Enzymatic Activities of Activated and Zymogen Forms of Human Hageman Factor (Factor XII)

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Pro-Phe-Arg chloromethylketone (PPACMK) at 5.26 μM inactivated the amidolytic activity of native human Hageman factor with an apparent first-order rate constant of 0.75 min⁻¹. The activated forms of Hageman factor, HFa and HFf, were also inactivated by PPACMK with rate constants 0.82 and 0.72 min⁻¹. These numbers indicate that the activity detectable in native Hageman factor is due to contamination with activated species. Uncleaved Hageman factor reacts slowly with 40 mM diisopropyl fluorophosphate with concomitant loss of its procoagulant activity. Incubation of native Hageman factor with PPACMK does not destroy its procoagulant activity, even in the presence of the activator dextran sulphate, but PPACMK inhibits autoactivation of Hageman factor, suggesting that no active site is formed in uncleaved, surface-bound Hageman factor. The activation of prekallikrein by Hageman factor under initial-rate conditions occurs after a lag and is prevented by an inhibitor of Hageman factor from corn. The kinetics of prekallikrein activation and the effects of inhibitors provide evidence that the amidolytic and proteolytic activities of human Hageman factor reside in the activated forms derived by limited proteolysis of the native molecule.

CONTACT ACTIVATION occurs when plasma is exposed to negatively charged surfaces such as glass, kaolin, or dextran sulphate. As a result of the contact with the surface, Hageman factor (factor XII) and prekallikrein become activated in a reaction for which high molecular weight (HMW) kininogen is a cofactor. During activation, both Hageman factor and prekallikrein are cleaved within disulfide loops to generate two-chain molecules [HFa (XIIa) and kallikrein], which are both trypsin-like proteases with the active site serines located on their light chains. A second form of activated Hageman factor is also formed that has lost the greater part of the heavy chain of HFa. This low molecular weight form of active Hageman factor is termed Hageman factor fragment or HFf (XIIa).

Studies performed with purified systems in vitro have demonstrated that the major activator of HF is kallikrein, whereas the major activator of prekallikrein is activated HF. Hageman factor becomes activated in prekallikrein-deficient plasma but at a much reduced rate, indicating that the activation of HF by kallikrein is a kinetically significant component of the reactions leading to the activation of factor XI.

It was originally proposed that interaction of Hageman factor and a surface created a conformational change sufficient to expose an active site in the uncleaved molecule and that this species was able to activate prekallikrein to kallikrein, which could feedback and cleave the HF. The autocorrection of prekallikrein-deficient plasma was explained by a less efficient feedback involving factor XIa instead of kallikrein. However, in a previous communication we demonstrated that pure Hageman factor autoactivates when exposed to a surface. This autoactivation is accompanied by cleavage of the polypeptide chain and can be initiated by residual traces of activity remaining in Hageman factor preparations even after treatment with diisopropyl fluorophosphate (DFP). Similar autoactivation may account for the apparent generation of activity in Hageman factor preparations exposed to ellagic acid. Nevertheless, the constant generation of activated Hageman factor in preparations of purified zymogen could be initiated by intrinsic enzymatic activity of the zymogen. In support of this argument, it has been reported that zymogen is able to react slowly with DFP with consequent loss of its coagulant activity. Furthermore, the results of a recent study of bovine prekallikrein and Hageman factor were interpreted as evidence for a substrate-dependent, substrate-induced cleavage of prekallikrein by native Hageman factor. Indeed, it was proposed that native and cleaved Hageman factor were equally effective as prekallikrein activators.

In this article we have attempted to determine the initial source of enzymatic activity in contact activation and, in particular, to distinguish between traces of activated Hageman factor and intrinsic activity of the zymogen as that source. These studies were facilitated...
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by the availability of a reactive, active site-directed inhibitor, and of a polypeptide inhibitor specific for Hageman factor.

MATERIALS AND METHODS

Proteins

Prekallikrein and Hageman factor were purified from human plasma by previously published procedures and stored in plastic tubes at -80°C in the presence of 10 mM benzamidine. IgG was obtained from the SP-Sepahdex chromatography used to isolate prekallikrein from the γ-globulin fraction of human plasma. The IgG elutes from the column before either prekallikrein or factor XI. HFa and HFI were prepared from Hageman factor after digestion with kallikrein as described earlier. The Hageman factor inhibitor derived from corn was a generous gift of Drs. John Pisano and Yoshio Hozuma of the National Institutes of Health, National Heart, Lung and Blood Institute.

Chemicals

Pro-Phe-Arg chloromethyl ketone was a generous gift of Drs. Elliot Shaw and Charles Kettner of Brookhaven National Laboratories. H-D-Pro-Phe-Arg-pNA (S2302) and Benzoyl-Ile-Glu-Gly-Arg-pNA (S2222) were obtained from Kabi Inc. Greenwich, Conn. Benzoyl-Pro-Phe-Arg-pNA (Chromozym PK) was from Boehringer Mannheim. Other chemicals were obtained as follows: dextran sulphate (mol wt 500,000), Sigma Chemical Co. St. Louis Mo.; disisopropyl fluorophosphate, Calbiochem-Behring; reagents for gel electrophoresis, BioRad Laboratories, Hicksville, N.Y.; deficient plasmas, George King Bio-Medical Inc., Overland Park, Kans.

Methods

Plastic pipettes and tubes were used in all procedures. Enzyme assays were conducted in semimicro cuvettes containing 400 μl of 0.05 M Tris-Cl pH 7.8, 0.117 M NaCl (I = 0.15) and 500 μM chromogenic substrate. For routine assays of enzymatic activity we used plastic cuvettes (Walter Sarstedt Inc., Princeton, N.J.). Absorbance changes were recorded on a Gilford Model 250 recording spectrophotometer equipped with a cell changer thermostatted to 37°C.

Clotting assays were activated partial thromboplastin times using factor-XII-deficient plasma and calibrated with dilutions of normal human plasma.

Protein concentrations were determined using the method of Lowry et al. calibrated with human IgG.

H-D-Pro-Phe-Arg-chloromethyl ketone (PPACMK) was dissolved in 0.001 N HCl at 1 mM and appropriate dilutions made for addition to protein solutions. The kinetics of inactivation of Hageman factor by PPACMK were determined as follows. Hageman factor or its activated forms were dialyzed into 0.1 M sodium phosphate, pH 7.0. One-hundred microliter aliquots were placed into 500 μl microcentrifuge tubes and incubated briefly at 37°C. Two 5 μl aliquots were removed for estimation of the initial activity and replaced with 10 μl of 56.2 μM PPACMK. Five-microliter aliquots were removed for assays at various times; the activity remaining at any given time was expressed as a percentage of the original activity after correction for the dilution due to the addition of inhibitor.

For the determination of the kinetics of inactivation of Hageman factor by DFP, a stock solution of 0.4 M DFP in dimethylformamide was made and 10 μl added to 90 μl of Hageman factor that had been dialyzed into 0.1 M NaPi, pH 7.0. Aliquots were removed at intervals, diluted 1000-fold in phosphate-buffered saline (PBS), and assayed for coagulant activity as described above. The activity present, in units per milliliter, was expressed as a percentage of the activity of a control sample, containing 10% DMF, incubated in parallel.

Prekallikrein used in studies of its activation by Hageman factor was prepared by treating aliquots at 0.5–1 mg/ml with 100 μM PPACMK for 1 hr at 37°C. The prekallikrein was not dialyzed before this treatment and therefore contained 10 mM benzamidine used as a preservative; this did not prevent the reaction with PPACMK under these conditions. At the end of the incubation the prekallikrein was desalted on a column of Sephadex G25 fine (10 cm × 0.7 cm) equilibrated in 0.1 M NaPi, pH 7.0. IgG was prepared in the same way for use as a diluent for Hageman factor.

Gel electrophoresis was performed on 9% polyacrylamide gels using the buffer system of Laemmli.

RESULTS

Since activated Hageman factor reacts with the kallikrein substrate H-D-Pro-Phe-Arg-pNA, we would expect it to be susceptible to inactivation by the
substrate analogue Pro-Phe-Arg-chloromethyl ketone, which is a potent inhibitor of kallikrein. HFa or HFf (40 μg/ml) were treated with 5.26 mM PPACMK at 37°C. As shown in Fig. 1, both forms of activated Hageman factor rapidly lost amidolytic activity in a pseudo first-order reaction. The apparent first-order rate constants were 0.85 and 0.72 min⁻¹ for HFa and HFf, respectively. As described in a previous communication, purified native Hageman factor always possesses some amidolytic activity. We measured the rate of inactivation of this activity by 5.26 μm PPACMK using a number of different preparations of Hageman factor with molar specific activities of 60–120 moles of substrate converted per minute per mole of protein. We also included a preparation that had been pretreated with 10 mM DFP and then dialyzed; this material had a molar specific activity of 2.4 mole substrate/min/mole. In spite of these variations in specific activity, all of the preparations reacted with PPACMK with the same apparent first-order rate constant of 0.75⁻¹ (Fig. 1), which is not significantly different from the values obtained for the two forms of activated Hageman factor, both of which had molar specific activities of 800 mole substrate/min/mole enzyme. Thus, we are led to conclude that the enzymatic activity readily demonstrable in preparations of Hageman factor is most likely due to contamination with activated HF.

The rate of reaction with PPACMK proceeds unchanged until no more than 1% of the initial activity remains. We can obtain from this an upper limit for any potential contribution made by the zymogen to the amidolytic activity of the initial preparation. Figure 2 shows the results obtained with a single preparation of Hageman factor that had an initial molar specific activity of 36.7 mole substrate/min/mole. The initial reaction rate falls off to almost zero, and it is possible that the apparent slow rate of inhibition in the second part of the curve represents the reaction of a different kind of active site with the inhibitor. By extrapolation of this second linear region back to the ordinate, we can estimate that only 0.5% of the initial activity could be due to a slow reacting site that might belong to the zymogen. As the initial activity represented 4.6% of the activity of pure HFa or HFf, these numbers indicate that the amidolytic activity of uncleaved Hageman factor in the fluid phase is about 4200-fold less than that of activated HF in the standard spectro.

Fig. 3. Reaction of Hageman factor zymogen with inhibitors. Coagulant activity was measured at the indicated times by diluting an aliquot 1000-fold and using 50 μl of the dilution for a PTT. The PTT was calibrated using dilutions of normal plasma and the activity of the aliquots in U/ml was expressed as a percentage of the activity of a control sample incubated without inhibitors. The control had an average specific activity in this assay of 90 U/mg. Hageman factor was 608 mg/ml and incubated at 37°C with 40 mM DFP (○), 200 μM PPACMK (△), or 100 μM PPACMK plus 10 μg/ml dextran sulphate (■).
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photometric assay. This figure represents an upper limit and depends on amidolytic measurements that are barely distinguishable from zero. When we examined the results of similar experiments with various preparations of Hageman factor, HFa and HFf, we found that all the curves of activity versus time leveled off, including those obtained with the activated forms. The apparent specific activities obtained after the initial phase of rapid inactivation varied by an order of magnitude. We concluded that the true enzymatic activities had become less than the inherent uncertainty of the assay baseline.

Hageman factor that had been treated with 5.26 \( \mu M \) PPACMK until its amidolytic activity was essentially too low to measure (Fig. 2) was still able to autoactivate when diluted into a glass cuvette containing chromogenic substrate. The autoactivation is characterized by a long lag phase followed by a rapid acceleration in the rate of amidolysis. Simulated autoactivation curves using a simple kinetic model showed a similar pattern when the initial concentration of active sites was equivalent to less than 0.01% of the total number of protein molecules. This value is of the same order of magnitude as the upper limit for the enzymatic activity of the zymogen obtained above.

Since it has been reported that native HF is able to react with DFP with loss of procoagulant activity, we compared the reaction of native Hageman with DFP and with PPACMK (Fig. 3). Hageman factor at 608 \( \mu g/ml \) was incubated with 40 mM DFP. At intervals, aliquots were removed and assayed for their ability to correct the PTT of Hageman-factor-deficient plasma. Over several hours there was a progressive decline of procoagulant activity in the presence of DFP, suggesting that the active site serine is reactive with DFP, even in the uncleaved zymogen form of Hageman factor. Hageman factor was also incubated with 200 \( \mu M \) PPACMK and assayed for procoagulant activity in the same way. There was an initial decrease, but after 5 hr, the procoagulant activity remained at least 70% of that of the control. Essentially the same results were obtained from the reaction of Hageman factor and PPACMK in the presence of 10 \( \mu g/ml \) dextran sulphate, which is an efficient promoter of contact activation. There is thus a clear difference in the reaction of the native enzyme with DFP and with an inhibitor that is a close analogue of a substrate of Hageman factor. The results obtained with the latter, and shown in Fig. 3, may be compared with those illustrated in Fig. 1 where it can be seen that, at \( \frac{1}{4} \) of the concentration of inhibitor, activated Hageman is inactivated with a half-time of approximately 1 min. Thus, the results obtained with PPACMK do not support the hypothesis that Hageman factor zymogen has appreciable enzymatic activity expressed toward either a substrate or an inhibitor.

Figure 4 shows SDS-polyacrylamide gels of Hageman factor after incubation with dextran sulphate in the presence or absence of PPACMK for 7 hr. The starting material (Fig. 4A) shows a small amount of cleaved forms; these arise by autoactivation when the Hageman factor is dialyzed in preparation for the experiment. This autoactivation proceeds almost to completion when the preparation is incubated with dextran sulphate (Fig. 4B). However, when PPACMK is present in the incubation, only a small amount of additional cleavage is seen after 7 hr (Fig. 4C). Since
any cleaved species produced would be rapidly inactivated by the PPACMK, the gel shown in Fig. 4C is consistent with the retention of 70% of the initial procoagulant activity measured at this time (Fig. 3). Comparison of gels B and C shows that autoactivation occurs only slowly, when the concentration of active sites is kept below the level of detectability. This result is consistent with the hypothesis of a steady state in which very low concentrations of active enzyme remain. Alternatively, zymogen may have very weak enzymatic activity, but the gels in Fig. 4 show that this is no more readily expressed against the cleavage site(s) of the Hageman factor polypeptide chain than against oligopeptide substrate or inhibitor.

We next examined the possibility that prekallikrein could induce enzymatic activity in surface-bound Hageman factor so that prekallikrein might be activated to kallikrein by Hageman factor zymogen. In these experiments, Hageman factor was used to activate prekallikrein at very low ratios so that the kinetics of the reaction could be observed under initial rate conditions. The prekallikrein for these experiments was treated with 100 μM PPACMK, which was then removed by gel-filtration as described in Materials and Methods. IgG was similarly prepared and was used to dilute the Hageman factor to minimize losses due to surface adsorption of the highly diluted protein. In our experiments, 100–200 μg/ml prekallikrein was mixed with an equal volume of 12–16 ng/ml HF in 3.8 mg/ml IgG. The Hageman factor stock solution had been treated with 100 μM PPACMK before dilution. Aliquots of the mixture were periodically assayed for kallikrein activity using the chromogenic assay. The results are shown in Fig. 5, plotted according to a first-order rate equation to eliminate the effect of differing substrate (i.e., prekallikrein) concentrations. In the fluid phase, prekallikrein became activated in an accelerating reaction, which after 3 hr, approached the rate of prekallikrein activation by preformed HFf of the same molar concentration (Fig. 5). In contrast, the activation of prekallikrein in the presence of 10 μg/ml dextran sulphate occurred rapidly from the beginning after a very short lag phase. Although this lag was not very pronounced, it was seen in the results obtained from three separate experiments. We repeated the experiment using an inhibitor derived from corn, which is specific for Hageman factor and does not inhibit kallikrein. This inhibitor forms a complex with HF that is stable on alkaline disc gel electrophoresis but is not stable in the presence of SDS. We found that this inhibitor did not decrease the procoagulant activity of Hageman factor zymogen in an experiment similar to those performed with DFP or PPACMK, suggesting that a complex was not formed with uncleaved Hageman factor. In the presence of 0.05 mg/ml corn inhibitor, the activation of prekallikrein was com-

![Fig. 5. Activation of prekallikrein by Hageman factor under initial rate conditions. At indicated times, 5 μl aliquots of the reaction mixtures were assayed for amidolytic activity against chromozym-PK. The activity obtained at time t, in ΔA/min, is used as the value of Kₚ, and the value of PK is calculated as the activity that would be expressed upon total conversion of prekallikrein to kallikrein. This figure was checked by the addition to some samples of 5 μl HFf (76 μg/ml) and assay of the kallikrein formed after 1–2 hr incubation. The composition of the reaction mixtures was as follows: (Δ) 36.3 μg/ml prekallikrein, 6.1 ng/ml Hageman factor, 10 μg/ml dextran sulphate; (A) 58.5 μg/ml PK, 6.1 μg/ml HF, 10 μg/ml DS, 0.05 mg/ml corn inhibitor; (■) 107.5 μg/ml PK, 8 ng/ml HF; (●) 58.5 μg/ml PK, 6.1 ng/ml HF, 10 μg/ml DS. Note the different time scales in the left and right panels.]
pletely prevented (Fig. 5A), indicating that activated Hageman factor was required for prekallikrein activation. Thus, it would appear that in the presence of dextran sulphate, Hageman factor was rapidly converted to HFa by traces of kallikrein present in the prekallikrein. Such traces would be equimolar with the Hageman factor if they comprised only 0.01% of the total prekallikrein.

DISCUSSION

The experiments described in this paper and the results of earlier studies demonstrate that purified prekallikrein and Hageman factor become activated in the presence of certain negatively charged substances. As described in the introduction, Hageman factor is the probable location of the first enzymatically active sites. We have considered four hypotheses that might account for the initiation of contact activation in vitro: (1) Hageman factor zymogen has intrinsic enzymatic activity. (2) Hageman factor zymogen acquires enzymatic activity when bound to a surface prior to any proteolysis. (3) An active conformation is induced by the substrate in surface-bound Hageman factor zymogen. (4) Autoactivation and the reciprocal activation by kallikrein are initiated by traces of active cleaved Hageman factor that are always present.

The first and second hypotheses arose from experiments in which the preparations of Hageman factor used had not been treated with effective inhibitors to remove all traces of HFa and Hff. Since Hageman factor autoactivates in a reaction catalyzed by traces of activated enzyme, it seems likely that the activities ascribed to Hageman factor zymogen actually belonged to traces of activated derivatives that were formed in the experiments. The cleavage of Hageman factor by HFa was demonstrated earlier and has been treated in detail in another publication.

In our studies we have made extensive use of inhibitors to eliminate, or at least minimize, the effects of activated forms of Hageman factor. In particular, we have used Pro-Phe-Arg-chloromethyl ketone, which is an active-site-directed irreversible inhibitor of Hageman factor and has a close structural similarity to a synthetic Hageman factor substrate. Our results with PPACMK and chromogenic substrates indicate that Hageman factor zymogen can have only very weak reactivity with these oligopeptides and we estimated an upper limit for the hydrolysis of 500 \( \mu M \) substrate as 4200-fold less than that of HFa or Hff. Attempts to estimate a \( K_m \) for this reaction were not successful and our best estimate would be in the mM range.

The reactivity of the zymogen with PPACMK is also very low as demonstrated by the retention of procoagulant activity after treatment with high concentrations of the inhibitor. This result is unchanged by the presence of an activating surface. It is important, however, to consider the possibility that a macro-molecular substrate might be able to induce significant activity in a manner not possible for low molecular weight compounds. However, the data of Fig. 4 show that although the Hageman factor polypeptide chain is a satisfactory substrate for activated Hageman factor, it is barely, if at all, cleaved by the zymogen. We therefore considered the possibility that the other natural substrates of Hageman factor, namely factor XI and prekallikrein, could be activated efficiently by the zymogen. Since it is clear that contact activation can proceed in prekallikrein-deficient plasma, one would have to postulate the factor XI would be the substrate that might be cleaved by Hageman factor zymogen. However, contact activation of normal plasma depends on a positive feedback reaction in which kallikrein enzymatically activates Hageman factor and bradykinin generation is normal in factor-XI-deficient plasma (suggesting that factor XI is not required for Hageman factor activation). We, therefore, concentrated on the interactions of Hageman factor and prekallikrein.

The interaction of prekallikrein with Hageman factor possesses the property of positive feedback and therefore is subject to rapid acceleration. It thus becomes difficult to investigate the initiating events. We attempted to avoid this difficulty by reducing the concentration of Hageman factor several thousand fold relative to prekallikrein. This sufficiently reduced the rate of prekallikrein activation to allow us to measure kallikrein activity as a function of time. In these experiments, both proteins were pretreated with PPACMK to reduce the concentrations of active sites to below the level of detectability with synthetic substrates. Nevertheless, prekallikrein became activated indicating that enzymatic activity must still be present in some form. Significantly, the initial rate of activation of prekallikrein by Hageman factor in the fluid phase was negligible and took a period of hours to approach that of activated Hageman factor (Fig. 5B), indicating that the zymogen has at most a small fraction of the prekallikrein activating activity of the activated enzyme. In the presence of a surface, the activation of prekallikrein proceeded rapidly, but the effect of the surface was to shorten the lag phase, not eliminate it. This is consistent with the hypothesis that the surface induces a conformation in Hageman factor more susceptible to proteolytic cleavage. All of our experiments showed evidence of lag phases at early times, but even at these dilutions of Hageman factor, the acceleration of the activation of prekallikrein was
too great to permit estimation of the true rate at zero time. It is this rate that would reflect the possible activity of the zymogen or the concentration of active enzyme present. In either case, we are led to conclude that activation of Hageman factor must occur before it can express maximal activity in the activation of prekallikrein.

We attempted to distinguish between activated enzyme and zymogen activity by repeating the experiment in the presence of an inhibitor of Hageman factor derived from corn.12 In the presence of this inhibitor, no activation of prekallikrein occurred for over an hour, even in the presence of dextran sulphate. The most probable explanation for this result is that activated Hageman factor is the species required for prekallikrein activation and that the zymogen has no such activity.

If Hageman factor zymogen is inactive, it is important to consider how PPACMK-treated preparations are nevertheless able to initiate both autoactivation and contact activation. It seems likely that since Hageman factor is able to autoactivate there will arise a steady state where the rate of generation of new active sites will equal their rate of removal through reaction with PPACMK. This amount of activated Hageman factor, which is undetectable using synthetic substrates, is able to activate prekallikrein rapidly enough to initiate contact activation because the value of $k_{cat}/K_m$ for this reaction is much higher than the values found with synthetic substrates.16 Corn inhibitor, on the other hand, inhibits Hageman factor with a $K_i$ of $2.5 \times 10^{-8}\text{ M}$, two orders of magnitude lower than the $K_m$ for prekallikrein ($5 \times 10^{-6}\text{ M}$). Thus, corn inhibitor is effective in preventing prekallikrein activation. However, it should be noted that corn inhibitor is a reversible inhibitor, and therefore, we would expect that even in its presence the equilibrium level of activated Hageman would eventually produce detectable prekallikrein activation.

These considerations also apply to the macromolecular inhibitors of plasma which, with the exception of $\alpha_1$-macroglobulin, are reversible and permit low equilibrium concentrations of active enzyme.23 Our results suggest that the residue of active enzymes that escape inhibition in vivo can have a significant physiologic role. Although it is not possible to rigorously exclude enzymatic activity in the zymogen, our conclusions are that unactivated Hageman factor has, at best, several thousand fold less enzymatic activity than proteolytically cleaved forms of the protein.

REFERENCES

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