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Detection by Fluorescence of Structural Changes Accompanying the Activation of Hageman Factor (Factor XII) (39773)

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Introduction. The intrinsic pathway of blood coagulation has been likened to a "cascade" (1) or "waterfall" (2) in which at each step a clotting factor is activated and in turn activates another clotting factor until ultimately a clot is formed. *In vitro,* the initiation of the pathway is brought about by the activation of Hageman factor (HF; factor XII) (3), a plasma protein with a molecular weight of approximately 80,000. Vromon (4) suggested that, upon activation, HF undergoes a conformational change such that its hydrophobic sites are exposed. Other workers (5–7) have lent support to this theory, but the available evidence has been indirect.

Recent work with fluorescent compounds that probe the hydrophobic regions of proteins (8) and cell membranes (9) has suggested that fluorescence spectroscopy might be used to determine whether hydrophobic sites are exposed when blood-clotting proteins are activated. This technique involves the use of extrinsic probes that fluoresce strongly in a hydrophobic environment. In this communication we describe the interaction of an extrinsic probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), with the unactivated and activated forms of HF and provide substantial evidence that hydrophobic sites are exposed upon activation.

Materials and methods. Buffer. Barbital-saline buffer (0.025 *M* barbital in 0.125 *M* sodium chloride, pH 7.5) was used throughout.

Preparation of HF. Hageman factor (factor XII) was isolated from human plasma as described earlier (10). The specific activity of the preparation was 53 units/mg of protein, 1 unit being arbitrarily defined as that present in 1 ml of standard pooled plasma. The clot-promoting activity of HF was measured as described previously (10).

Cleaning and coating of fluorimeter cells. Ultraviolet quartz fluorimeter cells (Precision Cells, Hicksville, N. Y.) were cleaned by soaking in warm concentrated nitric acid, rinsed thoroughly with distilled water, and dried with a stream of N_2. The clean cells were then filled with a 2 mg/ml aqueous solution of hexadimethrine bromide (Polybrene, Aldrich Chemical Co., Milwaukee, Wis.), emptied, rinsed thoroughly with distilled water, and dried with dry N_2. This procedure coated the cells with Polybrene to prevent the activation of the HF by their surfaces. Ellagic acid (10^{-4} *M*; J. T. Baker, Co., Phillipsburg, N. J.) in barbital-saline buffer was dissolved by homogenization for several minutes, followed by boiling for 15 min. The solution was then filtered through Whatman no. 1 paper and used within the next 2–3 hr. A portion of the 10^{-4} *M* solution was diluted to make a 10^{-5} *M* solution.

Preparation of DPH probe. A stock solution of 2×10^{-3} *M* 1,6-diphenyl-1,3,5-hexatriene (DPH; Sigma Chemical Co., St. Louis, Mo.) was dissolved in tetrahydrofuran (Fisher Scientific Co., Inc., Pittsburgh, Pa.). Portions of this stock were diluted 1000-fold with barbital-saline buffer, and the 2×10^{-6} *M* solution was used as a probe for hydrophobic sites.

The usefulness of DPH as a hydrophobic probe is demonstrated in Fig. 1. A mixture of 35% ethanol in barbital-saline buffer (v/v) displayed a nearly fivefold greater DPH fluorescence at 450 nm than the buffer alone. The slight change in solvent polarity resulted in a substantial change in the probe's fluorescence. This sensitivity gives

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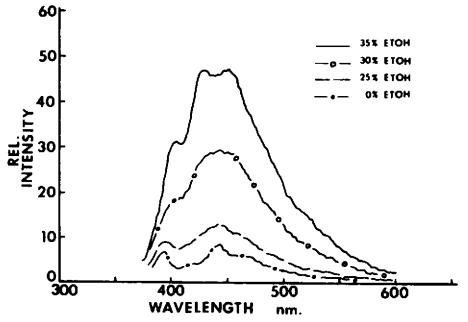

FIG. 1. The fluorescence spectra of 1,6-diphenyl-1,3,5-hexatriene in solutions of varying polarity. Solutions of DPH (2×10^{-6} *M*) in barbital-saline displayed increased fluorescence intensity as the ethanol content was increased from 0 to 35% by volume. Emission at 450 nm was effected by excitation at 350 nm, using 5.5-nm bandpasses.

DPH a distinct advantage over other types of hydrophobic probes.

Preparation of samples. All samples were mixed and final dilutions were made with barbital-saline buffer in clean Polybrene-coated fluorimeter cells. The concentration of HF was 0.027 mg/ml in each sample. To activate HF, samples were dissolved in 10^{-5} or 10^{-6} *M* ellagic acid. Extrinsic fluorescence measurements were made from samples containing activated or unactivated HF and a 2×10^{-7} *M* concentration of DPH.

Esterolytic activity. The esterolytic activity of a mixture of HF and ellagic acid was measured utilizing *N*-α-acetylglycine lysine methyl ester (AGLMe, Cyclo Chemical, Los Angeles, Calif.) as a substrate (11). One-tenth-milliliter samples of HF (0.27 mg/ml) were incubated with 0.1 ml of 10^{-4} *M* ellagic acid and 0.8 ml of barbital-saline buffer in 12 × 75-mm polystyrene tubes at 37° for 3 min. After 1 and 61 min, 2 ml of 0.015 *M* AGLMe were added to each tube and 1-ml aliquots were added to tubes containing 0.5 ml of 0.75 *M* perchloric acid at 37°. The methanol release was measured by the method of Siegelman *et al.* (12).

Fluorescence spectra. All measurements were made with an Aminco Bowman corrected spectra spectrophotofluorometer (American Instrument Co., Silver Springs, Md.). The corrected emission spectra were obtained for both the intrinsic fluorescence of the protein and extrinsic fluorescence of the DPH probe. Intrinsic fluorescence was derived from fluorescent amino acid residues in the protein molecule, whereas external probe molecules gave rise to extrinsic fluorescence. The excitation wavelength for the intrinsic fluorescence was 280 nm with emission at 335 nm (tryptophan), whereas the excitation wavelength for DPH was 350 nm with emission at approximately 450 nm. In both cases, the excitation and emission bandpasses were 5.5 nm.

Results. HF displayed intrinsic fluorescence attributed to tryptophan residues (280-nm excitation, 335-nm emission) in the protein (Fig. 2). When HF was activated with 10^{-6} *M* ellagic acid, fluorescence at 335 nm decreased 16% and, when HF was activated with 10^{-5} *M* ellagic acid, fluorescence at 335 nm decreased 47%. Ellagic acid alone did not display any fluorescence nor did it act to impede tryptophan fluorescence under these conditions.

The DPH probe (350-nm excitation, 450-nm emission) induced extrinsic fluorescence of HF as demonstrated by a spectral change (Fig. 3). Fluorescence of unactivated HF at 450 nm increased 100% over a blank of barbital-saline buffer and DPH. DPH at this concentration (2×10^{-7} *M*) did not appreciably change the clot-promoting ac-

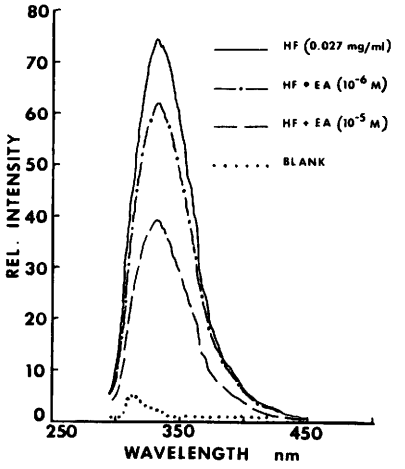

FIG. 2. The intrinsic tryptophan fluorescence of unactivated and activated Hageman factor. The concentration of HF in each sample was 0.027 mg/ml of saline-barbital. Activation was achieved through interaction of HF with 10^{-6} and 10^{-5} *M* ellagic acid (EA). The blank represents barbital-saline buffer with and without ellagic acid. The excitation wavelength was 280 nm with 5.5-nm bandpasses.

tivity of HF. When HF was activated with 10^{-5} *M* ellagic acid and treated with DPH (2×10^{-7} *M*), fluorescence at 450 nm increased 638% over a blank of barbital-saline buffer, ellagic acid (10^{-5} *M*), and DPH (2×10^{-7} *M*).

In these conditions, a mixture of HF and ellagic acid digested AGLMe (21 μmol of methanol released/hr/ml of HF). Neither HF nor ellagic acid alone hydrolyzed AGLMe.

Discussion. The decreased intrinsic fluorescence observed when HF was activated by ellagic acid suggests that activation is accompanied by a conformational change in the environment of at least some of the tryptophan residues such that their quantum efficiency is significantly lowered. The activation of HF by ellagic acid was monitored by the generation of esterolytic activity for AGLMe. Although this property of HF on AGLMe is not necessarily the same as that of the clot-promoting function, this is the best evidence to indicate that HF acquires a new enzymatic property when exposed to ellagic acid. The activation of HF by ellagic acid, studied in terms of clotting activity (13), is dependent upon the concentration of ellagic acid. The decrease in tryptophan fluorescence observed in the present study is also inversely proportional to the concentration of ellagic acid. Thus, it is tempting to speculate that the decrease in fluorescence at 335 nm reflects the degree of the activation of HF. The intrinsic fluorescence of HF may therefore be a simple measure of the degree of activation of this protein.

The relatively slight increase in the fluorescence of DPH with unactivated HF (Fig. 3) may be attributed to the presence in the native unactivated protein of a few hydrophobic pockets that are readily accessible to the solvent. Alternatively, some of the protein molecules may have been altered from their native conformation *in vivo* or during purification, thus exposing hydrophobic regions to the environment. In any event, the low intensity of fluorescence indicates that there are few binding sites for DPH on unactivated HF. The large increase in fluorescence of DPH exposed to ellagic acid-activated HF suggests that in these experiments ellagic acid interacted with the protein, al-

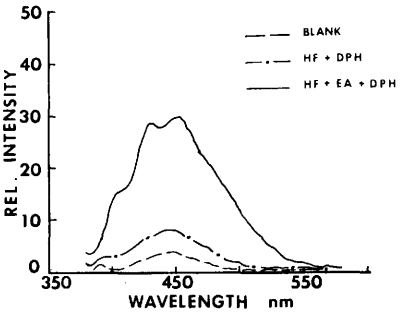

FIG. 3. The extrinsic fluorescence of the DPH probe bound to unactivated and ellagic acid-activated HF. In each sample, the HF concentration was 0.027 mg/ml with 2×10^{-7} *M* DPH. HF was activated by 10^{-5} *M* ellagic acid (EA). The blank represents DPH in barbital-saline buffer with and without ellagic acid. Excitation for DPH was at 350 nm with a 450-nm emission; 5.5-nm bandpasses were used.

tering its conformation and exposing hydrophobic sites to the solvent environment. Presumably DPH was then energetically attached to these hydrophobic pockets, accounting for the greatly increased fluorescence.

The decreased intrinsic fluorescence of tryptophan in conjunction with increased extrinsic fluorescence of DPH in activated HF lends solid evidence to the supposition that this blood-clotting protein undergoes a conformational change exposing hydrophobic sites when mixed with ellagic acid. The significance of this exposure of hydrophobic sites is not clear at this point. Whether there is a sequential exposure of hydrophobic groups in other clotting proteins as they become active, as postulated earlier (14), remains to be seen. The technique described herein will lend itself nicely to such a problem.

Summary. The activation of Hageman factor (factor XII) by ellagic acid may involve a conformational change in which at least some of its hydrophobic regions, previously buried, are exposed to the surrounding environment. The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), when added to a solution of ellagic acid-activated Hageman factor, displayed substantially more fluorescence compared to solutions of the probe and the unactivated form of the protein. These studies imply that the hy-

drophobic regions of activated Hageman factor are exposed in such a way that the DPH probe has easy access to the hydrophobic environment to which it binds.

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