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Enzymes produced by autoactivation of blood factor XII in buffer A contribution from the Hematology at Biomaterial Interfaces Research Group

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ABSTRACT

High-resolution electrophoresis of *FXII*-derived proteins produced by contact activation of *FXII* in buffer solutions (*i.e.* in absence of plasma proteins) with hydrophilic and silanized-glass activators spanning the observable range of water wettability (hydrophilic to hydrophobic), shows no evidence of proteolytic cleavage of *FXII* into *αFXIIa* or *βFXIIa*. The autoactivation mixture contains only a single-chain protein with a molecular weight of ~80 kDa, confirming Oscar Ratnoff's previous finding of a single-chain activated form of *FXII* that he called 'HF_{ea}'. Functional assays have shown that these autoactivation products exhibit procoagulant potential (protease activity inducing clotting of blood) or amidolytic potential (cleaves amino bonds in s-2302 chromogen but do not cause coagulation of plasma) or both amidolytic potential and procoagulant potential. Some of these proteins also have the remarkable potential to 'suppress autoactivation' (*i.e.* suppress creation of enzymes with procoagulant potential). It is thus hypothesized that autoactivation of *FXII* in the absence of plasma proteins generates not just a single type of activated conformer, as suggested by previous researchers, but rather an ensemble of conformer products with collective activity that varies with activator surface energy used in contact activation of *FXII*. Furthermore, reaction of *αFXIIa* with *FXII* in buffer solution does not produce additional *αFXIIa* by the putative autoamplification reaction *FXIIa* + *FXII* → 2*FXIIa* as has been proposed in past literature to account for the discrepancy between chromogenic and plasma-coagulation assays for *αFXIIa* in buffer solution. Instead, net procoagulant activity measured directly by plasma-coagulation assays, decreases systematically with increasing *FXII* solution concentration. Under the same reaction conditions, chromogenic assay reveals that net amidolytic activity increases with increasing *FXII* solution concentration. Thus, although autoamplification does not occur it appears that there is some form of "*FXII* self reaction" that influences products of *αFXIIa* reaction with *FXII*. Electrophoretic measurements indicate that no proteolytic cleavage takes in this reaction leading us to conclude that change in activity is most likely due to change(s) in *FXII* conformation (with related change in enzyme activity).

1. Introduction

Potentiation of the blood-plasma coagulation cascade by surface contact activation of the blood zymogen Factor XII (*FXII*, Hageman factor) is thought to be a cause of the relatively poor hemocompatibility of all currently known cardiovascular biomaterials. Two enzymes, *αFXIIa* and *βFXIIa*, have been identified as products of *FXII* activation with protease activity inducing clotting of blood plasma (procoagulant activity). These enzymes initiate the intrinsic pathway of coagulation that ultimately causes blood or blood plasma to undergo a phase transition from liquid to gel (i.e. coagulate or clot, see Ref. [1] and citations therein that generally support this section). And yet, in spite of extensive research effort over the last five decades, *FXII* surface activation (a.k.a. autoactivation) remains a mysterious reaction with unusual characteristics recently revealed by functional assays measuring enzyme activity

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of the mixture produced by *FXII* autoactivation with particulate activators spanning a full range of surface chemistry/energy [2–5].

1. *FXII* autoactivation in buffer and protein solutions produces a highly variable burst of procoagulant enzyme(s) with no measurable kinetics, sensitivity to mixing, or solution-temperature dependence. Autoactivation thus appears to be a stochastic, mechanochemical-like reaction that does not exhibit characteristics of an ordinary (bio)chemical reaction.
2. Although autoactivation is definitively initiated by surface contact, no measurable dependence on activator surface area can be discerned in buffer or protein solutions. That is to say, serial addition of activator surface area does not measurably increase procoagulant-enzyme yield.
3. Autoactivation appears to be a self-limiting reaction with an apparent procoagulant-enzyme yield converting less than 10% of starting *FXII* (at 30 μg/mL physiologic concentration) in the continuous presence of activator surface area. Thus, it appears that surface activation is somehow "shut off" after an initial catalytic event.
4. The above observations are to be contrasted to the fact that activation of blood-plasma coagulation *in vitro* by contact with particulate activator is demonstrably dependent on plasma-volume-to-activator-surface-area ratio.
5. *FXII* autoactivation in buffer produces an ensemble of as-yet unidentified proteins represented herein by $FXII_{act}$ in the descriptive chemical formula $FXII \xrightarrow[surface]{activator} FXII_{act}$. $FXII_{act}$ includes proteins exhibiting either procoagulant potential (protease activity inducing clotting of blood) or amidolytic potential (cleaves amino bonds in s-2302 chromogen but do not cause coagulation of plasma) or both amidolytic potential and procoagulant potential. Apparent yield of procoagulant enzymes was less than purely amidolytic enzymes under all conditions studied. $FXII_{act}$ also includes proteins that remarkably suppress production of procoagulant enzymes but does not suppress production of purely amidolytic enzymes.
6. The relative reactive proportions of proteins subsumed by $FXII_{act}$ were found to depend on activator surface chemistry/energy. Thus it appears that *FXII* undergoes some kind of chemical transformation by contact with any kind of activator surface, quite independent of hydrophilicity or surface chemistry; at least in buffer solution.

The *FXII*-derived proteins mentioned in the above item (6) appear to be in a complex statistical ensemble of a large number of states, with some states of low local stability and others with high stability. Phenomena such as allosteric regulation are often ascribed to arise from equilibrium between two major conformations of dynamic proteins [6]. It is not proven herein that *FXII* exists in more than one conformer state, but change in conformation with various perturbants has been observed [7].

Precise biochemical mechanisms for reactions involving *FXII* are not well understood, but *in-vitro* activation of *FXII* has been variously attributed to proteolytic cleavage [8–14], change in conformation [7,15–21], or a combination of these mechanisms [10,22]. Speculation on the nature of these conformation changes is hampered by the fact that 3-D crystal structures of *FXII* and its cleavage products are not clear, with only a few studies conducted using computer models [23].

In light of the above, it has become evident that a molecular inventory of proteins and conformers resulting from *FXII* autoactivation under different experimental conditions with activators spanning a full range of surface chemistry/energy is a necessary step in the formulation of testable mechanisms of autoactivation. In turn, formulation of testable mechanisms is essential to the long-sought ability to prospectively surface engineering cardiovascular biomaterials with improved hemocompatibility. This inventory will compliment purely functional assays; hopefully leading to an identification of those specific protein(s) comprising $FXII_{act}$ that is (are) responsible for the above-mentioned amidolytic, procoagulant, and procoagulant-suppressive activity. Present work also sheds light on putative "self reactions" between *FXII* and *αFXIIa* in buffer solution in the absence of activator surfaces (other than that of test tubes and cuvettes in which experimental solutions are contained). Herein, we report results from a two-prong research strategy using high-resolution electrophoresis coupled with chromogenic and plasma-coagulation functional assays.

2. Methods and materials

2.1. Plasma and coagulation proteins

Citrated human platelet poor plasma (PPP) was prepared from unexpired pooled lots obtained from the M.S. Hershey Medical Center Blood Bank and prepared as described previously [24–26]. This work was performed with a single batch of PPP aliquoted into 15 ml polypropylene tubes (Falcon, Becton Dickinson) and frozen at −20 °C until use. Experiments for this work were conducted within a month of PPP preparation. Repeat trials yielded no significant variations in coagulation times within this experimental time frame.

Human *FXII* and *αFXIIa* were used as received from Enzyme Research Laboratories (South Bend, IN). Activity of both *FXII* and *αFXIIa* was specified by the vendor in mg/mL or traditional units of plasma equivalent units per mL (PEU/mL) [27]. Neat-buffer solutions of *FXII* and *αFXIIa* solutions were prepared in phosphate buffer saline (PBS; Sigma; 0.14 M NaCl, 3 mM KCL prepared from powder in 18 MΩ de-ionized water, pH 7.4).

2.2. Preparation and characterization of particulate activators

Test activator surfaces used in this work were 425–600 μm diameter glass particles (Sigma Aldrich) in either cleaned or silanized form. The nominal specific area used in this work was 5×10^{-3} m^2/g (based on 512.5 μm mean diameter and 168 μg/particle). The surface area measured by the Brunauer-Emmett-Teller (BET) method (Micromeritics ASAP 2000 using liquid nitrogen as the probe gas) was $(8.5 \pm 0.1) \times 10^{-3}$ m^2/g. Nominal surface area was used throughout this work as a matter of consistency with prior work and in recognition of the fact that surface area did not influence conclusions based on comparisons among experiments described herein using the same source of particulate activator with a fixed specific surface area.

Silanes (used as received from Sigma Aldrich) applied in this work were octadecyltricholorosilane (OTS) and 3-aminopropyltriethoxysilane (APTES). OTS-treated glass particles were coated in a 0.2% solution of 1,1-pentadecaflurooctylmethacrylate in tricholorotrifloroethane ("Nyebar", Nye Lubricants, Fairhaven, MA) by immersion followed by air drying. Glass cover slips (Fisher 22 × 30 × 0.1 mm) were subjected to all surface treatments for particles as described above and further below, providing a substrate suitable for reading phosphate buffer saline (PBS, Sigma) contact angles.

Glass particles and cover slips were first cleaned and activated by 30 min immersion in heated piranha solution (30% H_2O_2 in concentrated H_2SO_4 at approximately 80 °C) followed by 3X sequential washes in each of 18 MΩ de-ionized water and 100% ethanol. Piranha-solution oxidized glass was air dried and subsequently oxidized by air-plasma treatment (10 min at 100 W plasma; Herrick, Whippany, NY) of a single layer of particles (or cover slip) held in a 15 mm Pyrex glass Petri dish, directly before use in silanization procedures or blood-plasma coagulation activation measurements. Glass surfaces treated in this manner were found to be fully water wettable and designated "clean glass". Clean glass particles and cover slip samples were silanized by 1.5 h reaction with 5% v/v OTS in chloroform. Silanized samples were 3X rinsed with chloroform before curing in a vacuum oven at 110 °C for 12 h. Cured OTS samples were optionally immersed in Nyebar solution for 10 min and air dried to produce a surface slightly more hydrophobic than rendered by OTS treatment alone (see Table 1). APTES silanization was carried out by 20 min reaction of clean glass with 95:5 v/v ethanol-water solution with 5% APTES that had been hydrolyzed overnight in the ethanol-water mixture before use. APTES treated glass was 3X washed with ethanol and cured overnight in a vacuum oven at 110 °C.

PBS (see below) contact angles on glass cover slip witness samples were measured using an automated contact-angle goniometer (First Ten Angstroms Inc., Portsmouth, VA) that employed the captive-drop method of measuring advancing/receding contact angles (see Refs. [28,29] for a comparison of goniometric techniques and discussion of experimental errors). Contact angles could not be read directly on glass particles but optical microscopy of the shape of the liquid meniscus of particles partly immersed in water on a microscope slide qualitatively confirmed that the treated particles were not different from the cover slip witness samples. Surface chemistry of glass-particle surfaces has been previously assayed using a