978a; Hashimoto et al., 1977; Kato et al., 1976, 1977a) have been reported. Studies on the amino acid sequence of the heavy chain portion are now in progress. Bovine plasma kallikrein liberates bradykinin and fragment 1.2 (histidine-rich peptide) and a carbohydrate-free fragment 1.2 from bovine HMW kininogen (Han et al., 1978 a,b). The carbohydrate structure of fragment 1.2 has been determined recently by Endo et al. (1977).

Waldmann et al. (1976) and Matheson et al. (1976) in collaboration with our laboratory have reported that bovine HMW kininogen corrects the abnormality of human kininogen deficient plasma, Fitzgerald trait, and Flaujeac trait plasmas. These studies have also revealed that kinin-free protein and a polypeptide consisting of fragment 1.2 and light chain corrected kininogen-deficient plasmas, while kinin and fragment 1.2 free protein and light chain did not show any activity (Scicli et al., 1979; Wuepper et al., 1978). On the other hand, Oh-ishi et al. (1977) have found that fragment 1.2 has a strong inhibitory activity on the contact-mediated activation of factor XII. These results suggest that a region containing fragment 1.2 and the light chain of HMW kininogen plays an important role in the activation of factor XII.

In the present paper we report the role of HMW kininogen and various derivatives of HMW kininogen in the kaolin-mediated activation of factor XII and prekallikrein using highly purified reagents. This has included the development of a fluorogenic peptide substrate for a sensitive and quantitative method to evaluate the activation of factor XII.

Experimental Procedures

Materials. Z-Phe-Arg-MCA was a product of the Protein Research Foundation (Minoh, Osaka, Japan). Diisopropyl phosphofluoridate (iPr_2PF) was purchased from Katayama Chemical Industries Co., Ltd. (Osaka, Japan). Kaolin (acid washed, American Standard) was a product of Fisher Scientific Company. Five kinds of Celite, Filter Cel (4 gal per ft^2 per h), Hyflo-Supercel (15 gal per ft^2 per h), Celite 503 (30 gal per ft^2 per h), Celite 535 (45 gal per ft^2 per h), and Celite 545

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[1] Abbreviations used: iPr_2PF, diisopropyl phosphofluoridate; HMW, high molecular weight; LMW, low molecular weight; MCA, 4-methylcoumaryl-7-amide; AMC, 7-amino-4-methylcoumarin; Z, carbobenzoxy; $NaDodSO_4$, sodium dodecyl sulfate.

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(65 gal per ft^2 per h), were purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). Glass powder (Jencons Ltd., No. 14 N 102/1, approximately 0.1 mm in diameter) was kindly provided by Professor M. Katori of Kitasato University (Kanagawa, Japan). Ellagic acid from chestnut bark was purchased from Sigma Chemical Company (St. Louis, MO) and a 10^{-3} M solution was prepared by the method of Kluft (1978). Bovine serum albumin was a product of Sigma Chemical Co.

Polybrene (hexadimethrine bromide) was a product of Aldrich Chemical Co., (Milwaukee, WI). Benzamidine hydrochloride was purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). Bovine HMW kininogen was prepared by a method previously described (Komiya et al., 1974a). Trace contaminants of prekallikrein in the HMW kininogen preparation were removed by chromatography on *p*-chlorobenzylamine-ε-aminocaproyl–Sepharose (Kato et al., 1979). Derivatives of HMW kininogen were prepared as follows: Kinin-free protein was isolated after the incubation of HMW kininogen with human urinary kallikrein (Han et al., 1978b). Kinin and fragment 1.2 free protein and fragment 1.2 were prepared by the incubation of HMW kininogen with bovine plasma kallikrein (Han et al., 1976). Heavy and light chains were obtained by the reduction and carboxymethylation of kinin and fragment 1.2 free protein. A polypeptide consisting of fragment 1.2 and light chain was obtained by the reduction and carboxymethylation of kinin-free protein (Han et al., 1978b). Low molecular weight (LMW) kininogen was prepared as reported previously (Yano et al., 1967). Prekallikrein was purified from bovine plasma by the method of Takahashi et al. (1972). Factor XIIa was isolated by the method of Fujikawa et al. (1977b). The specific activity of kallikrein prepared by the activation of prekallikrein with factor XIIa was calculated to be 0.77 μmol of AMC per min per mg of protein at 37 °C using Z-Phe-Arg-MCA as substrate.

Methods. Protein concentrations for kininogens, prekallikrein, and factor XII were calculated from the values of molecular weight and absorption at 280 nm using the following data: Molecular weight and $E_{280nm}^{1\%}$ were 76 000 and 7.4 for HMW kininogen (Komiya et al., 1974a), 48 000 and 6.7 for LMW kininogen (Kato et al., 1977b), 82 000 and 10.9 for prekallikrein (Heimark et al., 1978; Heimark & Davie, 1979), and 74 000 and 14.2 for factor XII (Fujikawa et al., 1977a). Protein concentrations for the various derivatives of HMW kininogen were determined by dry weight using the following values for molecular weight (Kato et al., 1976): kinin-free protein, 75 000; kinin and fragment 1.2-free protein, 60 500; fragment 1.2 light chain, 30 500; fragment 1.2, 14 500; light chain, 16 000; and heavy chain, 48 500. Factor XII composed of a single polypeptide chain was prepared by a modification of the method of Fujikawa et al. (1977a). Briefly, bovine plasma (5.2 L) was applied to a DEAE-Sephadex A-50 column (23 × 7 cm), which was equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.04 M NaCl, benzamidine (3 mM), and polybrene (0.5 mg/L). After we washed the solution with 18 L of the same buffer, gradient elution was performed with 9 L of the same buffer and 9 L of buffer containing 0.6 M NaCl. Factor XII was measured by the kaolin-activated partial thromboplastin time using factor XII deficient plasma. The deficient plasma was kindly supplied by Dr. E. W. Davie of the University of Washington (Seattle, WA) and Dr. T. Kamiya of Nagoya University School of Medicine (Nagoya, Japan). The fractions containing factor XII were collected and dialyzed overnight against distilled water. One-half of the dialysate (4 L) was applied to a DEAE-Sephadex column (9 × 26 cm), which had been previously equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing polybrene (0.5 mg/L), 3 mM benzamidine, and 0.04 M NaCl. After the column was washed with 5.5 L of the same buffer, gradient elution was performed with 5 L of the same buffer and 5 L of buffer containing 0.3 M NaCl. Fractions containing factor XII were pooled and dialyzed overnight against distilled water. The dialysate was applied to a *p*-chlorobenzylamine-ε-aminocaproyl–Sepharose column (6 × 12.5 cm) which had been previously equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl. Factor XII appeared in the breakthrough fraction. Further purification of factor XII was performed according to the method of Fujikawa et al. (1977a), using heparin–agarose, CM-Sephadex C-50, and benzamidine–Sepharose. Homogeneity of prekallikrein and factor XII was confirmed by $NaDodSO_4$–polyacrylamide gel electrophoresis. Solutions of prekallikrein, factor XII, and HMW kininogen were stored at −70 °C after the addition of iPr_2PF to a final concentration of 10^{-3} M. The effect of iPr_2PF in the solution on the activation of factor XII was examined before and after dialysis of the solution, and we found that iPr_2PF in the solution did not appreciably affect the activation of factor XII under the conditions described in this paper.

The contact-mediated activation of factor XII was measured by mixing factor XII with prekallikrein, HMW kininogen, and kaolin. Kallikrein generated was measured using Z-Phe-Arg-MCA as substrate as reported previously (Morita et al., 1977). In these experiments, plastic tubes or siliconized glassware was used. Siliconization was performed with Drifilm SC-87 (Pierce Chemicals, Rockford, IL). To 0.475 mL of 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and bovine serum albumin (0.1 mg/mL), 5 μL of factor XII (0.24 nmol/mL) was added. HMW kininogen solution (13.2 nmol/mL) was serially diluted with the above buffer, and each 5 μL was added to the above solution to give a final concentration of 0 to 13.2 pmol/mL. To the mixture of factor XII and HMW kininogen, 10 μL of 0.125% kaolin suspended in the buffer was added, and the suspension was incubated at 37 °C for 15 min. Prekallikrein (5 μL containing 3.6 nmol/mL) was then added, and the mixture was further preincubated for 15 min at 37 °C. Kallikrein generated was measured by adding 5 μL of 10 mM Z-Phe-Arg-MCA (dimethylformamide solution). The amounts of AMC liberated were estimated with excitation at 380 nm and emission at 460 nm, using a Hitachi fluorescence spectrophotometer, Model MPF-2A. The instrument was standardized so that a 10 μM solution of AMC in 0.1% dimethyl sulfoxide gave 1.0 relative fluorescence unit. The initial velocity of kallikrein activity to hydrolyze Z-Phe-Arg-MCA was measured in a 0.5-mL cuvette which is kept at 37 °C using a Hitachi recorder, Model 065. One milliunit of kallikrein activity was defined as nanomoles of AMC liberated per minute. The effects of the derivatives of HMW kininogen on the activation of factor XII and prekallikrein were examined by the same method as HMW kininogen.

Results

Activation of Factor XII and Prekallikrein with Kaolin, Celite, Glass, and Ellagic Acid. As shown in Table I, the amidase activity toward Z-Phe-Arg-MCA was generated when factor XII was mixed with HMW kininogen, kaolin, and prekallikrein. In the absence of HMW kininogen, kaolin, prekallikrein, or factor XII, little activity was generated. Since each of the three protein components employed in these studies was pretreated with 10^{-3} M iPr_2PF, it seems unlikely that these preparations were contaminated with kallikrein or activated

Table I: Generation of Kallikrein Activity after the Incubation of Factor XII with Prekallikrein, HMW Kininogen, and Kaolin[a]

	factor XII	HMW kininogen	kaolin	prekallikrein	Z-Phe-Arg-MCA hydrolyzing act. (mU)
(1)	+	+	+	+	0.42
(2)	+	−	+	+	0.003
(3)	+	+	−	+	0
(4)	+	+	+	−	0
(5)	−	+	+	+	0

[a] In 0.5 mL of 0.02 M Tris-HCl buffer, factor XII (1.2 pmol) was mixed with 2.6 pmol of HMW kininogen and 12.5 μg of kaolin. After 15 min at 37 °C, 17.9 pmol of prekallikrein was added, and the mixture was further incubated for 15 min at 37 °C. Kallikrein generated was measured by incubation with 0.1 mM Z-Phe-Arg-MCA for 15 min at 37 °C.

Table II: Relative Activity of Kallikrein Generated with Kaolin, Celites, Glass, and Ellagic Acid[a]

	concn (μg/0.5 mL of buffer)	rel activ. rates of factor XII and prekallikrein (%)
kaolin	6.25	100
	12.5	100
	25	95.8
	100	5.0
Filter Cel	25	16.9
Hyflo-Supercel	25–100	11.0
Celite-503	25–100	5.9
Celite-535	250	6.8
Celite-545	100	9.3
glass tube[b]		1.7
ellagic acid	2×10^{-9}–10^{-6} M	3.4
glass powder	100–250	1.5

[a] In 0.5 mL of buffer, 3.3 pmol of HMW kininogen and 1.2 pmol of factor XII were mixed with each reagent with the final concentration shown in the first column. After 15 min of incubation at 37 °C, 17.9 pmol of prekallikrein was incubated, and the mixture was further incubated for 15 min. Kallikrein generated was estimated by measuring the amounts of AMC after the incubation with 0.1 mM Z-Phe-Arg-MCA for 15 min at 37 °C. Relative activation rate of factor XII was expressed by taking kallikrein activity generated by 12.5 μg of kaolin as 100. [b] New and unwashed 12 × 75 mm borosilicate glass tube.

factor XII. These results show that the activation of factor XII and prekallikrein was induced by the interaction of these two proteins with HMW kininogen and kaolin.

Other substances can also activate factor XII, including Celite, glass, and ellagic acid (Nossel, 1972). Table II shows the relative activity of kallikrein generated with these reagents, setting the value for kaolin as 100. Various types of Celite, the glass surface of a test tube, glass powder, and ellagic acid were examined in these experiments. The relative rates, however, were very small compared with that of kaolin. The activation rates with kaolin were dependent on the amounts of kaolin. With 100 μg of kaolin in the reaction mixture, the activation was dramatically decreased. Higher activation rates were not observed with increasing concentrations of Celite and ellagic acid. These results show that kaolin is the most potent surface tested in the activation of factor XII and prekallikrein.

The optimum conditions for the activation reaction were then examined, using kaolin at a concentration of 12.5 μg/0.5 mL. Figure 1 shows the effect of time on kallikrein generation with increasing concentrations of factor XII. In these experiments, factor XII and HMW kininogen were preincubated with kaolin for 10 min and prekallikrein was then added. At time intervals, Z-Phe-Arg-MCA was added, and the initial velocity of kallikrein activity was measured. Figure 1 shows

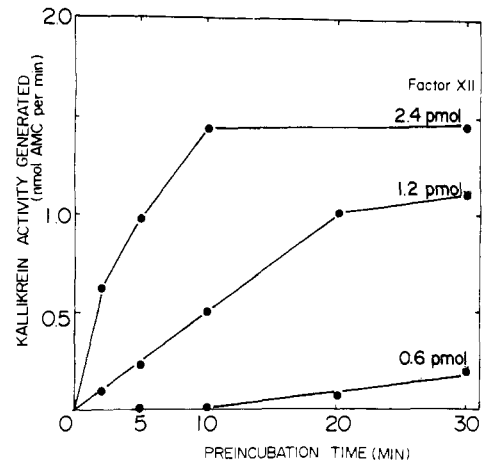

FIGURE 1: Activation of factor XII in the presence of prekallikrein. Factor XII was preincubated with HMW kininogen and kaolin for 10 min under the same conditions as described under Experimental Procedures. To the reaction mixture 5 μL of prekallikrein (3.6 nmol/mL) was added, and the kallikrein generated was measured after 0–30 min of incubation at 37 °C. Three different concentrations of factor XII were used. The values on the three curves show the amounts of factor XII employed.

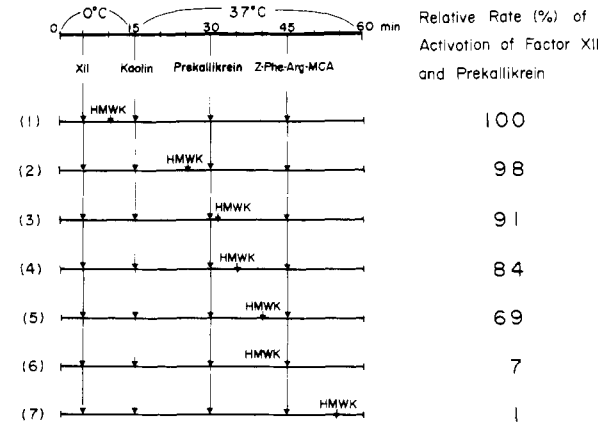

FIGURE 2: Effect of the order of addition of HMW kininogen. HMW kininogen was added at various times during incubation, fixing the order of the addition of other components. As a control, 5 μL of HMW kininogen solution (1.52 nmol/mL) was dissolved in 0.475 mL of buffer before the addition of 5 μL of factor XII solution (0.24 nmol/mL). After 5 min at 0 °C, 10 μL of kaolin (0.125%) was added to the mixture, and incubation was started at 37 °C. To the incubation mixture 5 μL of prekallikrein (3.6 nmol/mL) and 5 μL of 10 mM Z-Phe-Arg-MCA were added at the 15-min interval. The kallikrein generated was measured by the end point method. The additions of HMW kininogen were: (1) 5 min after the addition of factor XII; (2) 10 min after the addition of kaolin; (3) 15 s after the addition of prekallikrein; (4) 5 min after the addition of prekallikrein; (5) 10 min after the addition of prekallikrein; (6) 15 s before the addition of Z-Phe-Arg-MCA; (7) 10 min after the addition of Z-Phe-Arg-MCA.

that prekallikrein was completely converted to kallikrein within 10 min, when 2.4 pmol of factor XII was initially added. With 1.2 pmol of factor XII, kallikrein activity increased linearly up to 20 min. With 0.6 pmol of factor XII, the activation of prekallikrein was very slow.

In the above experiments, factor XII and HMW kininogen were preincubated with kaolin prior to the addition of prekallikrein. Since the contact-mediated activation of factor XII involves protein–protein interaction and the interaction with a surface, the order of the addition of each component will affect the surface activation rate of factor XII. In Figure 2, experiments are shown where HMW kininogen was added at various times during the incubation time, fixing the order of

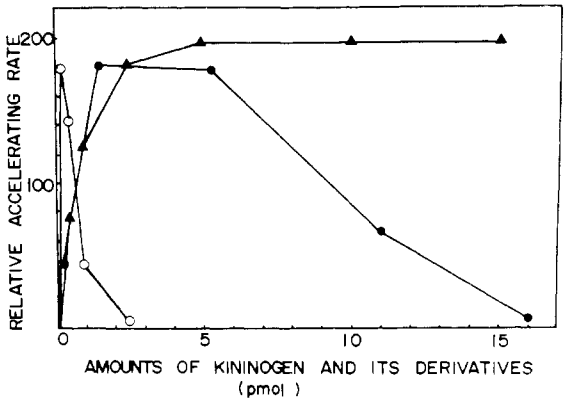

FIGURE 3: Accelerating effect of HMW kininogen and its derivatives on the kaolin-mediated activation of factor XII and prekallikrein. Factor XII was preincubated with kaolin and the various amounts of HMW kininogen and its derivatives for 15 min and with prekallikrein for 15 min under the same conditions as described in Figure 2. Relative accelerating rates of HMW kininogen and its derivatives were calculated by comparing with the amounts of kallikrein generated in the absence of HMW kininogen: (●) HMW kininogen; (○) kinin-free protein; (▲) fragment 1.2-light chain.

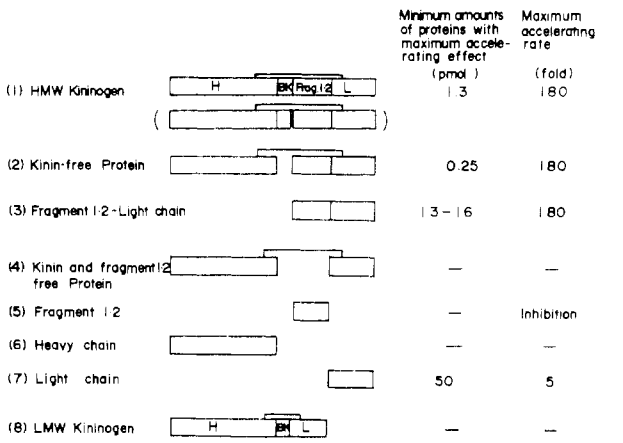

FIGURE 4: Summary of the relative accelerating rates of the activation of factor XII and prekallikrein with HMW kininogen and its derivatives. From the data shown in Figure 3 accelerating effects of each protein were expressed as the maximum accelerating rates, which were given by minimum amounts of proteins: BK, bradykinin moiety; Frag. 1·2, fragment 1.2; H, heavy chain; L, light chain.

the addition of other components.

The amount of kallikrein generated in the experimental conditions described in Figure 1 was taken to be 100. When HMW kininogen was added before or after the addition of kaolin, but before the addition of prekallikrein, the relative activation rate of factor XII and prekallikrein did not change appreciably. However, when HMW kininogen was added after the addition of prekallikrein, the activation rate of factor XII and prekallikrein decreased, and the decrease seems to be proportional to the lapse of the time after the addition of prekallikrein. These results suggest that HMW kininogen should be added prior to the addition of prekallikrein.

Effects of HMW Kininogen and Its Derivatives on the Kaolin-Mediated Activation of Factor XII and Prekallikrein. When the rate of the activation of factor XII and prekallikrein was measured as described in the previous section, kallikrein generation was dependent on the amount of HMW kininogen. Only minimal activity (0.0033 mU) was generated in the absence of HMW kininogen. With 1 to 5 pmol of HMW kininogen, maximal activity (0.6 mU) was obtained. In Figure 3, the accelerating effect was expressed as the relative activity of kallikrein generated in the presence of HMW kininogen, as compared to that in the absence of HMW kininogen. The maximum accelerating rate was calculated to be 182-fold with 1 to 5 pmol of HMW kininogen. The accelerating effect of HMW kininogen decreased with more than 5 pmol of kininogen and disappeared with 16 pmol of HMW kininogen.

The fragment of HMW kininogen consisting of fragment 1.2 and light chain showed the same accelerating effect as HMW kininogen. A high concentration of this fragment (up to 15 pmol) did not lead to inhibition. The kinin-free protein also showed the same stimulating effect, but the dose dependency was quite different from that of HMW kininogen. With 0.25 pmol of kinin-free protein, maximum kallikrein formation was observed, and the effect disappeared with more than 1 pmol. Kinin and fragment 1.2-free protein, fragment 1.2, heavy chain, and LMW kininogen did not show the accelerating effect on the activation of factor XII and prekallikrein. The light chain showed very weak accelerating activity. However, a mixture of fragment 1.2 and light chain did not show appreciable accelerating activity.

The ability of plasma kallikrein to hydrolyze Z-Phe-Arg-MCA was neither accelerated nor inhibited by HMW kininogen and its derivatives within the concentration range used in Figure 3. Since HMW kininogen is a natural substrate for plasma kallikrein, it can inhibit the amidase activity of plasma kallikrein. However, the molar concentrations of HMW kininogen (10^{-9}–10^{-8} M) employed in these experiments are far less than that of Z-Phe-Arg-MCA (10^{-4} M). Accordingly, the failure of HMW kininogen to inhibit the activity of plasma kallikrein can be explained by the difference in the molar concentration of two substrates.

Figure 4 summarizes the effects of HMW kininogen and its derivatives on the activation of factor XII and prekallikrein in the presence of kaolin. The accelerating effects of these proteins were compared by employing the level of these proteins which show the maximum accelerating effects. These data indicate that only the HMW kininogen derivatives containing both fragment 1.2 and the light chain accelerate the activation of factor XII and prekallikrein by kaolin. Among the four proteins which accelerated the reaction, kinin-free protein was the most efficient.

Inhibition of the Reaction by Derivatives of HMW Kininogen. In previous papers (Oh-ishi et al., 1977; Han et al., 1978a), we have reported that fragment 1.2 strongly inhibits kinin formation in bovine plasma exposed to ballotini glass and also prolongs the calcium clotting time of citrated rat plasma analogous to hexadimethrine bromide. It also inhibited the formation of activated factor XII in kaolin-treated human plasma. In the purified system containing factor XII, prekallikrein, and HMW kininogen, the activation of factor XII and prekallikrein with kaolin was also markedly inhibited by fragment 1.2 (Figure 5). Complete inhibition was observed with 20 pmol of fragment 1.2 under the conditions used. In contrast, kinin and fragment 1.2 free protein or heavy chain or light chain and LMW kininogen showed no effect on the kaolin-mediated activation reaction in the presence of HMW kininogen. These results clearly indicate that fragment 1.2 is strongly inhibitory, while kininogen derivatives without fragment 1.2 neither accelerate nor inhibit the kaolin-mediated activation reaction.

Discussion

It has been known that HMW kininogen is an essential cofactor for the kaolin-mediated activation of factor XII. It is of interest to define the functional sites of HMW kininogen, since the partial amino acid sequence of bovine HMW kini-

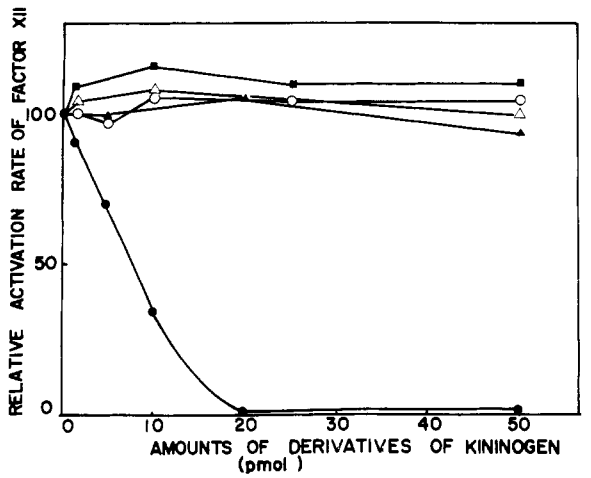

FIGURE 5: Inhibition of kaolin-mediated activation of factor XII and prekallikrein by HMW kininogen derivatives. Various amounts of derivatives were mixed with 10 μL of kaolin (0.125%) in 0.5 mL of buffer. After 2.5 min at 0 °C, 5 μL of HMW kininogen (1.52 nmol/mL) was added to the mixture. After further standing of the mixture for 2.5 min at 0 °C, 5 μL of factor XII (0.24 nmol/mL) was added, and the activation of factor XII was measured as described under Experimental Procedures. The inhibitory effect of derivatives on the activation of factor XII was expressed by taking the amounts of kallikrein generated in the absence of derivatives as 100: (●) fragment 1.2; (○) kinin and fragment 1.2-free protein; (■) heavy chain; (▲) light chain.

nogen has been determined. Before starting these investigations, we had to solve two important problems: the preparation of HMW kininogen free of prekallikrein and the development of the sensitive assay method to measure quantitatively factor XII activity. It has been quite difficult to remove a trace amount of prekallikrein in HMW kininogen (Komiya et al., 1974b). Recently, we found that the prekallikrein contaminating HMW kininogen preparations can be successfully removed by the application of *p*-chlorobenzylamine-ε-aminocaproyl–Sepharose (Kato et al., 1979). The activation of factor XII has been examined by measuring kallikrein generated from prekallikrein using N^{α}-tosyl-L-arginine methyl ester (Cochrane et al., 1973; Chan et al., 1976), Z-Phe-Val-Arg-*p*-nitroanilide (Meier et al., 1977), or Z-Pro-Phe-Arg-*p*-nitroanilide (Griffin, 1978) and by measuring activated factor XI using factor XI deficient plasma (Meier et al., 1977). We have previously developed the sensitive fluorogenic substrate for plasma kallikrein, Z-Phe-Arg-MCA (Morita et al., 1977). Using this substrate, we could follow quantitatively the activation of factor XII and prekallikrein in the presence of HMW kininogen, prekallikrein, and kaolin by measuring the initial velocity of kallikrein activity generated.

In this paper, we have presented the evidence that kallikrein was generated by mixing the precursor forms of serine proteases, factor XII, and prekallikrein in the presence of kaolin and HMW kininogen. No appreciable contamination of the active forms in the above components has been detected. What triggers the proteolytic process after the mixing of the zymogens with kaolin is still a controversial point. Negatively charged surfaces rendered factor XII much more susceptible to proteolytic activation by kallikrein or plasmin (Griffin, 1978). Griffin & Beretta (1979) reported that factor XII and prekallikrein incorporated iPr_2PF at a very slow rate in their zymogen forms. Consequently, based on these observations, either factor XII or prekallikrein might function as a weakly "active zymogen" (Griffin & Cochrane, 1979). However, Ratnoff & Saito (1979) reported that factor XII acquires the enzymatic activity upon contact with ellagic acid without proteolysis, although further experiments seem to be necessary to substantiate the results.

The activation of factor XII and prekallikrein was very much dependent on the concentrations of kaolin, factor XII, and HMW kininogen and the activation of factor XII, and prekallikrein was apparently accelerated 180-fold by HMW kininogen. Our assay system described here includes two reactions, the activation of factor XII and the activation of prekallikrein. Recently, we measured two reactions separately and found that HMW kininogen accelerated both of the reactions ~20-fold (Sugo et al., 1979b). Therefore, the effects of HMW kininogen examined in this paper may be the total effects on both reactions. Since this reaction includes a protein–protein interaction and a protein–surface interaction, not only the ratio of the amounts of each component but also the concentration of each component may strikingly affect the rate of the reaction. Therefore, it is probable that the accelerating effect of HMW kininogen on the activation of factor XII differs under different experimental conditions.

The activation of factor XII and prekallikrein was inhibited by the excess amounts of HMW kininogen. A similar inhibitory effect of excess HMW kininogen has been noted with human proteins (Griffin & Cochrane, 1976; Meier et al., 1977). The inhibitory effect may be due to the competitive binding of factor XII and HMW kininogen on the surface of kaolin.

The activation of factor XII and prekallikrein was accelerated not only by HMW kininogen, but also by kinin-free protein, which was derived from HMW kininogen by removing kinin by urinary kallikrein. The same accelerating effect as HMW kininogen was obtained by the kinin-free protein, with about 10 times less HMW kininogen. Thus, some derivatives of HMW kininogen such as kinin-free protein may be more favorable than HMW kininogen for the interaction with prekallikrein and kaolin. In relation to these results, it is quite interesting to note that a polypeptide consisting of fragment 1.2 and light chain has the same accelerating effect as HMW kininogen, while light chain alone has a very weak effect and fragment 1.2 has inhibitory activity. The accelerating effect of HMW kininogen was abolished only by fragment 1.2. These results strongly indicate that both fragment 1.2 and light chain regions are essential for the accelerating effect of HMW kininogen, which is consistent with the results obtained using kininogen-deficient plasmas (Scicli et al., 1979; Wuepper et al., 1978).

Since HMW kininogen is found to make a complex with prekallikrein (Mandle et al., 1976), it has been speculated that a trimolecular complex of factor XII, prekallikrein, and HMW kininogen will be formed on the surface of kaolin, which leads to the activation of factor XII. We have recently found that HMW kininogen binds on kaolin through the fragment 1.2 region (Kato et al., 1979; Ikari et al., 1978). The inhibitory activity of fragment 1.2 can be explained by its competitive binding with HMW kininogen on kaolin. Light chain accelerated very weakly but did not inhibit the activation of factor XII. The light chain seems to be essential for the accelerating effect, because fragment 1.2–light chain has the same accelerating effect as HMW kininogen. The role of the light chain remains to be established, but it may be essential for the interaction with prekallikrein (Ikari et al., 1978). HMW kininogen in the contact-mediated activation of factor XII is thought to concentrate prekallikrein on a foreign surface. Through the fragment 1.2–light chain region, HMW kininogen binds to kaolin and prekallikrein, so that factor XII interacts with prekallikrein on kaolin.

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