Structural Changes Accompanying Enzymatic Activation of Human Hageman Factor

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ABSTRACT The structure of Hageman factor, isolated from human plasma, was analyzed before and after enzymatic activation. The purified molecule is a single polypeptide chain of 80,000 molecular weight (mol wt) sedimenting at 4.5S. An amino acid analysis has been performed. The concentration of Hageman factor in normal human plasma was found to be 29 μg/ml with variation between individuals ranging from 15 to 47 μg/ml. Treatment of the molecule with kallikrein, plasmin, or trypsin resulted in cleavage at two primary sites, yielding fragments of 52,000, 40,000, and 28,000 mol wt. No further changes occurred in the fragments with subsequent reduction. Prekallikrein-activating ability was associated exclusively with the 28,000 moiety.

INTRODUCTION

The participation of Hageman factor in the kinin-forming, intrinsic clotting, and fibrinolytic systems has been clearly established (1–9). Two mechanisms of activation of the Hageman factor molecule in vitro have been described (10): solid-phase activation involving contact with a negatively charged surface, and fluid-phase activation in which proteolytic enzymes such as kallikrein, plasmin, or trypsin activate the Hageman factor. The transformation of Hageman factor on a negatively charged surface to a biologically active form does not appear to involve any cleavage of the molecule (10). By contrast, activation of Hageman factor by the action of proteolytic enzymes is accompanied by cleavage of the native molecule although the actual sites have not been defined nor all the resulting fragments identified.

In the present study we have investigated the structure of Hageman factor isolated from human plasma. With a realization of the importance of kallikrein and plasmin as primary activators of Hageman factor in fluid phase (8, 10, 11), we have endeavored to determine the changes in the molecule which accompany these interactions. The results are compared with those obtained by treatment with trypsin. The presence of a radiolabel on the purified molecule provided a means of detecting and characterizing fragments not possessing biologic activity that appear with enzymatic activation. This investigation was facilitated by a purification technique that produced a yield of Hageman factor sufficient to perform amino acid analyses and to determine quantitatively the amount of Hageman factor present in normal human plasma.

METHODS

In all stages of purification, storage, assay, and experimentation, contact of Hageman factor with glass was avoided. Where plastic pipettes or containers could not be used, glassware was coated with a 1% silicone solution (Siliclad, Clay-Adams, Inc., Parsippany, N. J.). Column eluates were monitored by measuring their resistances which were then, for simplicity, converted to ionic strengths based on NaCl standards.

Preparation of human Hageman factor. Human Hageman factor (HHF) [1] was prepared by a modification of the technique previously described (7). Human blood was collected into one-sixth volume acid citrate dextrose (ACD) anticoagulant, centrifuged free of formed elements, and stored in plastic at −70°C until used. For the preparation of a batch of Hageman factor, 1,600 ml plasma was dialyzed overnight against 0.01 M sodium phosphate plus 0.005 M EDTA pH 8.1 ("starting buffer") plus 50 μg/ml hexadimethrine bromide (HBr, Polybrene, Aldrich Chemical Co., Inc., Milwaukee, Wis.). The dialyzed plasma was

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[1] *Abbreviations used in this paper:* ACD, acid citrate dextrose; BAEe, benzoyl L-arginine ethyl ester; BSA, bovine serum albumin; CM, carboxymethyl; HBr, hexadimethrine bromide; HHF, human Hageman factor; OMTI, ovomucoid trypsin inhibitor; SDS, sodium dodecyl sulfate.

TABLE I

Purification of Human Hageman Factor

	Volume	Total protein*	Total HHF‡	Specific activity
	ml	*mg*	*mg*	*mg HHF/mg protein*
Normal human plasma§	1,600	94,000	40.0	0.0004
Starting material for first DEAE-Sephadex column	1,730	84,338	34.5	0.0004
Starting material for second DEAE-Sephadex column	2,222	67,082	25.4	0.0004
Starting material for CM-Sephadex column	730	41,522	18.8	0.0005
Peak tube of activity on CM-Sephadex column	20	1.8	1.9‡	1.05

* Protein concentrations were based on nitrogen determinations (Kjeldahl)

‡ Hageman factor concentrations were determined by Mancini quantitative immunodiffusion assays.

§ Includes dilution with one-sixth volume ACD anticoagulant.

‡ Note that value is for peak tube only; total recovery of column was estimated to be 14–16 mg HHF.

applied to 2,500 ml packed volume DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) which had been pretreated with starting buffer containing 50 μg/ml HBr in a 10 × 50-cm plastic column. Elution was effected with a linear salt gradient of 3 liters each of starting buffer and starting buffer plus 0.6 M NaCl. Two major protein peaks were found, with Hageman factor occurring in the second in the ionic strength range of $\mu = 0.1$–0.3. By functional and/or immunologic assays using specific antibodies, this region was found to be free of prekallikrein, clotting factor XI, fibrinogen, α_2-macroglobulin, and kininogen. The region was pooled (approximately 2 liter volume) and dialyzed against starting buffer. The euglobulin precipitate which formed was removed by centrifugation before the material was applied to a second DEAE-Sephadex A-50 column prepared identically to the first but packed to a volume of 2 liters. Elution this time was with an increasing salt and decreasing pH gradient of 3 liters each starting buffer and terminal buffer of 0.15 M NaH_2PO_4 plus 0.25 M NaCl plus 0.001 M EDTA pH 4.6. Hageman factor was found in the second half of a single protein peak between $\mu = 0.065$ and 0.129 and pH 8.10–6.85 in a total volume of approximately 1,200 ml. The absence of α_1-antitrypsin from this region was confirmed by double immunodiffusion assays. The pool was dialyzed against 0.01 M PO_4 plus NaCl to $\mu = 0.1$ pH 6.5 and applied to a 3.7 × 62-cm column of carboxymethyl (CM)-Sephadex C-50 (200 ml packed volume) with the same buffer. After the sample was applied, the column was washed with 750 ml of buffer and eluted with a gradient of 0.01 M PO_4 pH 6.5 $\mu = 0.1$ (starting) to $\mu = 0.5$ M NaCl (terminal), 2.5 liters each. A large amount of protein eluted in the wash while the gradient itself yielded two more peaks, with Hageman factor following the second at an ionic strength of $\mu = 0.30$–0.35. The absence of C1 esterase inhibitor and plasminogen was confirmed at this point by the lack of precipitation with the corresponding antibodies in double immunodiffusion assays, as well as the failure of the material to lyse fibrin upon addition of streptokinase. Electrophoresis of protein from this region on polyacrylamide gels with and without sodium dodecyl sulfate (SDS) yielded a single band upon staining. Further evidence of purity was obtained by radiolabeling the protein in the Hageman factor region with ^{125}I and passing it over a column of antihuman Hageman factor covalently bound to Sepharose 4B (Pharmacia Fine Chemicals, Inc.). Such a procedure resulted in absorption of all the ^{125}I onto the column while passage over an identically prepared column of normal goat serum yielded minimal loss of counts, supporting the assertion that all the protein present was Hageman factor. Clot-promoting activity was measured in plastic tubes by the addition to 100 μl sample±50 μg kaolin of 50 μl cephalin (Sigma Chemical Co., St. Louis, Mo., diluted 1:50) and 50 μl Hageman-deficient plasma. After 6 min incubation at 37°C, 50 μl 0.05 M $CaCl_2$ was added and the time elapsed until clot formation was recorded. A dilution of the purified Hageman factor used in these experiments containing 0.23 μg protein was capable of reducing the clotting time of Hageman-deficient plasma from 26.86 to 9.60 min (65% reduction), with kaolin the reduction was from a control time of 7.67 to 1.61 min (79% reduction). There was no prekallikrein-activating ability until the preparation was activated by negatively charged surfaces or enzymes. Specific activity and yields during the purification procedure are shown in Table I. Protein assays were performed by nitrogen determinations on a Technicon AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N. Y.). Hageman factor concentrations were based on the quantitative antigenic presence of the molecule as determined by the technique of Mancini, Carbonar, and Heremans (12). As shown in the table, the specific activity increase is almost entirely on the CM-Sephadex column; elimination of either of the DEAE-Sephadex columns, however, results in impure preparations obtained after chromatography on CM-Sephadex. The primary changes in this method over that previously used in this laboratory are the elimination of an initial fractionation with saturated ammonium sulfate and chromatography on a Sephadex G-200 gel filtration column before separation with CM-Sephadex. Elimination of these two steps has increased yields 25- to 50-fold.

Antibody to HHF. Hageman factor prepared by an earlier method (7) was used for the preparation of antibody. Weekly injections totaling 10–40 μg protein in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.), were administered to a year-old goat in multiple subcutaneous sites. After the 4th week serum was collected and tested. Trace contaminants were adsorbed with either Hageman-deficient plasma or purified contaminant yielding a monospecific antibody. For use in Mancini radial immunodiffusion tests, the purified antibody was diluted 1:25 and the technique followed as published (12).

Preparation of rabbit prekallikrein. For assay purposes, semipurified rabbit prekallikrein at a concentration of 31 or 12.5 μg/ml was generally employed. This material was prepared according to previously published methods (13), being used after the second DEAE-Sephadex column.

Preparation of human kallikrein. Prekallikrein from human plasma was prepared according to a modification of the technique used for the purification of rabbit prekallikrein (13). Briefly, the globulin portion of human plasma soluble in 25% saturated ammonium sulfate and precipitated at 55% saturation was applied to a column of DEAE-Sephadex A-50 equilibrated with 0.01 M phosphate buffer plus 0.001 M EDTA pH 8.2 after dialysis against this buffer. Proteins were eluted with a linear salt gradient.

Prekallikrein was found from $\mu = 0.03$ to 0.09, the region pooled and dialyzed against a $\mu = 0.04$ citrate-phosphate buffer pH 6.2 plus NaCl to $\mu = 0.07$ and applied to a column of CM-Sephadex C-50. Prekallikrein was eluted by a gradient of NaCl in the region of $\mu = 0.22$–0.24 well separated from factor XI which eluted from $\mu = 0.29$–0.31. Plasminogen proactivator was not assayed. Contaminating proteins present in the prekallikrein fractions were identified as β_2-glycoprotein I and gamma globulin. The prekallikrein was concentrated by ultrafiltration using an Amicon UM-10 filter (Amicon Corp., Lexington, Mass.) to a concentration of approximately 20–50% that present in normal human plasma. Activation to kallikrein was accomplished by incubation of 0.7 ml prekallikrein with 100 μl suspended Enzite agarose trypsin beads (Miles-Seravac Ltd., Berkshire, England) for 30 min at 37°C. The beads were removed by centrifugation and the supernate assayed for activity by hydrolysis of benzoyl-L-arginine ethyl ester (BAEe). 25 μl (approximately 15 μg protein) hydrolyzed 5 μmol BAEe/min. Confirmation of complete activation of the prekallikrein to kallikrein was tested by further treatment with trypsin.

Plasmin. Plasmin was obtained from purified human plasminogen (kindly supplied by Doctors J. A. Brockman, E. C. DeRenzo, and P. H. Bell of Lederle Laboratories, Pearl River, N. Y.) by incubation with 180 U streptokinase (Lederle Laboratories)/40 μg plasminogen for 10 min at 37°C. This material had a specific activity of 34 CTA[2] U/mg using the casein assay described by Johnson, Kline, and Alkjaersig (14). Before activation the plasminogen gave a single band on SDS-polyacrylamine gel.

Assay of the ability of Hageman factor to activate prekallikrein. The ability of Hageman factor or its fragments to activate prekallikrein was tested as follows: samples not exceeding 0.2 ml were brought to neutral pH with 0.1 M Tris buffer pH 7.6 in plastic tubes before the addition of 0.1 ml containing 1.25 μg rabbit prekallikrein (3.1 μg when minimal activity was expected). After incubation at 37°C for 20 min, 3 ml of 1 mM BAEe in 0.01 M Tris buffer pH 7.6 containing 0.15 M NaCl was added to each assay tube. At 20 min and again at later times if necessary, absorbance of the solutions was measured at 253 nm which is specific for the benzoyl arginine cleaved from BAEe by kallikrein, thus giving a qualitative measurement of the amount of active Hageman factor in the assay tube. If unactivated Hageman factor were to be assayed, it was first converted to an active form. Generally this was accomplished by adding 0.2 μg trypsin (2× crystallized, Worthington Biochemical Corp., Freehold, N. J.) to the sample (pH 7–8), incubating 20 min at 37°C, and blocking the trypsin with at least 10 μg ovomucoid trypsin inhibitor (OMTI, Worthington Biochemical Corp.), before the addition of prekallikrein. Alternatively, the molecule could be converted to a form also capable of cleaving prekallikrein by absorption onto 500 μg kaolin for 15 min at 22°C, removal of the supernate after a brief centrifugation, followed by addition of prekallikrein to the kaolin precipitate. (It must be noted that the ratio of kaolin to Hageman factor is critical and often a dose-response curve must be made if optimal activation is desired [10]). This latter method of activation was used for assays of the first two columns in the purification procedure or other instances where trypsin inhibitors may have been present.

Radiolabeling. Proteins were radiolabeled with iodine-131 or -125 as noted according to the method of McConahey and Dixon (15). Hageman factor was dialyzed after the radiolabeling procedure against 0.15 M sodium chloride buffered with 0.01 M phosphate pH 7.0.

Sucrose density gradients. 5-ml linear gradients of 5–20% sucrose in 0.01 M Tris buffer plus 0.15 M NaCl pH 7.4 were employed. Sample volumes of up to 0.3 ml were applied to the top of the gradients and centrifugation carried out in a Beckman model L ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) at 45,000 rpm (165,000 *g*) for 16–17 h. At the termination of the run, the gradients were harvested dropwise from the bottom of the tubes in 10-drop fractions (approximately 0.17 ml) into plastic or siliconized glass tubes as noted.

Disk electrophoresis. 7% alkaline polyacrylamide gels were prepared according to the method of Davis (16) modified as follows: 2 ml of small pore gel was used in tubes measuring 6 × 100 mm. A single layer of 0.25 ml large pore gel was used as a spacer and a sample of 0.2–0.3 ml in 10% sucrose was applied above it. Electrophoresis was carried out for approximately 1½ h at 6 mA/tube in a Tris-glycine buffer pH 8.2, or until a bromophenol blue marking dye added to the top buffer had reached the end of the gels. Gels containing radioactive proteins were frozen on solid CO_2 and sectioned into 1.2-mm segments with a wire cutter. Radioactivity of the segments was then counted. Sections from gels to be eluted were placed in siliconized tubes coated with bovine serum albumin (BSA, 5X crystallized, Armour Pharmaceutical Co., Kankakee, Ill.) and buffer added as noted.

SDS-acrylamide gel electrophoresis. 7% acrylamide gels containing 0.1% SDS were prepared and run according to the method of Weber and Osborn (17) in glass tubes measuring 4.5 × 100 mm. Gel was added to a height of 80 mm and the samples applied in glycerol. Electrophoresis differed from the reported method only in the elimination of mercaptoethanol and use of one-half the recommended amperage for the first 45 min. Gels to be counted for radioactivity were treated in the same manner as the polyacrylamide gels (see above). Gels containing marker proteins were stained for 1 h in a Coomassie blue staining solution and destained with repeated changes of the acetic acid-methanol destaining solution. Mobilties of all bands and radioactive fragments were always computed relative to BSA and unknown mol wt determined by a semilogarithmic graph of the relative mobilities vs. mol wt of the marker proteins. Reducton of proteins before electrophoresis was performed as noted.

Amino acid analysis. A 3-ml sample containing approximately 200 μg purified Hageman factor was brought to pH 8 with 0.1 M $NaHCO_3$ after the addition of guanidine hydrochloride (Ultra-pure, Mann Research Labs, Inc., New York) to a final concentration of 6 M. After 15 min at room temperature, 25 μl mercaptoethanol (Eastman Kodak Co., Rochester, N. Y.) was added, followed 15 min later by 50 mg recrystallized bromoacetic acid. Neutrality was restored with 1 N NaOH, the sample dialyzed against water, and lyophilized. After 24 h of HCl hydrolysis, the material was dried, brought up to 3 ml with pH 2.2 buffer and analyzed with a Beckman model 120 C amino acid analyzer (Beckman Instruments, Inc.) by a modification (18) of the method of Spackman, Stein, and Moore (19).

[2] 1 CTA plasmin U, as defined by the N.H.I. Committee on Thrombolytic Agents, Subcommittee for Standardization, is that amount of plasmin which releases 0.1 μeq of tyrosine/min from casein under standard assay condition (14).

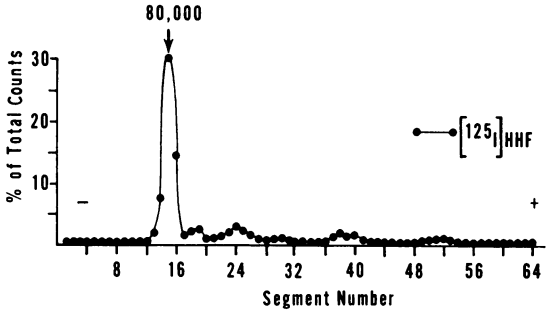

FIGURE 1 SDS-acrylamide gel electrophoresis of radiolabeled HHF. Protein was reduced and alkylated in 12 M urea before electrophoresis. Mol wt is based on comparison with marker proteins run in parallel gels.

RESULTS

Characterization of HHF

It was essential to characterize the native HHF molecule before examining how it was affected by various enzymes. Mol wt was determined by subjecting ^{125}I-labeled HHF to SDS-acrylamide gel electrophoresis after reduction in the presence of 12 M urea by 0.02 M dithiothreitol for 10 min at 37°C followed by 0.05 M iodoacetate for 10 min. A single peak of radioactivity appeared at a position of 80,000 mol wt as compared with standard marker proteins run in parallel gels (Fig. 1). Although the molecule appeared to be a single polypeptide chain, it was possible that cysteines involved in interchain disulfide bonds might be positioned within the three-dimensional structure in such a way that they might have resisted reduction under the conditions used. A stronger denaturant, 6 M guanidine, was employed, and reduction of labeled HHF was performed with mercaptoethanol followed by alkylation with bromoacetate (see Methods). A mol wt of 80,000 was again observed upon SDS-acrylamide electrophoresis. Utilization of the denaturant guandine should afford reduction of all interchain disulfides. Furthermore, amino acid analysis of HHF reduced under these conditions showed no half-cysteines. It was concluded that the 80,000 mol wt HHF consisted of a single polypeptide chain.

Ultracentrifugation of [^{125}I]Hageman factor in a sucrose density gradient revealed a sedimentation rate of 4.5S (Fig. 2). The HHF was identified by both the ^{125}I label and the ability to activate prekallikrein after treatment with trypsin.

An amino acid analysis was performed on approximately 200 μg purified HHF after reduction with mercaptoethanol in 6 M guanidine. The results are given in Table II.

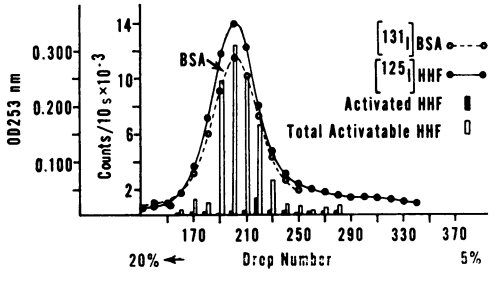

FIGURE 2 Sucrose density ultracentrifugation of purified HHF. Sedimentation at 45,000 rpm, 16 h. Fractions were assayed for spontaneously activated Hageman factor by the addition of rabbit prekallikrein followed by BAEe. Hydrolysis of BAEe was measured by increased optical density at 253 nm. Potentially activatible HHF was determined by treatment of the fractions with trypsin before prekallikrein incubation.

Determination of the concentration of Hageman factor in human plasma

By using quantitative immune diffusion assays (12) with a purified preparation HHF as standard, it was possible to quantitate the amount of HHF in plasma. A pool of plasma collected into one-sixth volume ACD from eight individuals was made, aliquoted at −70°C, and thereafter referred to as "standard normal human plasma". This plasma was found to have a concentration of 29 μg Hageman factor/ml after correction for the dilution due to anticoagulant. Plasmas from 17 normal individuals were assayed and concentrations of HHF were found to vary from 15 to 47 μg/ml. Little variation (< 5 μg/ml) was seen in the concentration of Hageman factor in plasma from any one individual bled at different times over a 3-mo period. When tested with antibody not absorbed with Hageman factor-deficient

TABLE II
Amino Acid Composition of Human Hageman Factor

Amino acid	Residues per molecule	Amino acid	Residues per molecule
Lysine	47.9	Glycine	72.1
Histidine	26.5	Alanine	52.7
Arginine	41.0	Valine	37.9
CM-cysteines	26.4	Methionine	2.1
Aspartic acid	61.0	Isoleucine	20.6
Threonine*	41.4	Leucine	61.9
Serine*	72.2	Tyrosine	20.1
Glutamic acid	89.2	Phenylalanine	26.3
Proline	43.1	Tryptophan	ND‡
		Total§	742.4

* Threonine and serine were corrected for 5 and 10% destruction during 24 h of hydrolysis respectively (20).
‡ Not determined.
§ Total was based on a mol wt value of 80,000.

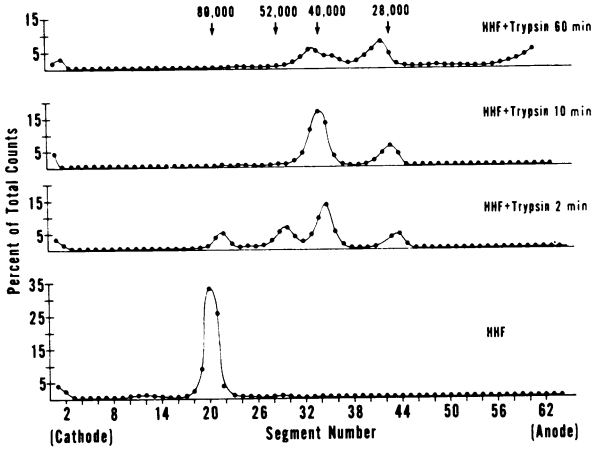

FIGURE 3 Electrophoresis on SDS acrylamide gels of HHF treated with a constant amount of trypsin for varying periods of time.

plasma, plasma from an individual with known Hageman factor trait failed to show the presence of Hageman factor protein in a concentration equal to that in a 1:20 dilution of normal human plasma. This would tend to confirm the conclusion by Smink in 1967 that Hageman factor trait reflects a deficiency in the plasma of protein with antigenic similarity to Hageman factor (21). Two Fletcher trait (prekallikrein-deficient), one clotting factor XI-deficient, and two hereditary angioneurotic edema plasmas all showed normal levels of Hageman factor.

Molecular changes in HHF associated with activation by various enzymes

Treatment with trypsin. Radiolabeled HHF was treated with 0.2 μg trypsin/50 μl reaction volume. At various times the reaction was stopped by the addition of 10 μg OMTI. The samples were then either placed directly on SDS gels or reduced and alkylated before the electrophoresis. No differences were observed in the results of the two different treatments except for slightly higher calculated mol wt for the nonreduced

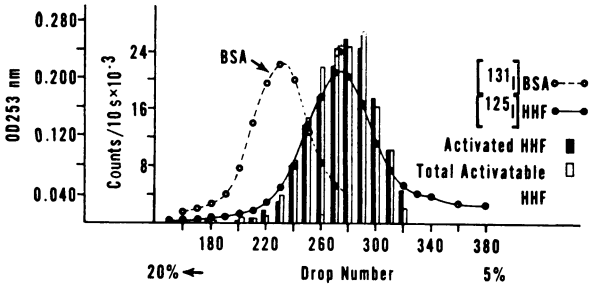

FIGURE 4 Sucrose density ultracentrifugation of HHF after treatment with trypsin for 20 min. Sedimentation and assay conditions same as for Fig. 2.

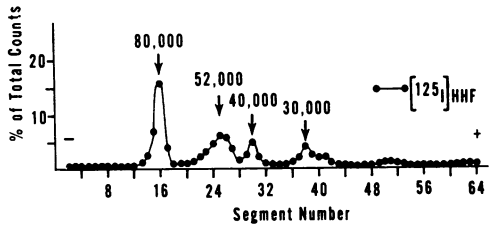

FIGURE 5 SDS-acrylamide gel electrophoresis of HHF treated with kallikrein.

samples. The results for three incubation times are shown in Fig. 3. (All mol wt pertain to reduced samples unless otherwise noted). Exposure to trypsin for short periods yielded fragments of mol wt 52,000, 40,000, and 28,000. The 52,000 fragment was quickly lost upon further trypsin treatment with a concurrent increase in the 40,000 peak being noted. Since no fragments of 12,000 mol wt or less were noted in the SDS gels, this presumably represents a region of the molecule unlabeled with ^{125}I. With prolonged periods of trypsin treatment, all the peaks were further cleaved into small fragments as noted in the uppermost panel in Fig. 3. That at least one of the fragments produced by trypsin treatment was biologically active was confirmed by sedimentation studies in sucrose gradients. As shown in Fig. 4, both prekallikrein-activating capacity and radiolabel appeared in the 2.6–3.0S region, a distinct shift from the 4.5S position of the native molecule (Fig. 2).

Treatment with kallikrein. Trace amounts of radiolabeled Hageman factor were added to 200 μl purified unlabeled HHF and incubated with 200 μl partially purified human kallikrein for 30 min at 37°C. At the end of the incubation period a 50-μl aliquot was removed, reduced and alkylated, and subjected to electrophoresis in an SDS acrylamide gel. The remainder was applied to a 5–20% sucrose gradient and centrifuged at 45,000 rpm for 17 h. The results of these two procedures are shown in Figs. 5 and 6. As can be seen, the SDS electrophoresis yields radioactive peaks at mol wt positions of 80,000, 52,000, 40,000, and 30,000 while

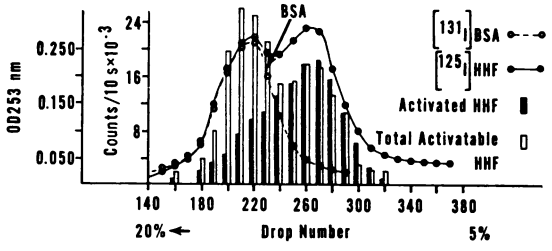

FIGURE 6 Sucrose density ultracentrifugation of HHF treated with kallikrein (same material as in Fig. 5). Sedimentation was for 17 h at 45,000 rpm. Assays as in Fig. 2.

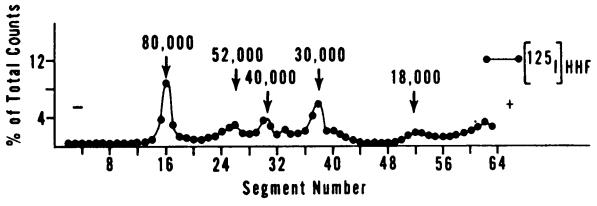

FIGURE 7 SDS-acrylamide gel electrophoresis of HHF treated with plasmin. See text for incubation details.

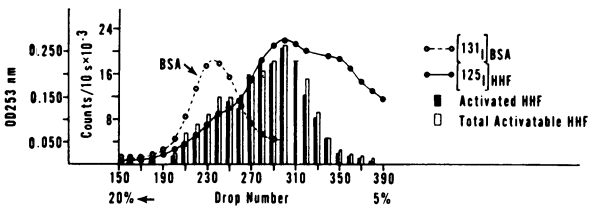

FIGURE 8 Sucrose density ultracentrifugation of plasmin-treated HHF. Sedimentation at 45,000 rpm for 17 h. Assays as in Fig. 2.

the sucrose gradient shows a biphasic profile with peaks at 4.5S and approximately 3.0S. Cleavage and activation under these conditions were not complete; that is, material remained which was 80,000 in mol wt and sedimented at 4.5S. Although this uncleaved portion of the sample had little prekallikrein-activating ability, such activity could be generated by treating the 4.5S region with trypsin as noted in Fig. 6. The material which did contain the biologic activity sedimented at approximately 2.6–3.0S but whether this represented the 30,000, 40,000, or 52,000 mol wt fragment observed on SDS gels could not be determined from these experiments.

Treatment with plasmin. 80 μg plasmin (0.2 ml) was incubated with 0.2 ml Hageman factor containing trace quantities of [^{125}I]HHF for 30 min at 37°C. A small aliquot was removed, treated with dithiothreitol in the presence of 12 M urea, alkylated with iodoacetamide, and subjected to electrophoresis in SDS acrylamide gels. The result is shown in Fig. 7. Peaks are observed at 80,000, 52,000, 40,000, 30,000, and < 18,000. The remainder of the plasmin-treated sample was sedimented at 45,000 rpm for 17 h in a 5–20% sucrose gradient. As shown in Fig. 8, the radioactivity appeared spread from the top of the gradient to the 4.5S position defined by the [^{131}I]BSA marker. The fractions were tested with and without treatment with trypsin for their ability to cleave prekallikrein. Such ability appeared in the regions from approximately 2.6 to 4.5S and was not increased by the trypsin treatment. The possibility of plasmin being responsible for the cleavage of prekallikrein was ruled out by negative controls of appropriate quantities of plasmin directly added to prekallikrein. No biologic activity was associated with the very small fragments (< 2.6S). Neither plasminogen or streptokinase alone had any effect on Hageman factor.

Identification of the Hageman factor fragment containing prekallikrein-activating capacity after enzymatic degradation

To determine whether the active site for prekallikrein activation resided in the 52,000, 40,000, or 28,000 mol wt peak obtained with enzymatic treatment, it was necessary to separate these fragments without destroying the activity of the molecule (gel filtration and sucrose gradient ultracentrifugation proved unsuccessful in this regard). Electrophoresis in 7% polyacrylamide gels yielded a multipeak pattern of [^{125}I]HHF treated with trypsin for 10 min (Fig. 9, bottom panel). The presence of the three fragments as well as a small amount of uncleaved 80,000 mol wt material was confirmed by SDS-acrylamide electrophoresis before the sample was applied to the polyacrylamide gels not containing SDS. Elution of the polyacrylamide gel slices with 0.2 ml Tris-buffered saline into BSA-coated siliconized tubes enabled us to perform assays to determine

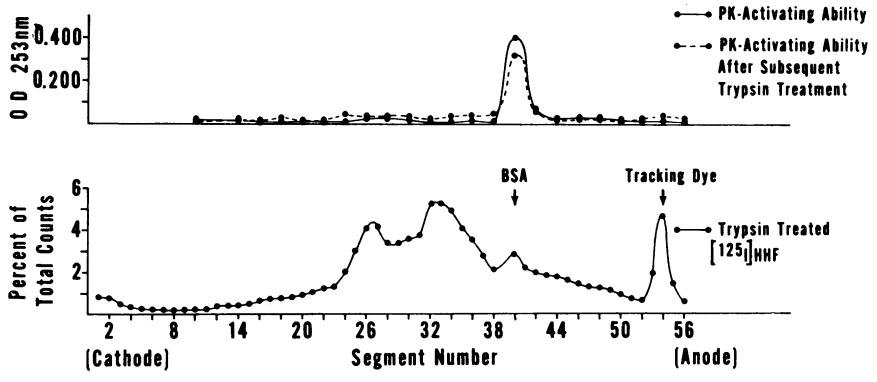

FIGURE 9 Electrophoresis of HHF in 7% alkaline polyacrylamide gel after treatment with trypsin for 10 min. Bottom panel shows the position of the radioactive fragments. Top panel shows ability of these fragments to activate prekallikrein (PK).

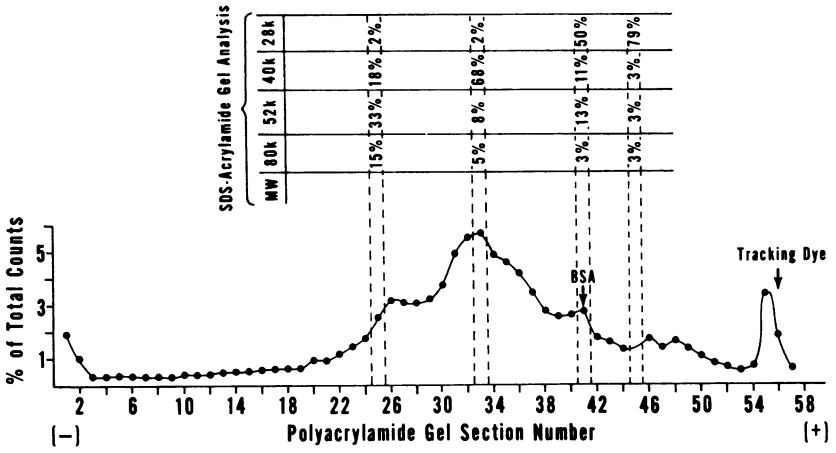

FIGURE 10 Analysis by SDS acrylamide gels of the molecular weights of radiolabeled protein fragments present in four slices from a polyacrylamide gel of trypsin-treated Hageman factor (similar sample as in Fig. 9). Each of the designated slices was placed on top of a regular SDS acrylamide gel, overlayed with SDS buffer, and subjected to electrophoresis as usual. The SDS gels were then sectioned and counted for radioactivity. The percent of the total counts occurring in the positions known to correspond with mol wt of 80,000, 52,000, 42,000, and 28,000 are indicated.

in which fragment the biologic activity resided. Ability to activate prekallikrein directly or after incubation with 0.1 μg trypsin was tested using 50-μl aliquots of the gel eluates. Both assays showed a single sharp peak associated with the fragment migrating coincident with BSA (Fig. 9, top panel).

The mol wt of the various peaks obtained on polyacrylamide electrophoresis were determined by placing individual gel slices directly on top of SDS acrylamide gels, overlaying with buffer, and conducting the electrophoresis in the usual manner. The percentage of total radioactivity in the mol wt regions of 80,000, 52,000, 40,000, and 28,000 were computed for each polyacrylamide slice tested. The results are shown in Fig. 10. By comparing the radioactivity profile with that of Fig. 9, it can be seen that the prekallikrein-activating potential lies in the 28,000 fragment.

DISCUSSION

The data show HHF to be a single polypeptide chain. A mol wt value of 80,000 was obtained by electrophoresis in acrylamide gels containing SDS under reducing conditions. Using this mol wt, a computation of the number of residues of each amino acid per Hageman factor molecule was made after an amino acid analysis of a purified preparation (Table II). Numerous basic amino acids are present which may account for the binding of the molecule to negatively charged surfaces. The very low number of methionines (approximately two residues per molecule) may prove useful in further studies of the structure by virtue of the ability of cyanogen bromide to cleave a polypeptide chain specifically at this residue. It is of interest to note than an amino acid composition reported in 1965 by Speer, Ridgway, and Hill (22) for HHF has generally the same relative amounts of the different amino acids, with the exception of cysteine, although the actual values differ. It was unclear, however, whether the analysis reported by these workers was for the uncleaved 80,000 mol wt molecule or some fragment of it.

The concentration of Hageman factor in human plasma was found to be 29 μg/ml, with a range among 17 individuals of from 15 to 47 μg/ml. These values, based on immunologic assay techniques, are in reasonable agreement with the value on can extrapolate from the purification data for "activated" Hageman factor reported by Ratnoff and Davie in 1962 (23) in which a functional assay was used. The current studies also show relatively little variation (± 5 μg/ml) in the concentration of the protein in any one person's plasma obtained at different times. These data are preliminary to studies on the variation in Hageman factor concentration that may occur during the course of specific diseases.

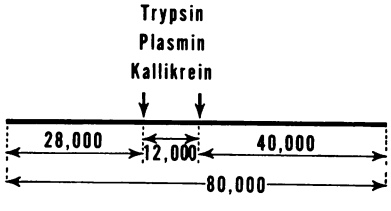

FIGURE 11 Proposed sites of cleavage by enzymatic activators of HHF.

The HHF molecule has been shown to be cleaved at two primary sites during activation by various enzymes: trypsin, plasmin, kallikrein (the possibility exists of potential contamination of the kallikrein with plasminogen activator contributing to the results obtained with this enzyme). This is presented diagramatically in Fig. 11. Knowledge of the patterns of cleavage of HHF by enzymes from the plasma will be of considerable help in defining the mechanisms of activation of the molecule in vivo. Activation in solid phase by negatively charged surfaces is not accompanied by cleavage of the molecule (10), while enzymes from the plasma activate and cleave Hageman factor (8, 10, 11). The importance of the cleavage in directing activity along a particular pathway, i.e. the kinin forming, intrinsic clotting, or fibrinolytic systems, has yet to be defined.

The active site on Hageman factor, as defined by the ability to activate prekallikrein to kallikrein, is clearly associated with the 28,000 mol wt fragment that results from enzymatic activation. Studies to show that this fragment. exclusively, also contained the region responsible for activation of clotting factor XI were not definitive. Clotting assays performed on the polyacrylamide gel eluates showed clot-promoting ability associated with the most cathodal peak as well as the anodal 28,000 mol wt peak. Since the cathodal peak contained both the uncleaved 80,000 mol wt molecule, which was known to have clotting potential, and the 52,000 mol wt fragment, the absence of a factor XI-activating site on the 52,000 mol wt fragment could not be confirmed. The association of some clotting activity with the 28,000 mol wt peak, however, supports earlier reports of a Hageman factor-derived molecule of this size possessing the capacity to induce clotting in Hageman trait plasma (7, 24). The ability of the fragments to activate plasminogen proactivator was not tested in these experiments. However, Kaplan and Austen have reported such capacity associated with low mol wt fragments of Hageman factor (9).

The two sites of trypsin cleavage appear to be equally sensitive to the enzyme. When the initial cleavage yields the 52,000 and 28,000 fragments, however, the 52,000 is rapidly cleaved at the 40,000 position. The remaining fragment of 12,000 daltons is usually not seen as a discrete peak implying either a further breakdown of the peptide or perhaps a deficiency in this region of tyrosine residues which would couple the iodine-125.

That the overall charges of the fragments produced by trypsin treatment of HHF are similar is suggested by the observance that they migrate in alkaline polyacrylamide gels in order of decreasing mol wt.

Hageman factor of rabbit origin differs structurally from that of the human molecule (25). It has been found to be a molecule of approximately 90,000 mol wt composed of three polypeptide chains linked by disulfides near the ends of the chains. Upon either reduction or trypsin treatment, it yields fragments of 30,000 mol wt. Why the structure of these two functionally similar molecules should differ so greatly is of considerable interest. Several possible explanations present themselves: the rabbit Hageman factor may be initially synthesized as a single polypeptide chain but upon release from its RNA template or secretion from its cell of origin becomes cleaved between disulfide linkages. Such a situation exists with several other proteins, such as insulin (26). Alternatively, the rabbit Hageman factor could undergo partial cleavage during purification at points between intrachain disulfide bonds; or, the molecules from the two species may indeed differ structurally in their native form.

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