

## THE AUTOACTIVATION OF RABBIT HAGEMAN FACTOR\*

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The rapid activation of Hageman factor (HF)<sup>1</sup> which occurs when plasma comes into contact with a negatively charged surface, results from the interaction of HF with high molecular weight kininogen and prekallikrein (1). Under these conditions, rapid proteolytic cleavage of each of these molecules takes place as HF and prekallikrein are converted from zymogen to active enzyme (1-3).<sup>2</sup> However, the critical question of what provides the triggering event leading to reciprocal activation between surface-bound HF and prekallikrein has not been answered.

Early studies pointed to the possibility that HF might become activated by merely binding to the negatively charged surface and thereby undergoing some conformational change which exposed an active site (4-10). Ratnoff and Saito (11) have also recently suggested that Sephadex-Ellagic acid mixtures may activate HF without proteolytic modification. That conformational changes do occur in surface-bound HF was supported by finding increased hydrophobicity (12) and a change in the circular dichromatic spectrum (13) of HF upon binding to negatively charged substances. Other workers, however, have concluded that binding of HF to a surface per se, is not alone sufficient to activate the molecule but that limited cleavage is also required for HF activation. In plasmas which are deficient in prekallikrein or high molecular weight kininogen, the HF binds to added negatively charged particles at the same rate as it does in normal plasma, but is only slowly cleaved and activated (1). The uptake of [<sup>3</sup>H]diisopropylfluorophosphate (DFP) into zymogen HF occurs only slowly, and this slow rate of incorporation remains unchanged when the HF is surface bound (14, 15). Using purified components, bovine single-chain HF, unlike the two chain form, is not procoagulant (16) and the rapid activation of surface-bound HF is only achieved in the presence of prekallikrein and high MW kininogen (17-19). Thus, it appears to be the assembly of this complex of molecules on the surface (20) that leads to the normal rapid burst of activity of HF which is associated with activation of the contact system.

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<sup>1</sup> *Abbreviations used in this paper:* HF, Hageman factor; HF<sub>a</sub>, activated HF; α-HF<sub>a</sub>, the 82,000 dalton form of activated HF; SBTI, soybean trypsin inhibitor; DFP, diisopropylfluorophosphate; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

<sup>2</sup> Bouma, B. N., and J. H. Griffin. 1979. Human plasma prekallikrein. Purification, characterization and identification as a Factor XII-dependent plasminogen proactivator. Submitted for publication.

Therefore, although the reciprocal activation between HF and kallikrein accounts for the rapid activation of these enzymes, the initial triggering event which leads to conversion of zymogen to enzyme remains obscure. Because HF activation does proceed, albeit slowly, in prekallikrein-deficient plasma (1, 21), we have explored the possibility that zymogen HF might be capable of activating other HF molecules in the absence of prekallikrein.

### Materials and Methods

*Purification of Proteins from Rabbit Plasma.* HF and  $\alpha$ -HF<sub>a</sub> were purified from citrated rabbit plasma by combined affinity and ion-exchange chromatography as described elsewhere.<sup>3</sup> The preparations of purified HF and  $\alpha$ -HF<sub>a</sub> used for the experiments described are shown in Fig. 1. Factor XI was purified by ion-exchange chromatography from rabbit plasma as described elsewhere (22). Prekallikrein, kindly provided by Dr. Richard Ulevitch, was purified from rabbit plasma as described elsewhere (23). Both factor XI and prekallikrein were homogeneous single protein bands on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). High molecular weight kininogen was purified from rabbit plasma as previously reported for human high molecular weight kininogen (20).

*SDS-Polyacrylamide Gel Electrophoresis.* SDS-polyacrylamide slab gels were prepared according to Laemmli (24). Reactions were stopped by boiling in the presence of 2% SDS and 2.5%  $\beta$ -mercaptoethanol. The running gels (9% acrylamide) were fixed, dried in a slab gel drier (Hoefer Scientific Instruments, San Francisco, Calif.) and placed on Kodak X-omat film (Eastman-Kodak Co., Rochester, N. Y.) to localize the radioactive protein. The radioactivity on the gel was quantitated by cutting up the gel and counting the radioactivity in the regions indicated by the autoradiogram. These results were expressed as the percent change in the proportion of radioactivity present at native molecular weight. In all experiments, at least 80% of the radioactivity was present at the native molecular weight.

#### *Experimental Protocols*

**PROTEOLYTIC CLEAVAGE OF KAOLIN-BOUND DFP-TREATED HF IN THE PRESENCE AND ABSENCE OF DFP.** All reagents used were freshly prepared using water filtered by a Barnstead Nanopure system (Barnstead Co., Sybron Corp., Boston, Mass.). Kaolin (400  $\mu$ g) was washed three times in 1 ml of 0.1 M Tris, pH 7.4. HF (2.4  $\mu$ g plus <sup>125</sup>I-HF, 1  $\mu$ g, 0.7  $\mu$ Ci) was added to 10  $\mu$ l of 0.1 M Tris buffer, pH 7.4. 15  $\mu$ l of 50 mM DFP (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) in water (total vol 60  $\mu$ l) was added to this mixture. After 5 min at 22°C, the HF-DFP mixture was added to the washed kaolin and incubated for a further 5 min at 22°C. This mixture was then split into two halves. The kaolin in one-half was washed as previously described in 0.1 M Tris buffer containing 5 mM DFP and the second half was washed with 0.1 M Tris buffer alone. Each kaolin sample was washed four times in 1 ml of cold buffer and separated by centrifugation at 12,000 *g* for 1 min. After the last wash, the samples were suspended in 60  $\mu$ l of either 0.1 M Tris buffer pH 7.4 alone or 0.1 M Tris buffer pH 7.4 containing 5 mM DFP. Samples were placed in a water bath at 37°C and at the times shown in Fig. 4, aliquots were removed for analysis on SDS-polyacrylamide gels.

**EFFECT OF PROLONGED INCUBATION WITH DFP ON CLEAVAGE OF KAOLIN-BOUND HF.** DFP (50 mM in water) was added to HF (1.6  $\mu$ g plus 0.5  $\mu$ g <sup>125</sup>I-HF) in the presence of 1 M Tris pH 7.4 to a final concentration of 20 mM DFP in a plastic 1.5 ml capped tube. Samples were incubated for 48 and 6 h at 37°C and for 24, 4, and 0 h at 22°C. For the samples incubated more than 4 h, DFP was added to the incubation mixture twice every 24 h. The pH was maintained by addition of 1 M Tris buffer. For each HF sample an identically treated sample of 0.1 M acetate buffer pH 5.0 (the buffer against which the HF and <sup>125</sup>I-HF had previously been dialyzed) was incubated (hereafter called DFP control). A 20- $\mu$ l subsample of each HF and DFP-control mixture was removed and added to 180  $\mu$ l of Tris-buffered saline containing bovine serum

<sup>3</sup> Wiggins, R. C., and C. G. Cochrane. 1979. Rabbit Hageman factor. Activation of Factor XI and prekallikrein by  $\alpha$ -HF<sub>a</sub>. The role of the negatively charged surface. Submitted for publication.

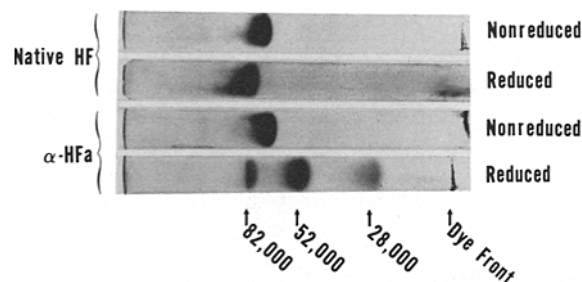


FIG. 1. SDS-polyacrylamide gel analysis of the preparation of purified single-chain HF and the two-chain 82,000 mol wt form of activated Hageman factor ( $\alpha$ -HF<sub>a</sub>) used for the studies described. Before electrophoresis, samples (30  $\mu$ g protein) were boiled with sodium dodecyl sulfate (2%). Reduction was effected with  $\beta$ -mercaptoethanol (2%).

albumin (BSA) (1 mg/ml). The HF procoagulant activity was measured using human HF-deficient plasma. The clotting times were compared to a standard curve that was constructed using dilutions of normal citrated rabbit plasma. An adjustment for residual DFP was made by measuring the prolongation of the clotting time caused by the DFP-control solution on a 1/10 dilution of normal rabbit plasma. The proportion of HF procoagulant activity present was calculated on the basis of non-DFP-treated HF having a value of 100%. That no significant (<15%) loss of HF occurred during the incubation procedure was confirmed by measuring the recovery of radioactivity. The DFP-treated HF mixture, from which a 10  $\mu$ l aliquot had previously been removed for analysis on SDS-polyacrylamide gels (control), was added to 200  $\mu$ g of kaolin. The kaolin was resuspended in the HF-containing solution for 5 min at 22°C. The kaolin was then washed four times with 0.1 M Tris buffer, by centrifugation at 12,000 *g*, to remove the DFP and non-bound HF (~30% of <sup>125</sup>I-HF bound to kaolin). After washing, the kaolin was suspended in 60  $\mu$ l of 0.1 M Tris buffer and a 20- $\mu$ l aliquot was removed for SDS-polyacrylamide gel analysis. The remaining kaolin was incubated at 37°C for 35 min and then a final 20  $\mu$ l aliquot was removed for analysis by SDS-polyacrylamide gel electrophoresis. The extent of proteolytic cleavage of <sup>125</sup>I-HF was calculated as the proportion of radioactivity remaining in the native 82,000 dalton band in relation to the control removed before the addition of kaolin for each sample (81–86% of the radioactivity in the gel).

**PREPARATION OF NORMAL HUMAN PLASMA AND PREKALLIKREIN-DEFICIENT PLASMA DEPLETED OF HF.** The IgG fraction of normal goat globulin or goat-anti-human HF was coupled to Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) by the cyanogen bromide method (25). The beads were suspended in 0.1 M Tris 0.15 M NaCl, pH 7.4 so that 80% of the settled volume consisted of beads. Normal human plasma or prekallikrein-deficient plasma (140  $\mu$ l) was incubated with an equal volume of the beads for 20 min at 22°C, and then separated by centrifugation at 12,000 *g* for 2 min. The plasmas absorbed with anti-HF beads contained 10% of the amount of HF present in the plasma absorbed with goat globulin beads as measured by clotting assay in human HF-deficient plasma. An aliquot of the HF-depleted plasmas were then reconstituted to the same HF-content as the goat-globulin absorbed plasma by addition of purified human HF (provided by Ms. Susan Revak, Department of Immunopathology, Scripps Clinic and Research Foundation), as assessed by clotting assay.

<sup>125</sup>I-rabbit HF (5  $\mu$ l, 400 ng, 0.5  $\mu$ Ci) was added to 50  $\mu$ l of each plasma-diluted one-half in Tris-buffered saline. Kaolin (4  $\mu$ l, 200  $\mu$ g) was added to the plasma and subsamples were withdrawn for analysis by polyacrylamide gel electrophoresis at 0, 1, 2, 8, and 20 min. The results are expressed as the percent <sup>125</sup>I-HF cleaved from the native 82,000 dalton band at each time point compared with the 0 time point for each plasma.

**Miscellaneous.** Radiolabeling of protein with <sup>125</sup>I was performed by the chloramine-T method (26) to a specific activity of 0.5  $\mu$ Ci/ $\mu$ g. BSA (recrystallized five times), was purchased from Reheis Chemical Co., Division of Armour Pharmaceutical Co., Chicago, Ill.

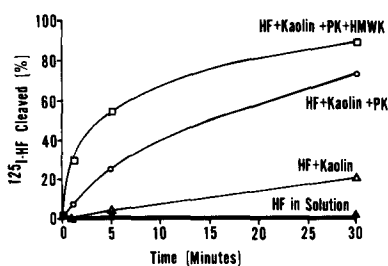


FIG. 2. The rate of proteolytic cleavage of  $^{125}\text{I}$ -HF incubated at  $37^\circ\text{C}$  in solution ( $\blacktriangle$ ), in the presence of kaolin ( $\triangle$ ), in the presence of kaolin plus prekallikrein ( $\circ$ ), in the presence of kaolin, prekallikrein, and high molecular weight kininogen ( $\square$ ).

## Results

*Proteolytic Cleavage of HF in the Presence and Absence of Kaolin, Prekallikrein, and High Molecular Weight Kininogen.* The effect of kaolin ( $100\ \mu\text{g}$ ), prekallikrein ( $0.8\ \mu\text{g}$ ), and high molecular weight kininogen ( $1.5\ \mu\text{g}$ ) upon the proteolytic cleavage of  $^{125}\text{I}$ -HF ( $300\ \text{ng}$ ,  $0.4\ \mu\text{Ci}$  plus HF  $1.2\ \mu\text{g}$ ) in a purified system ( $120\ \mu\text{l}$ ) containing  $0.1\ \text{M}$  Tris, pH 7.6 ( $20\ \mu\text{l}$ ) and BSA ( $20\ \mu\text{g}$ ) was studied (Fig. 2). Incubation of HF alone in solution for 30 min at physiological concentration ( $12\ \mu\text{g}/\text{ml}$ ) resulted in no detectable cleavage of  $^{125}\text{I}$ -HF. However, when kaolin was added to this incubation mixture, proteolytic cleavage of  $^{125}\text{I}$ -HF did occur so that in a typical experiment, as shown in Fig. 2, 16% of the molecules were cleaved after 30 min. This observation has also been made with human HF in the presence of both glass and kaolin. The rate of cleavage of  $^{125}\text{I}$ -HF was enhanced  $\sim 10$ -fold when prekallikrein ( $6.5\ \mu\text{g}/\text{ml}$ ) was present in the incubation mixture, and further enhanced when high molecular weight kininogen ( $12\ \mu\text{g}/\text{ml}$ ) was also present. In parallel experiments, radiolabeled prekallikrein and factor XI did not undergo proteolytic cleavage when incubated alone in the presence or absence of kaolin. However, rapid cleavage of both these molecules was observed in the presence of kaolin when HF was present in the reaction mixture.

The cleavage pattern of  $^{125}\text{I}$ -HF observed on autoradiography was the same for all experiments described in this report. The 82,000 dalton native HF was cleaved to fragments which migrated with apparent mol wt of 50,000 and 28,000 daltons in the presence of reducing agent.

*Proteolytic Cleavage of HF by  $\alpha$ -HF<sub>a</sub>.* Because HF appeared to be capable of undergoing slow proteolytic cleavage in the presence of kaolin alone, we considered the possibility that the two-chain form of activated HF ( $\alpha$ -HF<sub>a</sub>) might proteolytically cleave single-chain HF. To assess this, purified HF<sub>a</sub> ( $100\ \mu\text{g}/\text{ml}$  and serial dilutions thereof in  $10\ \mu\text{l}$   $0.01\ \text{M}$  acetate buffer, pH 5) was incubated with  $^{125}\text{I}$ -HF ( $800\ \text{ng}$  HF) in the presence and absence of kaolin ( $250\ \mu\text{g}$ ), and the extent of proteolytic cleavage of the  $^{125}\text{I}$ -HF after a 10-min incubation at  $37^\circ\text{C}$  was measured by SDS-polyacrylamide gel electrophoresis. Even at high concentrations of  $\alpha$ -HF<sub>a</sub> ( $50\ \mu\text{g}/\text{ml}$ ),  $<5\%$  cleavage of  $^{125}\text{I}$ -HF occurred in solution. In contrast, in the presence of kaolin,  $^{125}\text{I}$ -HF was cleaved in a dose-related fashion. At 50, 25, 12.5, 6.25, 3.13, and  $1.6\ \mu\text{g}/\text{ml}$  the extent of cleavage of  $^{125}\text{I}$ -HF observed was 42, 47, 29, 18, 8, and 2%, respectively. Thus,  $\alpha$ -HF<sub>a</sub> appeared to be capable of proteolytically cleaving HF, in the presence of kaolin.

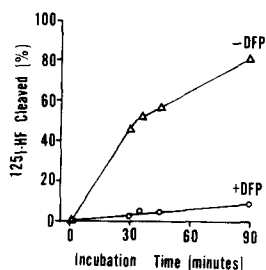


FIG. 3. The rate of proteolytic cleavage of kaolin-bound diisopropylfluorophosphate (DFP)-treated HF incubated in the presence (O) and absence ( $\Delta$ ) of 5 mM DFP.

**Inhibition of  $\alpha$ -HF<sub>a</sub> by Soybean Trypsin Inhibitor (SBTI) and DFP.** Because cleavage of HF by the  $\alpha$ -HF<sub>a</sub> preparation could have been a result of a contaminating protease, the ability of SBTI and DFP to inhibit  $\alpha$ -HF<sub>a</sub>-mediated cleavage of prekallikrein, factor XI and HF were compared.  $\alpha$ -HF<sub>a</sub> (4  $\mu$ g) was incubated with SBTI in 0.1 M Tris, pH 7.4, in various amounts (2, 0.5, 0.125, 0.03, and 0  $\mu$ g) for 10 min at 22°C in a total 80- $\mu$ l vol. <sup>125</sup>I-HF (50 ng) or <sup>125</sup>I-factor XI (45 ng) was added to kaolin (50  $\mu$ g) in Tris-buffered saline. <sup>125</sup>I-prekallikrein (50 ng) was added to an equal volume of Tris-buffered saline containing 1 mg/ml BSA. To plastic tubes containing 10  $\mu$ l of 0.1 M Tris buffer pH 7.4 and the above mixture of radiolabeled proteins, a 10- $\mu$ l aliquot of the  $\alpha$ -HF<sub>a</sub>/SBTI reaction mixture was added (500 ng of  $\alpha$ -HF<sub>a</sub> plus 250, 62.5, 15.6, 3.9, or 0 ng SBTI). After a 10-min incubation at 37°C the extent of proteolytic cleavage of the radiolabeled protein was measured by SDS-polyacrylamide gel electrophoresis. Compared to the control (no SBTI), the SBTI-treated  $\alpha$ -HF<sub>a</sub> (3.9, 15.6, 62.5, and 250 ng SBTI + 500 ng  $\alpha$ -HF<sub>a</sub>) cleaved, respectively, factor XI (90, 58, 24, and 6%), HF (79, 52, 18, and 5%) and prekallikrein (70, 48, 15, and 5%). Thus, SBTI inhibited  $\alpha$ -HF<sub>a</sub>-mediated cleavage of factor XI, HF, and prekallikrein in a dose-related fashion and to a similar extent, indicating that the same enzyme was responsible for their cleavage. In a similar experiment, preincubation of  $\alpha$ -HF<sub>a</sub> with DFP (2 mM for 10 min at 22°C) completely inhibited the observed  $\alpha$ -HF<sub>a</sub>-mediated cleavage of radiolabeled HF, factor XI, and prekallikrein.

**Proteolytic Cleavage of Kaolin-bound HF in the Presence and Absence of DFP.** Incubation of single-chain HF with kaolin resulted in slow proteolytic cleavage of HF molecules (Fig. 2). Such cleavage could have been caused either by the HF preparation containing a small proportion of  $\alpha$ -HF<sub>a</sub> or by some other contaminating protease. To investigate this possibility, HF was preincubated with DFP (12 mM) to inhibit active serine protease enzymes. Kaolin was then added to this mixture to inhibit any enzymes possibly associated with the kaolin (see Methods for details). The mixture was then divided into two equal aliquots. The kaolin-bound HF in one aliquot was washed in 0.1 M Tris buffer to remove DFP and nonbound HF; the kaolin-bound HF in the second aliquot was washed in 0.1 M Tris buffer containing 5 mM DFP. Both preparations of kaolin-bound HF were then incubated at 37°C and the rate of proteolytic cleavage of <sup>125</sup>I-HF measured by analysis of SDS-polyacrylamide gels. One of three similar experiments is shown in Fig. 3; rapid proteolytic cleavage occurred in the reaction mixture from which DFP had been removed by washing, but not in the reaction mixture in which DFP was present. Thus, although the observed rapid cleavage of HF was caused by an enzyme that was sensitive to DFP, the enzyme

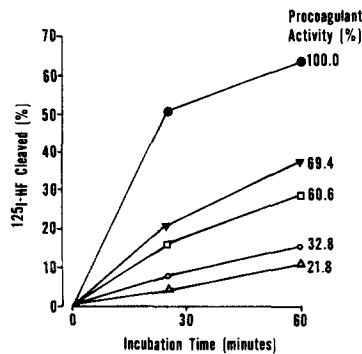


FIG. 4. The effect of prolonged preincubation of HF with DFP, resulting in inhibition of HF procoagulant activity by 78.2% ( $\Delta$ ), 67.2% ( $\circ$ ), 39.4% ( $\square$ ), 30.6% ( $\blacktriangle$ ), and 0% ( $\bullet$ ) upon the rate of cleavage of kaolin-bound  $^{125}\text{I}$ -HF.

could not be blocked by prior incubation with DFP. In addition, even in the presence of a 10,000-fold molar excess of DFP cleavage of HF was not inhibited altogether (Fig. 3). In fact, proteolytic cleavage under these conditions proceeded in a linear fashion so that  $\sim 9\%$  of the  $^{125}\text{I}$ -HF molecules were cleaved after 90 min, or 0.1%/min. A catalyst which was resistant to short-term DFP treatment must therefore have been present in the HF preparation.

*The Effect of Prolonged Incubation of HF with DFP.* Because zymogen single-chain HF slowly takes up and is inactivated by DFP (14, 15), the effect of prolonged incubation of HF with DFP on cleavage of kaolin-bound HF was measured. After incubation of HF for 48 h at  $37^\circ\text{C}$  with 10 mM DFP, only 21.8% of the HF procoagulant activity remained. Shorter incubations with DFP at 37 or  $22^\circ\text{C}$  (Methods) resulted in less inhibition of HF procoagulant activities (32.8, 60.6, 69.4, and 100% HF activity remaining). Kaolin was added to these HF preparations and subsequently washed to remove DFP and nonbound HF (Methods). The extent of  $^{125}\text{I}$ -HF cleavage was measured by SDS-polyacrylamide gel analysis immediately after the washing procedure and after a further 35-min incubation at  $37^\circ\text{C}$ . As shown in Fig. 4, the rate and extent of cleavage of  $^{125}\text{I}$ -HF varied with the amount of inhibition of procoagulant activity by DFP. Thus, HF which had been extensively inhibited by DFP underwent less proteolytic cleavage than HF which had not been inhibited by DFP. Although the true initial rates of cleavage of kaolin-bound HF cannot be calculated from these data, a plot of the proportions of HF cleaved (after the 25-min period of kaolin washing) against the proportion of remaining HF procoagulant activity on a linear scale (Fig. 5) suggests that at 0 procoagulant activity there would be no cleavage of HF. Such a relationship is consistent with the concept that it is single-chain HF which is responsible for HF cleavage, and not a contaminating DFP-insensitive enzyme.

*The Effect of Varying the Kaolin Concentration upon Cleavage of HF.* From the preceding experiment, it appeared that single-chain HF was capable of undergoing cleavage upon contact with kaolin. We then questioned whether cleavage could occur either from simple interaction of the HF with kaolin or alternatively by the action of HF molecules upon other HF molecules, converting them into the two-chain form. To differentiate between these possibilities, the amount of negatively charged surface (kaolin) was varied over an extensive range, resulting in binding of HF molecules either in close apposition, or widely separated. A mixture of  $^{125}\text{I}$ -HF (50 ng, 0.05  $\mu\text{Ci}$ )

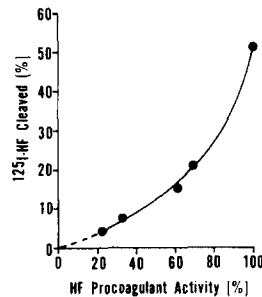


FIG. 5. Plot of the extent of proteolytic cleavage of kaolin-bound  $^{125}\text{I}$ -HF (after incubation for 25 min at  $22^\circ\text{C}$ ) with remaining HF-procoagulant activity after DFP treatment. The line approaches the 0 point of both the x and y axes indicating that the cleavage of HF is directly related to HF procoagulant activity.

and HF (300 ng) in the presence of 0.1 M Tris pH 7.4 and BSA (5  $\mu\text{g}$ ) in a 31- $\mu\text{l}$  final vol was incubated in the presence of various concentrations of kaolin, as noted in Fig. 6, for 10 min at  $37^\circ\text{C}$ . As shown in Fig. 6, at high concentrations, conditions where HF molecules would be widely separated on the kaolin surface, only 5–10% of HF molecules were cleaved. However, as the kaolin concentration fell and therefore the surface area available for binding of HF molecule diminished so that HF molecules would be bound closer together on the kaolin surface, the extent of cleavage of  $^{125}\text{I}$ -HF rose to a maximum of 25% at a kaolin concentration  $\sim 10$ -fold higher than the HF concentration (wt:wt). Further reduction in the kaolin concentration was accompanied by a fall of the total HF cleavage. In a parallel experiment done at the same time, addition of high mol wt kininogen (520 ng) to the reaction mixture inhibited cleavage of HF by about one-half at the optimal kaolin concentration. Similar results were obtained in three separate experiments, and an identical effect of surface area on human HF cleavage has been observed with both glass and kaolin. These findings are compatible with the concept that a close molecular association between kaolin-bound HF molecules is required for cleavage to occur. Such a relationship may be disturbed by high molecular weight kininogen.

*Factor XI Cleavage by Kaolin-bound HF and HF<sub>a</sub>.* To investigate whether kaolin-bound single-chain HF was capable of proteolytically cleaving factor XI, or whether HF<sub>a</sub> was required for this function, the following experiment was performed. Single-chain  $^{131}\text{I}$ -HF (250 ng, 0.6  $\mu\text{Ci}$  + HF 1.35  $\mu\text{g}$ ) was incubated with kaolin (250  $\mu\text{g}$ ) in the presence of 0.1 M Tris, pH 7.4, BSA 10  $\mu\text{g}$  in a final reaction vol of 85  $\mu\text{l}$  for up to 50 min to allow cleavage of HF to occur (Fig. 4). At intervals, a 12- $\mu\text{l}$  aliquot of kaolin-bound  $^{131}\text{I}$ -HF was removed from the reaction mixture and incubated for 2 min at  $37^\circ\text{C}$  with  $^{125}\text{I}$ -factor XI (120 ng, 0.5  $\mu\text{Ci}$ ) in the presence of 0.1 M Tris, pH 7.4 (10  $\mu\text{l}$ ) and BSA (10  $\mu\text{g}$ ). The extent of cleavage of the  $^{131}\text{I}$ -HF and  $^{125}\text{I}$ -factor XI were then measured by analysis on SDS-polyacrylamide gels.

After 3, 15, and 50 min of incubation, 4, 13, and 34% of the  $^{131}\text{I}$ -HF and 5, 11, and 27% of the  $^{125}\text{I}$ -factor XI molecules were cleaved, respectively. Thus, the extent of proteolytic cleavage of  $^{131}\text{I}$ -HF was paralleled by cleavage of  $^{125}\text{I}$ -factor XI, suggesting that for cleavage of factor XI to occur cleavage of HF was required. Furthermore, single-chain HF cleaved in the presence of kaolin apparently became biologically active with respect to factor XI cleavage. The cleavage fragments of factor XI

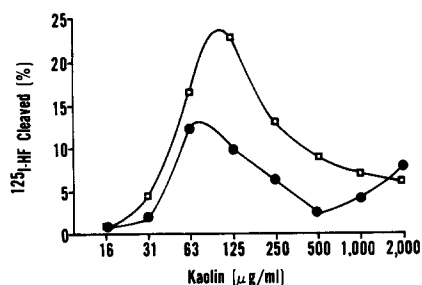


FIG. 6. The effect of varying the kaolin concentration upon the extent of proteolytic cleavage of HF (present at 12  $\mu\text{g/ml}$ ). The presence of high molecular weight kininogen (●) in the reaction mixture resulted in less cleavage of  $^{125}\text{I}$ -Hageman factor than when high molecular weight kininogen was absent (□) from the reaction mixture.

generated under these conditions were observed at 50,000 and 33,000 daltons, identical to those seen in kaolin-activated rabbit plasma (27).

*Proteolytic Cleavage of  $^{125}\text{I}$ -HF in Prekallikrein-deficient Plasma.* This experiment was performed to investigate the role of HF in the slow proteolytic cleavage of radiolabeled HF reported to occur in prekallikrein-deficient plasma (1). Normal human plasma or prekallikrein-deficient plasma were depleted to 10% of their normal concentration of HF (Methods). These depleted plasmas were also reconstituted by addition of purified human HF.  $^{125}\text{I}$ -rabbit HF was added to each plasma and after kaolin activation the extent of proteolytic cleavage was measured during a 20-min incubation at 37°C.

In normal human plasma (control, HF depleted, or HF reconstituted) the extent of cleavage of  $^{125}\text{I}$ -HF at 1, 2, 8, and 20 min was similar for all three plasmas, being 20, 63, 72, and 74%, respectively. No cleavage was observed in HF-depleted prekallikrein-deficient plasma. In contrast, control or HF-reconstituted prekallikrein-deficient plasma the values were 0, 1, 3, and 10% cleaved after 1, 2, 8, and 20 min, respectively. Thus, HF-depletion made no difference to the rate of cleavage of  $^{125}\text{I}$ -HF in the presence of prekallikrein (normal human plasma). However, in the absence of prekallikrein (prekallikrein-deficient plasma) the slow cleavage of  $^{125}\text{I}$ -HF was dependent upon the presence of HF.

### Discussion

The observation that single-chain zymogen HF became proteolytically cleaved when incubated in the presence of kaolin raised the question of whether HF molecules might be capable of proteolytically cleaving other HF molecules. This concept was supported by finding that two-chain 82,000-dalton activated HF ( $\alpha\text{-HF}_a$ ) cleaved single-chain HF in a dose-dependent manner, and that this occurred much more rapidly in the presence of kaolin. The possibility that this cleavage could have been caused by a contaminating protease in the  $\alpha\text{-HF}_a$  preparation is very unlikely because soybean trypsin inhibitor inhibited  $\alpha\text{-HF}_a$ -mediated cleavage of HF to the same extent as it inhibited  $\alpha\text{-HF}_a$ -mediated factor XI and prekallikrein cleavage.

Because  $\alpha\text{-HF}_a$  can cleave HF it was possible that small amounts of  $\alpha\text{-HF}_a$ , or some other enzyme, present in the HF preparation might cause the observed cleavage of kaolin-bound HF. However, this appears not to be the case, because preincubation of HF in the presence of a 10,000-fold molar excess of DFP, conditions which inhibited



$\alpha$ -HF<sub>a</sub>-mediated cleavage of kaolin-bound HF, did not inhibit subsequent HF cleavage once the DFP has been removed.

Single-chain HF is known to slowly take up and be inhibited by DFP (14, 15), and can be considered to be an active zymogen, like trypsinogen (28) and Factor VII (29). Our observation that long-term incubation of single chain HF prevented cleavage of kaolin-bound HF to an extent proportional to the inhibition of its procoagulant activity is quite compatible with the concept that it is single-chain HF, functioning as an active zymogen, which is responsible for the observed cleavage of kaolin-bound HF. The alternative hypothesis, that possible contaminating enzymes (e.g.,  $\alpha$ -HF<sub>a</sub>) might not be completely inhibited by the DFP treatment cannot be entirely excluded. However, the observation that slow cleavage of kaolin-bound HF proceeded in a linear rather than an exponential fashion even in the presence of a 10,000-fold molar excess of DFP (Fig. 3) makes this latter hypothesis most unlikely. Making the assumption that cleavage under these circumstances does indeed only reflect zymogen HF activity (as opposed to the added effect of  $\alpha$ -HF<sub>a</sub> superimposed upon zymogen activity as occurred in the absence of DFP) a calculation of the rate of effective zymogen activity can be made (0.1% of the kaolin-bound molecules expressing activity per minute). This number is similar to that previously measured for the rate of uptake of tritiated DFP by human single-chain HF (14).

The requirement for kaolin for HF cleavage was not surprising because kaolin-bound HF is more susceptible to cleavage by other enzymes (30). The fact that the amount of kaolin was critical suggests that a close molecular association on the kaolin-surface was required for cleavage to take place, and provides evidence that it is not the binding of HF to kaolin per se which leads to proteolytic cleavage of the molecule, but rather the interaction between surface-bound HF molecules which is required before cleavage will occur. High molecular weight kininogen which binds to negatively charged surfaces inhibited cleavage of HF probably by simply preventing the interaction between HF molecules.

The concept that single-chain HF does have enzymatic activity is therefore supported by its ability to take up tritiated DFP (14, 15) and to cleave a synthetic substrate (H-D-phenylalanyl-L-pipecobyl-L-arginine-*p*-nitroanilide) (31). In the present study we suggest that single-chain HF can also proteolytically cleave other HF molecules on a kaolin surface. That this cleavage is associated with the acquisition of enhanced activity was demonstrated by finding that cleavage of factor XI by kaolin-bound HF directly correlated with the extent of cleavage of the HF. This finding supports previous observations that emphasize the requirement for limited proteolytic cleavage for the activation of HF molecules into a form capable of generating procoagulant activity (1, 14-19), but is in contrast to the findings of Ratnoff and Saito (11, 31) who, using Sephadex-Ellagic acid mixtures, observed amidolytic activity and activation of Factor XI in the apparent absence of HF cleavage.

The importance of the concept that single-chain HF might possess low levels of enzymatic activity and be able to cleave other HF molecules, lies in understanding the events that take place during contact activation of plasma. The fact that proteolytic cleavage of HF does occur slowly in kaolin-activated prekallikrein-deficient plasma implies that prekallikrein is not essential for the slow activation of HF. Our finding that depletion of the HF in prekallikrein-deficient plasma to 10% of normal abolished detectable HF cleavage suggests that HF cleavage under these conditions

is dependent upon HF, and that it is unlikely that a quite different enzyme capable of independently activating HF is present in kaolin-activated prekallikrein-deficient plasma. These data are compatible with the concept that HF can activate HF in prekallikrein-deficient plasma.

The question of what actually initiates this well-described reciprocal activation between HF and prekallikrein (10, 17-19) when a negatively charged surface is added to plasma is not finally answered. However, one possible mechanism is suggested by the results of this study, namely, that if two or more HF molecules are bound in close association on a negatively charged surface, proteolytic cleavage of one or both can occur. This relatively inefficient mechanism of activating a few surface-bound HF molecules could trigger the explosive reciprocal activation between prekallikrein and HF.

### Summary

Proteolytic cleavage and activation of isolated, single chain, zymogen Hageman factor was observed in the presence of kaolin alone. The rate of cleavage of kaolin-bound Hageman factor was enhanced 50-fold by the presence of prekallikrein and high molecular weight kininogen. The two-chain 82,000 dalton form of activated Hageman factor ( $\alpha$ -HF<sub>a</sub>) also cleaved kaolin-bound single-chain Hageman factor in a dose-dependent manner, yielding fragments of 28,000 and, 50,000 daltons under reducing conditions. Cleavage of kaolin-bound single-chain Hageman factor was not inhibited by preincubation with diisopropylfluorophosphate (12 mM) for 10 min, but long-term incubation of Hageman factor with diisopropylfluorophosphate (up to 48 h) resulted in inhibition of cleavage of kaolin-bound Hageman factor to an extent proportional to the inhibition of procoagulant Hageman factor activity. Hageman factor cleavage was maximal when the kaolin concentration was ~10-fold greater than the Hageman factor concentration (wt:wt), and was partially inhibited by high molecular weight kininogen. Kaolin-bound Hageman factor cleaved clotting factor XI in an amount which correlated with the extent of cleavage of the Hageman factor. These findings are compatible with the concept that single-chain Hageman factor and  $\alpha$ -HF<sub>a</sub> are both capable of cleaving and activating kaolin-bound Hageman factor and that a close molecular association of kaolin-bound Hageman factor molecules is required for this reaction.

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