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Amidolytic properties of single-chain activated Hageman factor

(Factor XI/Factor XII/Fletcher factor/Fitzgerald factor)

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ABSTRACT **Activation of Hageman factor (Factor XII) upon exposure to negatively charged agents has been attributed to proteolytic cleavage of this molecule. To examine this question, purified Hageman factor was exposed to Sephadex gels to which ellagic acid had been adsorbed. Such Hageman factor, separated from the gels and studied in the fluid phase, was amidolytic. Nonetheless, no cleavage of Hageman factor treated in this way could be demonstrated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Thus, activation of Hageman factor by negatively charged agents was not necessarily accompanied by molecular scission.**

When Hageman factor (HF, Factor XII) is exposed to certain negatively charged substances, it acquires the property of activating plasma thromboplastin antecedent (PTA, Factor XI) and plasma prekallikrein (Fletcher factor). The nature of this activation of HF has been disputed. Early views, that negatively charged agents induce a conformational change in HF that exposes enzymatically active groups, have been supported by several indirect techniques (1–3). More recently, evidence has been provided that activation of HF depends upon its proteolytic cleavage within an internal disulfide loop (4, 5).

The present study examines the activation of HF in the fluid phase. Purified HF was mixed with Sephadex gels to which an activator, ellagic acid, had been adsorbed. HF treated in this way and separated from the gels activates PTA in the presence of high molecular weight kininogen (HMWK) (6). The experiments to be described demonstrate that HF that had been exposed to Sephadex-ellagic acid in the apparent absence of proteolytic agents was amidolytic, yet had not undergone significant cleavage, as though scission of HF were not required for its activation.

MATERIALS AND METHODS

Normal human plasma was separated from venous blood containing 1/50th vol of 0.5 M sodium citrate buffer (pH 5.0). A standard pool of normal adult plasmas was said to contain HF, plasma prekallikrein, HMWK, and PTA, each at 1 unit/ml (7). Crude immunoglobulins were separated from normal rabbit serum or from monospecific rabbit antiserums to human HF (8), kallikrein (9), HMWK (10), and plasminogen (Behring Diagnostics, Somerville, NJ) (6, 11). Ellagic acid was dissolved as described (6).

Sephadex-ellagic acid was prepared by mixing Sephadex G-10 (Pharmacia) that had been swollen in barbital/saline buffer (pH 7.5) with 0.1 mM ellagic acid, as described (6). [Unless otherwise noted, the term buffer refers to barbital/saline buffer (25 mM sodium barbital/125 mM sodium chloride at pH 7.5)]. The Sephadex-ellagic acid was washed repeatedly in buffer, reducing the content of ellagic acid in the washes to less than 0.1 μM. Sephadex/buffer was prepared in the same way, omitting ellagic acid (6).

Purified HF (12) varied in specific activity from 63 to 83 units/mg of protein. ^{125}I-labeled HF [^{125}I-HF, 30 μCi/μg (1 Ci = 3.7×10^{10} becquerels)] was dissolved in buffer or in 0.1% bovine albumin in buffer (8). Partially purified bovine thrombin, depleted of Stuart factor (Factor X), contained 41 National Institutes of Health units/ml (1250 units/mg of protein) in 50 mM sodium phosphate buffer (pH 7.0) and was diluted in buffer before use (13).

Bovine serum albumin (crystallized; Miles) was dissolved at a concentration of 1% in buffer.

H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide (S2238, Ortho Diagnostics, Raritan, NJ) was dissolved in water at 1 mM and stored at 4°C. Imidazole/Tris/saline buffer (pH 8.2) contained 25 mM Tris·HCl (Sigma) and sufficient sodium chloride to provide an ionic strength of 0.15.

Purified HF was "activated" by exposure to Sephadex-ellagic acid in the presence of bovine serum albumin as described (6).

Amidolytic activity was measured in the supernatant of mixtures of purified HF, albumin, and Sephadex-ellagic acid, as noted in the footnote to Table 1.

The molecular size of ^{125}I-HF that had been exposed to Sephadex-ellagic acid was measured by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis after reduction by 2-mercaptoethanol. A sample of 0.3 ml of ^{125}I-HF (approximately 2×10^5 cpm) was mixed with 1.0 ml of settled Sephadex-ellagic acid or Sephadex/buffer and centrifuged at $1800 \times g$ for 15 min at 2°C. Supernatant (0.15 ml) was incubated with 0.15 ml of 4% $NaDodSO_4$ in 2% 2-mercaptoethanol/10 mM sodium phosphate buffer (pH 7.0) or $NaDodSO_4$ alone overnight at room temperature. Samples (0.2 ml) were then subjected to gel electrophoresis (14). In other experiments, 0.2 ml of a mixture of ^{125}I-HF (approximately 8×10^4 cpm) and unlabeled HF [final concentration 2.5 units (4.6 μg/ml)] was mixed with 2.0 ml of settled volume of Sephadex-ellagic acid or Sephadex/buffer, and the mixture was centrifuged at 30,000 $\times g$ for 15 min at 2°C. Supernatant (0.1 ml) was incubated with 0.1 ml of 4% $NaDodSO_4$/2% 2-mercaptoethanol/10 mM sodium phosphate buffer (pH 7.0) for 3 min at 100°C. Samples were then subjected to 6% polyacrylamide gel electrophoresis. The gels were fixed and stained with Coomassie blue and quick-frozen on blocks of solid carbon dioxide. Slices 2 mm in width were cut with a gel slicer (Mickel Laboratory Engineering, Co., Surrey, England), and radioactivity was measured in a Nuclear Chicago gamma counter (model 1085). Molecular weight was estimated in comparison to bovine serum albumin (M_r 67,000), human transferrin (Behringwerke, A. G., West Germany, M_r 88,000), and pepsin (Worthington, M_r 35,000).

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Abbreviations: HF, Hageman factor; PTA, plasma thromboplastin antecedent; HMWK, high molecular weight kininogen; S2238, *H*-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide; $NaDodSO_4$, sodium dodecyl sulfate.

Table 1. Amidolytic properties of HF exposed to Sephadex-ellagic acid

Enzyme	Antiserum added	*p*-Nitroaniline released: nmol per ml/60 min	*p*-Nitroaniline released: mol of substrate per mol of enzyme/min
HF	Barbital/saline buffer	8.8	28
HF	Normal rabbit immunoglobulin	11.2	—
HF	Anti-HF immunoglobulin	2.7	—
HF	Antiplasmin immunoglobulin	10.8	—
HF	Antikallikrein immunoglobulin	10.1	—
HF	Anti-HMWK immunoglobulin	9.9	—
Thrombin	Barbital/saline buffer	900.0	16,740

One-tenth milliliter each of purified HF [7 units (0.1 mg of protein per ml)] and 1% bovine serum albumin in buffer were mixed with 1.0 ml of Sephadex-ellagic acid that had been washed 15 times. The mixture was then diluted with the same buffer to 2.0 ml, and the supernatant was separated by centrifugation. The supernatant contained 0.8 units of activated HF per ml. Supernatant (0.1 ml) was incubated for 15 min at 37°C in polystyrene tubes with 0.1 ml of buffer or undiluted immunoglobulin fractions of normal rabbit serum or antiserum. Thereafter, 0.3 ml of barbital/saline buffer, 1.0 ml of imidazole/Tris buffer (pH 8.2), and 1.0 ml of 1 mM S2238 were added, the mixtures were incubated at 37°C for an additional 60 min, the reaction was stopped by addition of 0.3 ml of glacial acetic acid, and the absorbance at 405 nm was read against blanks consisting of similarly treated solutions in which HF was replaced with 0.1 ml of barbital/saline buffer. As a control, 0.1 unit of bovine thrombin [2.8 units (2.2 μg of protein) per ml] was incubated with 0.4 ml of barbital/saline buffer, 1.0 ml of imidazole/Tris buffer, and 1.0 ml of 1 mM S2238 for 1 min; the assay was carried out in the same manner.

RESULTS

Amidolytic Properties of HF. In experiments described separately (6), HF, in plasma or in purified form, was so altered by exposure to Sephadex-ellagic acid that it activated PTA in a substrate of HF-deficient plasma. The alteration in HF induced by Sephadex-ellagic acid occurred in the apparent absence of HMWK, plasma prekallikrein, and plasminogen, but activation of PTA required the presence of HMWK.

To determine whether enzymatic properties emerged when HF was exposed to Sephadex-ellagic acid, purified HF was mixed with this gel, and, after centrifugation, the super atant solution was incubated with S2238. Weak amidolytic activity evolved; under the conditions tested, amidolysis by bovine thrombin was 600-fold greater on a molar basis (Table 1). Amidolysis was inhibited by immunoglobulins against HF, but not against plasminogen, plasma kallikrein, or HMWK. No amidolytic activity developed when HF was mixed with Sephadex/buffer.

Molecular Alterations in HF Exposed to Sephadex-Ellagic Acid. Recently, Revak *et al.* (4) reported that HF was severed within an internal disulfide loop when plasma was exposed to glass and suggested that activation of HF depended upon this proteolytic change. These experiments were readily confirmed (Fig. 1*A*). In contrast, significant cleavage of HF was not observed under the conditions used in the present study. When ^{125}I-HF in bovine-serum albumin was exposed to Sephadex-ellagic acid, a peak of radioactivity corresponding to a M_r of approximately 80,000 was found upon NaDodSO$_4$/gel electrophoresis (Fig. 1*B*); the yield was usually about 1/10th of that obtained when ^{125}I-HF was exposed to Sephadex/buffer (Fig. 1*C*), in agreement with experiments of the coagulant activity of HF exposed to Sephadex-ellagic acid (6). When HF treated in this way was reduced before electrophoresis, little or no evidence of cleavage within a disulfide loop was obtained. In four of six experiments, a small increment of radioactivity, approximately 1.5% of that of the 80,000 M_r peak, was seen in one slice of the gel at a site corresponding to a M_r of 53,000 (Fig. 1*B*); none was seen in the other two trials. No increment in radioactivity was observed in slices corresponding to a M_r of about 30,000. In these experiments, the proportion of HF to Sephadex-ellagic acid was less than in the functional experiments described earlier (6). When sufficient unlabeled HF was mixed with ^{125}I-HF to duplicate these functional studies, no significant cleavage of HF was observed. ^{125}I-HF was also added to *normal* plasma and exposed to Sephadex-ellagic acid, and the supernatant was reduced before NaDodSO$_4$/gel electrophoresis. Under these conditions, as much as 15% of the HF appeared to be converted to a 53,000 M_r species.

DISCUSSION

How negatively charged substances convert HF to an enzymic form is uncertain. Two views, not mutually exclusive, have been proposed: (*i*) HF undergoes a conformational change upon exposure to negatively charged substances that bring enzymatically active groups to its surface (1–3), and (*ii*) activation of HF requires its proteolytic cleavage (4, 5). The present study approaches this question by utilizing a novel technique for preparation of fluid-phase HF that has been altered by a negatively charged agent, ellagic acid. HF, treated in this way, activates PTA, but only if HMWK is present in the substrate, HF-deficient plasma (6). Such experiments suggest that HMWK may be needed for expression of the coagulant properties of activated HF rather than for activation of HF. Support for this view came from studies of purified HF. This agent, when exposed to Sephadex-ellagic acid, hydrolyzed S2238 as well as correcting the clotting defect of HF-deficient plasma. HMWK was not needed for conversion of HF to its amidolytic form. These studies do not rule out an undetected change in HF during purification, nor that the amidolytic properties of HF belonged to molecules that were not clot-promoting.

Human HF is a single-chain molecule of about M_r 80,000 (1, 15). When partially digested by plasmin (5, 16), trypsin (17, 18), or plasma kallikrein (19), a subunit of M_r about 30,000 is separated that readily converts plasma prekallikrein to kallikrein but is much less effective in activating PTA. Revak *et al.* (4) reported that purified ^{125}I-HF, mixed with human plasma, was cleaved upon exposure to glass, first within an internal disulfide loop, changing HF to a two-chain species, and then more slowly into its high and low molecular weight subcomponents. Revak *et al.* did not report clotting studies. In somewhat similar experiments, Fujikawa *et al.* (5) separated a two-chain species of bovine HF that was more active than the single-chain parent molecule in clotting assays.

That HF mixed with Sephadex-ellagic acid was amidolytic demonstrated that it had acquired enzymatic properties. To determine whether this activation of HF was accompanied by

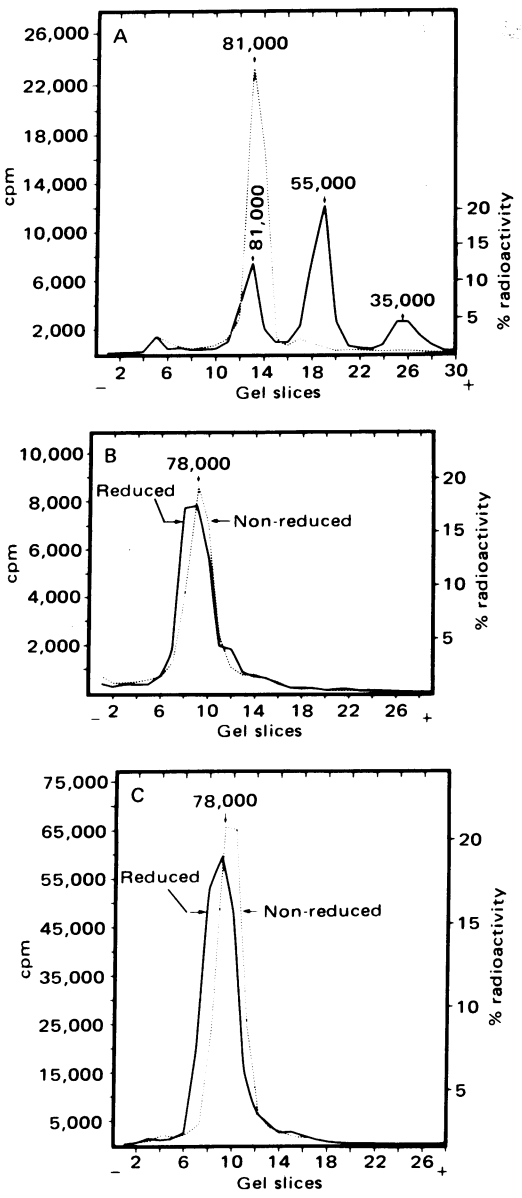

FIG. 1. (*A*) Analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis of ^{125}I-HF that had been exposed to glass in the presence of normal plasma. ^{125}I-HF in 0.1% bovine serum albumin was mixed in a 10 × 75 mm polystyrene tube with an equal volume of normal plasma that had been diluted 1:25 in buffer. Samples of 0.02 ml (approximately 7×10^4 cpm) were transferred to 10 × 75 mm glass tubes. At 0 min or after shaking these tubes for 20 min at room temperature, 0.05 ml of 4% $NaDodSO_4$ in 2% 2-mercaptoethanol was added to the tubes, which were then boiled for 3 min. Samples were subjected to $NaDodSO_4$/6% polyacrylamide gel electrophoresis. The gel was sliced and assayed for radioactivity. The vertical axes indicate cpm (left) and % radioactivity relative to the total added to the gel (right), and the horizontal axis, successive slices of gel with the anode at the right. Dotted line, electrophoretic pattern at 0 min; solid line, electrophoretic pattern after 20 min of exposure to the glass tube. (*B*) Analysis by $NaDodSO_4$/gel electrophoresis of ^{125}I-HF exposed to Sephadex-ellagic acid. Three-tenths milliliter of ^{125}I-HF in 0.1% bovine serum albumin was mixed with 1.0 ml of Sephadex-ellagic acid and centrifuged at 30,000 × *g* for 15 min at 2°C. Supernatant (0.15 ml) was incubated with 0.15 ml of 4% $NaDodSO_4$ with or without 2% 2-mercaptoethanol in 10 mM sodium phosphate buffer (pH 7.0) overnight at room temperature. Two-tenths milliliter was then subjected to $NaDodSO_4$/6% polyacrylamide gel electrophoresis. (*C*) Analysis by $NaDodSO_4$/gel electrophoresis of ^{125}I-HF exposed to Sephadex/buffer. The same procedure was used as in *B* except that Sephadex-ellagic acid was replaced by Sephadex/buffer.

its proteolytic scission, ^{125}I-HF was exposed to Sephadex-ellagic acid and studied by $NaDodSO_4$/gel electrophoresis. Cleavage of purified ^{125}I-HF upon exposure to Sephadex-ellagic acid was minimal, although this procedure activated coagulant and amidolytic properties. When HF treated in this way was reduced and subjected to $NaDodSO_4$/gel electrophoresis, neither separation of the M_r 30,000 fragment nor significant scission of HF within an internal disulfide loop was observed. In fact, the same result was obtained earlier in mixtures of purified HF and ellagic acid (10). Some cleavage—as much as 15%—was observed when ^{125}I-HF was mixed with normal human plasma containing plasma prekallikrein and other plasma proenzymes. In this experiment, plasma was diluted 50% with the ^{125}I-HF solution before exposure to Sephadex-ellagic acid. Revak *et al.* (4), in contrast, diluted plasma 1:6 before exposure to glass, and, in more recent studies, scission of HF was enhanced by still greater dilution (20). Thus, the scission observed by Revak *et al.* need not be necessary for development of clot-promoting properties in plasma. Alternatively, only a small fraction of HF need be split to provide clot-promoting activity. It should be emphasized that the activated HF we studied was in the fluid phase. Whether that fraction of HF adherent to Sephadex-ellagic acid was cleaved was not examined.

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