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Amidolytic, Procoagulant, and Activation-Suppressing Proteins Produced by Contact Activation of Blood Factor XII in Buffer Solution

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Abstract

The relative proportions of enzymes with amidolytic or procoagulant activity produced by contact activation of the blood zymogen factor XII (FXII, Hageman factor) in buffer solution depends on activator surface chemistry/energy. As a consequence, chromogenic assay of amidolytic activity (cleavage of amino acid bonds in s-2302 chromogen) does not correlate with the traditional plasma-coagulation-time assay for procoagulant activity (protease activity inducing clotting of blood plasma). Amidolytic activity did not vary significantly with activator-particle surface energy, herein measured as water adhesion tension $\tau^\circ = \gamma_{lv}^\circ \cos \theta_a$; where γ_{lv}° is pure buffer interfacial tension and θ_a is the advancing contact angle. By contrast, procoagulant activity varied as a parabolic-like function of τ° , high at both hydrophobic and hydrophilic extremes of activator surface energy and falling through a broad minimum within a $20 < \tau^\circ < 40 \text{ mJ/m}^2$ ($55^\circ < \theta_a < 75^\circ$) range, corroborating and expanding previously-published work. It is inferred from these functional assays that an unknown number of protein fragments are produced by contact activation of FXII (a.k.a. autoactivation) rather than just αFXIIa and βFXIIa as popularly believed. Autoactivation products produced by activator particles within the $20 < \tau^\circ < 40 \text{ mJ/m}^2$ ($55^\circ < \theta_a < 75^\circ$) surface-energy range suppresses production of procoagulant enzymes by activators selected from the hydrophobic or hydrophilic surface-energy extremes through as-yet unknown biophysical chemistry. Suppression proteins may be responsible for the experimentally-observed autoinhibition of the autoactivation reaction.

Keywords

Autoactivation; Contact activation; Plasma Coagulation; Hageman factor; FXII

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1. Introduction

Zymogen activation by contact with material surfaces is a fundamental problem of biomaterials surface science with considerable practical ramifications in the design of hemocompatible biomaterials. Activation of blood factor XII (FXII, Hageman factor) and C3 protein are arguably the most important examples, limiting development of advanced cardiovascular medical devices by the induction of blood coagulation and production of anaphylactic proteins, respectively.

Our studies of contact activation of the blood plasma coagulation cascade have revealed that the surface chemistry involved in the initial step, so-called “autoactivation” of FXII

($FXII \xrightarrow[\text{surface}]{\text{activator}} FXII_a$), where $FXII_a$ is the activated enzyme form, is far more complicated than suggested by this simple descriptive chemical reaction [1]. Not only do we find that autoactivation is not chemically specific for hydrophilic anionic activators, as proposed by the traditional paradigm of contact activation, but also we have discovered that autoactivation is strongly influenced by the overall protein composition of the fluid phase in which autoactivation occurs in a manner not contemplated by this paradigm [2].

The biochemistry of autoactivation is complex. Circular dichroism, fluorescence, and UV spectroscopy shows that FXII undergoes conformational changes upon binding to dextran sulfate, a “soluble activator surface”, and conversion to an active enzyme [3]. By contrast, significant conformational changes in FXII adsorbed to insoluble solid sulfonated polystyrene were not detected by sum frequency generation (SFG) and Fourier Transform Infrared (FTIR) spectroscopy [4]. Both cited studies suggest that aggregation and orientation of FXII molecules at an activating surface is important in autoactivation, at least for hydrophilic activator surfaces. Two enzymes are known to be produced by autoactivation, namely $\alpha FXII_a$ (variously referred to as HFa, factor $XII_{a_{HMW}}$, and Factor XII_a) and $\beta FXII_a$ (variously referred to as HFf, factor $XII_{a_{LMW}}$ and Factor XII_f). These enzymes have been well characterized [5] but the relative proportions produced by contact-activator surfaces with different surface chemistry/energy have not been measured. $\alpha FXII_a$ and $\beta FXII_a$ activate prekallikrein and factor FXI to different extents [6-8]. For example, it is reported that $\beta FXII_a$ activates prekallikrein at only 2-4% of the coagulant activity of $\alpha FXII_a$ [6, 9]. In addition, minor cleavage products of intermediate molecular weight between $FXII_a$ and $FXII_f$ have been observed, but the procoagulant activity of these minor fragments have not yet been well characterized, nor have the relative amounts produced by contact-activator surfaces with different surface chemistry/energy been mapped out (see further Section 4.1 and ref. [9]).

As a consequence of these findings, we are led to construe autoactivation as a biochemical

reaction better described by $FXII \xrightarrow[\text{surface}]{\text{activator}} FXII_{act}$, where $FXII_{act}$ represents a suite of protein fragments with relative yields that depend on contact activator surface chemistry/energy and the protein composition of the fluid phase in which autoactivation occurs. Furthermore, we have become suspicious that $FXII_{act}$ may include enzymes (see Fig. 1) that are amidolytic (cleavage of amino acid bonds in s-2302 chromogen) but not procoagulant (protease activity inducing clotting of blood plasma).

This work investigates the relative proportions of amidolytic and procoagulant enzymes produced by autoactivation of FXII in buffer solution by contact with glass-particle activators with surface energy incrementally sampling the full range of observable water wettability. A chromogenic assay is used to measure amidolytic activity and a plasma coagulation-time assay used to measure procoagulant activity. Results motivate assembly of a molecular inventory of protein fragments produced autoactivation and elucidation of the

dependence on activator surface energy. This inventory will provide insights into the mechanism of FXII autoactivation that will be useful in the surface engineering of materials with improved hemocompatibility.

2. Methods and Materials

The basic experimental strategy of this work was to compare the procoagulant activity of enzymes produced by autoactivation of FXII in buffer solution (Section 2.6) to the amidolytic activity of these enzymes (see Section 1 for definition of terms and Fig. 1). Silanized glass particles incrementally sampling the full range of water wettability (surface energy) at fixed surface area were used as test contact-activator surfaces. Extensive previous work has shown that contact activator surface energy as measured by contact angles correlates with contact activation of both FXII and plasma (see ref. [10] for a recent review). Activator particles at the hydrophobic extreme were referred to as “Type I” whereas activator particles at the hydrophilic extreme as “Type II”, with activators falling between these extremes as “Type 0” in recognition of low yield of procoagulant enzymes [2]. Physical mixtures of Type I or Type II activator particles with Type 0 activator particles were used in autoactivation experiments. Supernatant derived from Type 0 autoactivation experiments was used as the suspending fluid in Type I activation experiments (Section 2.5).

A plasma coagulation time assay was used to measure procoagulant activity whereas amidolytic activity was measured using an s-2302 chromogenic assay. These functional assays measured the net activity of enzyme(s) produced by FXII autoactivation in buffer solution in terms of “apparent α FXIIa activity”. Apparent α FXIIa activity was standardized by a calibration curve for each assay relating coagulation time (CT, Section 2.3) of platelet-poor plasma (procoagulation assay) or rate-of-color change (amidolytic assay, Section 2.4) to serially-increasing α FXIIa concentration [α FXIIa], expressed either in plasma-equivalent-units-per-milliliter (PEU/mL) or mg/mL [11, 12]. In this way, the outcome of the two assays was compared on the same basis – the concentration of α FXIIa required to evoke the same CT or rate-of-color change.

2.1 Preparation and characterization of particulate activators

Test activator surfaces used in this work were 425-600 μ m diameter glass particles (Sigma Aldrich) in either cleaned or silanized form. The nominal specific area used in this work was 5×10^{-3} m²/g (based on 512.5 μ m mean diameter and 168 μ g/particle). The actual surface area measured by the Brunauer-Emmett-Teller (BET) method (Micromeritics ASAP 2000 using liquid nitrogen as the probe gas) was 0.25 ± 0.09 m²/g. This was 50X larger than the nominal value, probably a reflection of the large disparity in particle size and possibly porosity. The BET surface area was used in this work. Choice of surface area used in calculations had no impact on conclusions drawn herein because qualitative and quantitative comparisons were made among silanized activators prepared from the same lot of glass particles with the same specific area.

Silanes (used as received from Sigma Aldrich) applied in this work to create activator particles with different surface chemistries/energies corresponding to each silane were octadecyltrichlorosilane (OTS), 3-aminopropyltriethoxysilane (APTES), n-propyltriethoxysilane (PTES), and vinyltriethoxysilane (VTES). OTS-treated glass particles were optionally coated in a 0.2% solution of 1,1-pentadecafluorooctylmethacrylate in trichlorotrifluoroethane (“Nyebar”, Nye Lubricants, Fairhaven, MA) by immersion followed by air drying. Glass cover slips (Fisher 22 $\times$ 30 $\times$ 0.1mm) were subjected to all surface treatments for particles as described below, providing a substrate suitable for reading phosphate buffer saline (PBS, Sigma) contact angles. Table 1 collects activators used in this work and identifies these activators as Type I, Type II, or Type 0 according to activation

behavior observed in previous studies [2] that relate to activator surface energy (contact angle, column 4).

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 M Ω de-ionized water and 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 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 hr 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 hr. 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. Silanization with PTES and VTES followed the APTES procedure, except that 90:10 v/v ethanol-water solution containing 0.5 % glacial acetic acid was used.

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 references [13, 14] 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. Water wettability of the s^{th} surface (Table 1) was expressed as (advancing) water adhesion tension τ_s° (where $\tau_s^\circ \equiv \gamma_{lv}^\circ \cos\theta_s$ is the product of pure-buffer interfacial tension $\gamma_{lv}^\circ = 71.97$ dyne/cm at 25 °C and cosine of the advancing contact angle θ_s observed on the s^{th} sample). Surface chemistry of glass-particle surfaces have been assayed using a Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT) collected on a Bruker IFS-66/S Tech as described in ref. [15].

Physical mixtures of APTES silanized activators (Type 0; $s=5$, Table 1) with hydrophobic activators (Type I; $s=1$ Table 1) and hydrophilic activators (Type II; $s=6$, Table 1) were prepared by gravimetry, incrementally sampling the 0.0-1.0 weight/weight fraction range. Particles were weighed directly into test tubes and mixed in the dry state by shaking. Particles were further mixed in suspension with PBS before use in activation experiments described below. A hypothetical “equivalent wetting” parameter was calculated using a linear combining rule as described previously [2].

2.2 Plasma and coagulation proteins

Citrated human platelet poor plasma (PPP) was prepared from outdated (within 2 days of expiration) lots obtained from the M.S. Hershey Medical Center Blood Bank and prepared as described previously [16-18]. This work was performed with two different lots of PPP aliquoted into 15 ml polypropylene tubes (Falcon, Becton Dickinson) and frozen at -20 °C until use. Whereas results with stored plasma are qualitatively consistent, minor quantitative variations arose within these lots over about one year of experimentation. Variations in stability of coagulation factors during storage may be responsible for these differences

[19-21]. Variation in coagulation properties of plasma was corrected for by use of an FXIIa titration curve (Section 2.3).

Human FXII and α FXIIa were used as received from Enzyme Research Laboratories (South Bend, IN). Activity of both FXII and α FXIIa was specified by the vendor in mg/mL or traditional units of plasma equivalent units per mL (PEU/mL) [11]. Buffer solutions of FXII and α FXIIa solutions were prepared in phosphate buffer saline (PBS; Sigma; 0.14M NaCl, 3mM KCL prepared from powder in 18M Ω de-ionized water, pH 7.4).

2.3 Coagulation time assay

Plasma coagulation time (CT) was used as the traditional hematology method to quantify procoagulant activity in terms of apparent α FXIIa concentration by use of a “ α FXIIa titration calibration curve” that relates CT to α FXIIa concentration ($[\alpha\text{FXIIa}]$), expressed either in PEU/mL or mg/ml [11, 12]). Protocol for the CT assay has been described in detail elsewhere [22-25]. Briefly, α FXIIa titrations were carried out by equilibrating 500 μ l of thawed PPP in 12 $\times$ 75mm polystyrene tubes (VWR), mixing with serially increasing $[\alpha\text{FXIIa}]$ in PBS, and diluting with sufficient additional PBS to bring the total volume to 900 μ l. Coagulation was induced by recalcification with 100 μ l of 0.1 M CaCl_2 , and tube contents were mixed on a slowly turning hematology mixer (Roto-shake Genie, Scientific Industries Inc.). CT after recalcification was noted by a distinct change in fluid-like rheology to gel formation, allowing determination of a coagulation endpoint to within 10 sec or so [24-26]. Measured CT was observed to be exquisitely sensitive to $[\alpha\text{FXIIa}]$ with a minimum quantifiable concentration of $\sim 9 \times 10^{-4}$ PEU/ml. α FXIIa titration curves in PPP provided reasonably linear calibration curves when scaled on a logarithmic concentration axis over the dynamic range of α FXIIa activity of interest in this work (see refs. [2, 23] for a detailed description). Linear calibration curves were fit to $CT = m \log_{10} [\alpha\text{FXIIa}] + c$ where m and c are adjustable parameters of the linear regression ($m = -7.40 \pm 0.42$, $c = 2.96 \pm 0.60$ with $R^2 = 0.91$ from triplicate measurements with plasma lot I, and $m = -13.52 \pm 0.72$, $c = 9.97 \pm 0.85$ with $R^2 = 0.94$ from triplicate generators using plasma lot II).

These calibration curves were used to quantify procoagulant activity of test solutions from FXII autoactivation experiments (Section 2.5), reported herein as apparent $[\alpha\text{FXIIa}]$ in PEU/mL. Strictly speaking, this assay actually measured net procoagulant activity of any number of proteins putatively produced by autoactivation of FXII in buffer solution. As such, the CT assay did not discriminate among various activated proteins or account for differential activity of different proteases that might be produced by autoactivation of FXII in buffer solution.

2.4 Chromogenic assay

A modified chromogenic assay that corrects for autohydrolysis of FXII in buffer solution ($\text{FXII} + \text{FXII}_{act} \rightarrow 2\text{FXII}_{act}$, where FXII_{act} subsumes all activated proteins types putatively produced by FXII autoactivation) was used to assay amidolytic activity [27]. The chromogenic substrate s-2302 (H-D-Pro-Phe-Arg-pNa $\cdots$ 2HCl, MW = 611.6 g/mole) [28-30], was used as received from Diapharma (Columbus Ohio). A 4.0 mM stock solution of chromogen was prepared in 18 M Ω de-ionized water (obtained from a Millipore Simplicity unit) and stored at 2-8 $^{\circ}\text{C}$ for no more than 6 months. A 0.4 mM working solution was prepared by diluting the stock solution 10-fold with buffer (0.05 M Tris/HCl, 0.012 M NaOH, 0.003 M EDTA, pH 7.8). Color development caused by cleavage of the chromogen was measured by mixing 450 μ l of working solution with 150 μ l of test solution in an ultra-microcuvette (VWR) held in the chamber of an automatically recording UV-VIS Spectrophotometer (DU $^{\circ}$ 800 Beckman Coulter Inc., Fullerton CA). Absorbance change (ΔA) at 405 nm was recorded for 5 minutes to determine the initial velocity ($\Delta A/\Delta t$) of color

development. A calibration curve was constructed by plotting $(\Delta A/\Delta t)$ for serially increasing $[aFXIIa]$ prepared PBS containing 20 $\mu\text{g/ml}$ FXII. Curvilinear calibration curves were quantified by non-linear best-fit to $(\Delta A/\Delta t) = ae^{b[aFXIIa]}$, where a and b were adjustable parameters of the fit ($a = 0.073 \pm 0.006$, $b = 4.35 \pm 0.22$, $R^2 = 0.99$ for duplicate measurements). The calibration curve exhibited good sensitivity over the dynamic range $0.005 < [aFXIIa] < 0.4$ PEU/ml.

These calibration curves were used to measure amidolytic activity of test solutions from FXII autoactivation experiments (Section 2.5), reported herein as apparent $[aFXIIa]$ in PEU/mL. Strictly speaking, this assay actually measured net amidolytic activity of any number of proteins putatively produced by autoactivation of FXII in buffer solution. As such, the chromogenic assay did not discriminate among these activated proteins or account for differential activity of different amidolytic fragments that might be produced by autoactivation of FXII in buffer solution.

2.5 Autoactivation of FXII in buffer solution

FXII activation in PBS solution was carried out as previously described [2, 22, 23]. Briefly, 200 μl of 30 $\mu\text{g/ml}$ FXII in PBS (nominal physiological concentration [1]) at 37 °C was brought into contact with 100 mg of test activators (Table 1) or mixtures thereof for between 2-30 min. At the BET surface area of 0.25 m^2/g given in Section 2.1, autoactivation experiments were performed at 0.24 mg FXII/ m^2 . To put this surface area in perspective, typical protein adsorbent capacity of hydrophobic particles of the type used herein fall within the 2-10 mg/ m^2 range, depending on protein molecular weight [15]. Autoactivation was therefore carried out at high surface-area-to-protein conditions.

Procoagulant activity was measured using the CT assay (Section 2.3) and amidolytic activity measured using the chromogenic assay (Section 2.4). The CT assay was performed by mixing 100 μl of autoactivation supernates with 500 μl of thawed PPP in 12 $\times$ 75mm polystyrene tubes (VWR) with sufficient additional PBS to bring total volume to 900 μl . Coagulation was potentiated by recalcification with 100 μl of 0.1 M CaCl_2 and tube contents were mixed on a slowly turning hematology mixer. The chromogenic assay was performed by mixing 450 μl of working solution with 150 μl of autoactivation supernatant in 20 $\mu\text{g/ml}$ FXII

2.6 Surface area titration of purified FXII in buffer solution and autoactivation suppression

The autoactivation protocol of Section 2.5 was carried out with incrementally varying mass between 0 and 100 $\pm$ 0.1mg (nominal surface area of $5.0 \times 10^{-4} \text{ m}^2$; BET surface area of $2.5 \times 10^{-2} \text{ m}^2$) of clean glass activator (Type 1; $s=6$, Table 1) weighed using a laboratory electronic balance. Autoactivation was carried out by 10 min. contact of 200 μl of 30 $\mu\text{g/ml}$ FXII in PBS. Apparent $[aFXIIa]$ was measured using CT assays (Section 2.3).

Autoactivation-suppression experiments were carried out as above except that the supernatant derived from autoactivation experiments using 100 mg of APTES particles (Type 0; $s=5$, Table 1) was used in place of PBS. An additional bolus of 30 $\mu\text{g/ml}$ FXII was added to the Type 0 supernatant before carrying out surface area titration with clean-glass Type II activator.

3. Results

Fig. 1 is a Venn diagram showing the three categories of protein fragments putatively produced by autoactivation of FXII in buffer solution considered in this work. Procoagulant fragments were not only amidolytic in the chromogenic assay of Section 2.4 but also procoagulant in the plasma coagulation assay of Section 2.3. Amidolytic fragments included

both procoagulant fragments and fragments that exhibited amidolytic activity in the chromogenic assay but not procoagulant activity in the plasma-coagulation assay. The “All Fragments” category potentially included autoactivation-suppression proteins, which may-or-may-not exhibit procoagulant and amidolytic activity, as well as protein fragments with no enzymic activity.

3.1 Autoactivation kinetics in buffer solution

Fig. 2 compares kinetics of FXII autoactivation in buffer solution by hydrophobic Type I activator particles (Panel A; $s=1$, Table 1) and hydrophilic Type II activator particles (Panel B; $s=6$, Table 1) as measured by the coagulation time (CT) assay (procoagulant yield, open symbols, Section 2.3) and chromogenic assay (amidolytic yield, closed symbols, Section 2.4). Procoagulant and amidolytic activity was measured in terms of apparent $[aFXIIa]$ in PEU/mL. Occasionally, activation yield appeared to increase with time (as shown in Panel B) but repeated measurements failed to confirm a trend at the 2σ confidence interval. Similar kinetics were obtained for other activator particles studied in this work (not shown, $s = 2-5$, Table 1). Amidolytic yield was between 4-10% of total FXII while procoagulant activity was less than 3.5% for all activator particles studied.

3.2 Autoactivation of FXII in buffer solution as a function of activator surface energy

Fig. 3 compares total amidolytic activity (Panel A) to procoagulant activity (Panel B) of FXII autoactivation products, reported as apparent $[aFXIIa]$ in PEU/mL, as a function of activator particle surface energy (Section 2.5). The trend in amidolytic activity was statistically flat (Panel A). By contrast, procoagulant yield (Panel B) exhibited a parabolic profile with activator surface energy that was consistent with our previously-published results (Panel B, open circles, [2]), with nearly equal autoactivation observed at the extremes of procoagulant water-wetting, falling through a broad minimum within the $20 < \tau^o < 40 \text{ mJ/m}^2$ ($55^\circ < \theta_a < 75^\circ$) range.

3.3 Suppression of autoactivation by physical mixtures of Type 0 activator particles

Physical mixtures of APTES (Type 0; $s=5$, Table 1) activators with either Nyebar (hydrophobic Type I; $s=1$, Table 1) or clean-glass (hydrophilic Type II; $s=6$, Table 1) activators were prepared by gravimetry in proportions that simulated different “equivalent adhesion tensions” (see ref. [2] for computation of the equivalent wetting parameter). The purely hypothetical equivalent wetting parameter τ^o was based on a linear combining rule that estimated the hydrophilicity of an imaginary surface populated by the surface-area fraction Type I or Type II surface energy represented by the w/w fraction of the physical mixture with Type 0.

Fig. 4 shows that mixing less than 20 w/w% APTES Type 0 activator with either Type I (Panel A) or Type II (Panel B) activator suppressed autoactivation yield of procoagulant proteins produced with 100% Type I or Type II activators (horizontal dashed line). Below this critical amount of Type 0 activator, activation yield rose sharply to levels observed with pure Type I and Type II activation. Amidolytic activity of particulate activator mixtures was not measured because results of the chromogenic assay were essentially invariant with activator surface chemistry/energy (see Fig. 3).

3.4 Suppression of autoactivation by Type 0 autoactivation fluid

Supernates prepared by autoactivation of FXII in buffer suspension with 100 mg of APTES activator particles (Type 0; $s=5$, Table 1) were prepared and used as the fluid phase for subsequent surface area titration of autoactivation by clean-glass activator (Type II; $s=6$, Table 1). An additional 30 $\mu\text{g/mL}$ bolus of FXII was added to the Type 0 supernatant prior

to incubation with Type II activator particles. Fig. 5 shows that autoactivation obtained with 100% Type II activator in buffer solution of FXII (filled circles) was nearly entirely suppressed when the fluid phase used in the autoactivation experiment was supernatant from Type 0 activation rather than PBS (arrow annotation).

4.0 Discussion

We begin this section with a brief review of the biochemistry of FXII activation that places experimental results of this work into context. Much of this literature is quite old at this writing but was expertly performed with techniques available at that time. It is hoped that our findings will encourage a reexamination of this early work with more modern tools of protein analysis. Following this review, results described in the preceding section will be discussed in order of presentation and then collectively interpreted in Sections 4.5 and 4.6 in terms of speculative hypotheses that will require testing by assembly of a molecular inventory of protein fragments produced by autoactivation.

4.1 Biochemistry of FXII and protein fragments formed on activation

FXII is a 596 amino acid, single-chain β -globulin with a molecular weight of approximately 80 kD [31]. The molecule is composed of two domains, an NH_2 -terminal heavy chain and a COOH -terminal light chain (Fig. 6, Panel A for a schematic diagram). The heavy chain contains fibronectin type I and type II domains, two epidermal growth factor (EGF)-like domains, a kringle domain (loops stabilized by 3 disulfide linkages), and a proline-rich region. The light chain contains the serine protease catalytic domain and a region homologous to the B-chain of the enzyme plasmin [32]. These domains are homologous to those found in other serine proteases, except for the proline-rich region that is unique to FXII. The catalytic domain of FXII is the single largest and best characterized region of the protein. The active site consists of 3 amino acids, H394, D442 and S544, indicating that the *in vivo* catalytic domain is globular, bringing these 3 residues in close apposition [33].

Activation of FXII results in the production of activated protein fragments [9]. Dunn *et al.* proposed six possible sites (see Fig. 6, Panel B) where FXII might be cleaved by either FXIIa (a.k.a. autohydrolysis or autodigestion) or kallikrein producing protein fragments which may have proteolytic activity [9, 34]. Autohydrolysis of site 1 in the area of a labile disulfide bridge results in the formation of αFXIIa . Revak *et al.* proposed that cleavage at sites 2 and 3 just beyond the disulfide bridge was responsible for formation of βFXIIa [35]. Based on additional evidence from Kaplan and Austen [36], Dunn further proposed that intermediate size fragments were produced by cleavage at site 4 (approximate center of the molecule) and site 6.

Cleavage by kallikrein was observed to be faster than autohydrolysis, producing predominantly αFXIIa and βFXIIa . Thus, it was concluded that cleavage at site 4 and 6 was relatively unimportant in kallikrein-mediated proteolysis, although a prolonged reaction of FXII or αFXIIa with kallikrein produced terminal digestion products by cleavage of site 5. Other proteolytic reactions producing smaller fragments were observed but not formally described.

It was also noted that complete hydrolysis of FXII was not achieved under any circumstance, even though the specific activity FXII preparations was >95%. Digestion of FXII by αFXIIa was <35% and increasing αFXIIa concentrations did not increase yield, suggesting that the autohydrolysis reaction was subject to autoinhibition, possibly limited by the conformation of FXII. This self-limiting feature of FXII activation is apparent in

autoactivation ($\text{FXII} \xrightarrow[\text{surface}]{\text{activator}} \text{FXII}_{act}$) as well, wherein total yield of procoagulant fragments

are found to be a small portion (< 5%) of the total FXII available [1, 2, 22, 23]. In order to produce a mathematical model of autoactivation kinetics and yield that accounted for all experimental observations, Zhuo *et al.* were forced to include a hypothetical autoinhibition reaction wherein increasing concentration of autoactivation products (putatively α FXIIa) increasingly inhibited autoactivation [22]. The biochemistry of autoinhibition, if it occurs at all, remains unknown.

4.2 Discontinuous autoactivation in the continuous presence of activator surfaces

Fig. 2 shows that autoactivation by Type I (Panel A) or Type II (Panel B) activator particles exhibit no statistically-discernable kinetic trend over 2-30 min., in agreement with previous work [1, 2, 22, 23]. Quite apparently, autoactivation of FXII in buffer solution is spontaneous within the experimental timeframe studied herein. Furthermore, autoactivation is found to be self limiting, activating only a small portion of available FXII in the continuous presence of activating particles. “Poisoning” of activator surfaces in the process of autoactivation was ruled out as a possible cause for this self-limiting feature [23-25, 37]. Instead, we have invoked autoinhibition mentioned in Section 4.1 to explain kinetics and steady-state yield of procoagulant proteins formed by autoactivation of FXII in buffer and plasma [1, 2, 22].

Furthermore, it is apparent from Fig.2 that yield of amidolytic protein fragments is higher than that of procoagulant proteins when compared on the equivalent basis of apparent α FXIIa activity deduced from α FXIIa titration calibration curves. That is to say, a higher level of amidolytic activity is produced by autoactivation of FXII in buffer solution than procoagulant activity, where activity is measured as the apparent [α FXIIa] required to obtain either chromogen color-development rate or plasma coagulation time expressed in PEU/mL. Although this functional assay does not identify the number or kind of proteolytic proteins produced by autoactivation, and proteolytic activity does not in itself measure concentrations of putative protein fragments, it seems reasonable to interpret Fig. 2 as evidence that both amidolytic and procoagulant protein enzymes are produced by autoactivation. The excess amidolytic activity over procoagulant activity indicates that a greater number or concentration of amidolytic enzymes are produced than procoagulant enzymes (Fig. 1).

Autoactivation may produce protein enzymes with neither amidolytic nor procoagulant properties that are not sensed by the coagulation-time or chromogen assays. Thus, it is quite possible that the activation yield calculated on the basis of apparent α FXIIa activity may significantly underestimate total conversion of FXII by autoactivation. It could also be hypothesized that autoactivation is not inhibited at all, and that the perception of autoinhibition is really due to the fact that most or all of the available FXII is converted into a form that can no longer undergo activation, possibly by denaturation, or other change in conformation of the FXII molecule (see Section 4.6).

4.3 Autoactivation as a function of activator surface chemistry/energy

Fig. 3 shows that autoactivation of FXII in pure buffer produces a higher yield of amidolytic enzymes than procoagulant enzymes across the full range of activator surface energy (water wettability). Amidolytic yield was effectively insensitive to contact-activator surface energy whereas procoagulant yield exhibited a parabolic trend in contact-activator surface energy (closed circles, Panel B), corroborating previously-published results (open circles) [2].

It is particularly interesting that yield of procoagulant enzymes falls almost to detection limits through the $20 < \tau^o < 40 \text{ mJ/m}^2$ ($55^\circ < \theta_a < 75^\circ$) Type 0 activator surface-energy range whereas yield of amidolytic protein enzymes remains high. This evidence supplements the

interpretation of Fig. 2 by showing conclusively that autoactivation produces amidolytic enzymes independent of procoagulant enzymes, at least within a particular activator surface-energy range. Taken together, Fig. 2 and Fig. 3 lead to the conclusion that both amidolytic and procoagulant protein enzymes are produced by autoactivation of FXII in buffer solution (Fig. 1).

4.4 Suppression of autoactivation by products of autoactivation

Physical mixture of as little as 10% w/w Type 0 activator particles with either Type I or Type II contact activator particles suppresses production of procoagulant protein enzymes by autoactivation of FXII in buffer solution, as shown in Fig. 4. This curious phenomenon is apparently mediated by fragments produced by autoactivation of FXII in buffer solution with Type 0 activator particles. If Type 0 autoactivation supernatant is used to suspend Type II activator particles in an FXII activation experiment, nearly no procoagulant enzymes can be detected, even at high surface-to-volume ratio, as shown in Fig. 5. Spiking the Type 0 autoactivation supernatant with additional FXII did not change this outcome, implying either that suppression is not due to a shortage of FXII or that FXII is very quickly denatured by Type 0 produced fragments.

4.5 Interpretation – autoactivation is surface catalyzed but solution phase mediated

Autoactivation is quite evidently a surface catalyzed reaction [1]. Contact activation of blood plasma is definitively contact activator surface-area dependent [37] and the initial rate of FXII activation in buffer also appears to be surface-area dependent [22], although additional work is required to be definitive in this latter regard. Our work has generally used relatively high contact activator surface-area-to-FXII-mass ratio as compared to typical protein adsorbent capacities for hydrophobic-particle activators (see Section 2.5). This latter feature is possibly responsible for spontaneous autoactivation observed herein and in prior work [2, 22, 23, 37].

Autoactivation is very unusual in that the activator surface-energy dependence is observed to be either flat or parabolic, depending on whether amidolytic or procoagulant enzyme yield is measured (Fig. 3). By contrast, other biological responses to materials exhibit a definitive trend in surface energy [38] as in, for examples; contact activation of blood plasma coagulation (nearly specific for water-wettable particulate activators [24, 37, 39]), adhesion of mammalian cells (low attachment efficiency to hydrophobic substrata but high attachment efficiency to hydrophilic substrata [40]), or adsorption of proteins (adsorbent capacity monotonically decreases with adsorbent hydrophilicity [15, 41-44]). We are thus inclined to interpret available results to mean that FXII autoactivation in buffer solution is an outcome of both activator-surface-catalyzed and solution-phase-mediated reactions. Initial surface reactions produce an ensemble of protein fragments that subsequently participate in solution-phase reactions in which the activating surface either does not play a direct role or is a mediator in the basic manner proposed by Zhuo [22]. The surface-energy trends shown in Fig. 3 are indirect evidence that the ensemble composition changes with activator surface energy. The observations summarized in Figs. 4-5 that Type 0 autoactivation fluid suppresses production of procoagulant fragments by Type I and Type II activators is strong evidence for solution-phase biochemistry independent of initial activation.

A seemingly inescapable conclusion is that some sort of FXII activation occurs by *contact with any surface* independent of chemistry or water wettability, producing activated fragments with some combination of amidolytic, procoagulant, or autoactivation-suppressive activity. At hydrophobic surfaces, contact presumably includes non-specific adsorption of FXII and/or activated fragments. Autoactivation by hydrophilic activators

presumably only involves “collisions” with surfaces because blood proteins do not adsorb to water-wettable surfaces (see, ref. [1] and citations therein).

We further interpret the collection of experimental results summarized by Figs. 2-5 to mean that at least three basic categories of fragments are produced by autoactivation of FXII in buffer solution; with each category possibly subsuming a family of fragments (see Fig. 1). One category includes procoagulant fragment(s) that exhibit proteolytic activity that is highly specific for FXI or prekallikrein in the plasma coagulation cascade and also cleave s-2302 chromogen. A second category includes fragments that exhibit only amidolytic activity toward s-2302 chromogen but not procoagulant activity. The entire ensemble of fragments contains proteins that suppress autoactivation, which might exhibit either-or-both procoagulant and amidolytic activity, and possibly fragments exhibiting neither procoagulant nor amidolytic activity.

4.6 Interpretation – autoinhibition or suppression of autoactivation

With discussion of Section 4.1 and experimental evidence disclosed herein in mind, three possible hypotheses come forward as possible explanations for suppression of FXII autoactivation in buffer solution:

- 1. Denaturation of FXII**—Amidolytic fragments denature FXII in some manner, as by conformational change or cleavage of labile groups/domains. FXII or a closely related molecular form persists in solution but in a non-functional state.
- 2. Autoinhibition by FXIIa**—Increasing $[\alpha\text{FXIIa}]$ increasingly inhibits autoactivation, possibly mediated by interaction with contact-activating surfaces as proposed by Zhuo [22].
- 3. Inhibition by amidolytic enzymes**—Amidolytic fragments engage in an enzyme-like inhibition wherein increasing concentration of activated FXII fragments inhibits further activation of FXII, possibly mediated by interaction with contact-activating surfaces.

The simplicity of Hypothesis 1 is attractive because no complex inhibitory biochemistry is required to explain suppression - suppression is simply due to the removal of native FXII. Hypothesis 1 explains the parabolic yield in procoagulant fragments shown in Fig. 3 by invoking high yield of suppression proteins produced by Type 0 activators compared to Type I and Type II activators. High yield of suppression proteins shuts down autoactivation by rapidly denaturing all FXII in solution. Hypothesis 1 does not, however, identify what these putative suppression proteins are or the means by which suppression proteins denature FXII. Dunn has shown that the 80 kDa band associated with FXII does not significantly decrease in intensity upon FXII activation [9]. Although presence of an electrophoresis band does not confirm protein activity, it is strong evidence that FXII denaturation is not due to wholesale proteolysis. Rather, denaturation must leave FXII more-or-less intact, perhaps with structural change(s) or decoration with low-molecular-weight proteins that do not significantly change band position. Furthermore, Hypothesis 1 does not identify the source of suppression proteins. Obviously suppression proteins must ultimately originate from FXII, the sole starting protein in buffer-autoactivation experiments. But Dunn's results suggest that most FXII remains as an 80 kDa band.

Zhuo's mathematical model suggests that Hypothesis 2 feasibly accommodates Type I and Type II autoactivation data obtained under experimental conditions similar to that employed herein with the important proposition that autoinhibition is a contact activator surface-mediated reaction. However, this model was based on phenomenological rate equations and did not specify how autoinhibition actually occurs. It is not immediately evident how the stoichiometric relationship between FXII and FXIIa required by enzyme-like inhibition

could arise. After all, autoactivation yield is low, creating a significant concentration imbalance between FXII and FXIIa. In principle, it is possible that FXIIa yield only appears to be low with the majority of FXIIa molecules becoming bound to FXII. However, this proposition is inconsistent with Dunn's experiments. Furthermore, FXIIa autoinhibition is inconsistent with suppression experiments described in Section 4.4 in which fresh FXII added to the autoactivation fluid phase fails to increase autoactivation yield of procoagulant fragments.

Hypothesis 3 is a generalization of Hypothesis 2 in which the inhibitory role of FXIIa is replaced with protein fragments of unknown identity that engage in enzyme-like inhibition of autoactivation. Hypothesis 3 is also a variant of Hypothesis 1 wherein unknown enzyme-like inhibition reactions replace FXII denaturation through unknown processes. In this regard, Hypothesis 3 suffers from some of the difficulties of both Hypothesis 1 and 2.

Experimental evidence at hand does not allow secure discrimination between the above hypotheses or proposition of alternative hypotheses with fewer unknown reactions/characteristics. We tentatively conclude that autoactivation suppression and autoinhibition are the same phenomenon manifested differently at different surfaces and that the parabolic yield of procoagulant factors observed in Fig. 3B is due to production of suppression proteins by Type 0 autoactivation rather than the result of a peculiarly non-activating surface chemistry postulated by Golas *et al.* [2].

5.0 Conclusion

Contact activation of the blood zymogen FXII in buffer solution produces at least three distinct categories of activated protein fragments, with each category possibly subsuming a family of fragments (see Fig. 1). One category includes procoagulant fragment(s) that either exhibit proteolytic activity for FXI in plasma and/or exhibit specificity for prekallikrein. Kallikrein produced by action of procoagulant fragments hydrolyzes produces FXIIa (possibly FXII_{act}) with high specificity for FXI. A second category includes fragments that exhibit amidolytic activity toward s-2302 chromogen but no procoagulant activity. A third category contains proteins that suppress autoactivation, which might exhibit either-or-both procoagulant and amidolytic activity. Protein fragments exhibiting neither procoagulant nor amidolytic activity may also be produced. The relative proportion of fragment categories depends on activator surface chemistry/energy. Suppression of autoactivation may be related to autoinhibition wherein autoactivation is observed to be self-limiting in a way that controls the amount of FXIIa (possibly FXII_{act}) produced. Results reported herein motivate the assembly of a molecular inventory of enzymes produced on factor XII autoactivation and elucidation of the dependence on activator surface energy. We anticipate that this inventory will provide insights into the mechanism of FXII autoactivation that will be useful in the prospective surface engineering of cardiovascular biomaterials with improved hemocompatibility.

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Impact Statement

This work shows that contact activation of the blood zymogen FXII is a complex surface-mediated reaction that produces a number of different kinds of protein fragments that can influence the hemocompatibility of cardiovascular biomaterials.

Figure 1.

Venn diagram proposing a distribution of protein fragments arising from autoactivation of FXII in neat-buffer solution. Procoagulant fragments (protease activity that induces coagulation of blood plasma and specifically cleaves chromogen s-2302) are shown as a smaller portion of the total fragments produced at any activator surface energy (water wettability). A larger portion of autoactivation fragments express amidolytic activity (cleaves chromogen s-2302) but not procoagulant activity. The “All Fragments” category includes proteins that suppresses autoactivation, which may or may not exhibit procoagulant and/or amidolytic activity, as well as putative fragments with no enzymic activity. The relative proportions of procoagulant and amidolytic fragments (relative sizes of circles) produced by activators with different surface energy are different.

Figure 2. Autoactivation kinetics of 30 µg/ml FXII in neat-buffer solution in contact with (A) hydrophobic octadecyltrichlorosilane treated particles coated with 0.2% solution of Nyebar (Type I; $s=1$, Table 1) and (B) hydrophilic clean-glass particles (Type II; $s=6$, Table 1). The left-hand ordinate plots apparent α FXIIa solution concentration in plasma equivalent units per milliliter (PEU/ml). The right-hand ordinate converts apparent α FXIIa concentration to percent conversion of the initial FXII. Solid symbols represent amidolytic fragment yield whereas open symbols represent procoagulant fragment yield. Error bars represent uncertainty in apparent α FXIIa concentration calculated by propagation of error from calibration curves. Solid lines are guides to the eye.

Figure 3.

Yield of amidolytic (A) and procoagulant (B) protein fragments as a function of activator particle surface energy obtained by autoactivation of 30 $\mu\text{g/ml}$ FXII in neat-buffer solution. Left and right ordinates measure activated protein fragment yield as in Fig. 1. Surface energy of activator particles is expressed as (advancing) water adhesion tension τ_s^0 (where $\tau_s^0 \equiv \gamma_{lv}^0 \cos \theta_s$ is the product of pure-buffer interfacial tension $\gamma_{lv}^0 = 71.97$ dyne/cm at 25 °C and cosine of the advancing contact angle θ_s observed on the s^{th} activator type, see Table 1). Activator Type I, Type 0, and Type II are functionally defined by the production of factors with procoagulant activity (Panel B) denoted by vertical dashed lines spanning Panels A,B. Solid and open symbols in (B) collect data from this and previous studies reported in ref. [2], respectively. Data and error bars represent mean and standard deviation of $N=5$ measurements made over 2-30 minutes of continuous activator particle-solution contact. Note difference in ordinate scaling between (A) and (B). Solid lines are guides to the eye.

Figure 4.

Procoagulant fragment yield obtained by autoactivation of 30 $\mu\text{g/ml}$ FXII in neat-buffer solution with binary mixtures of activator particles. Panel (A) mixes the APTES activator (Type 0; $s=5$, Table 1) with the hydrophobic Nyebar activator (Type I; $s=1$ Table 1). Panel (B) mixes the APTES activator with the hydrophilic clean glass activator (Table II; $s=6$, Table 1). Left and right ordinates measure activated protein fragment yield as in Fig. 1. Lower ordinate is the computed equivalent water-wettability of a hypothetical composite surface bearing molecularly-dispersed chemistries at surface coverage equal to weight fraction of the physical mixtures. Top ordinate axis scales the same data in terms of weight fraction of APTES in the physical mixture. Dashed horizontal lines running across Panel A and B represent αFXIIa yield corresponding to 100% Type I (Panel A) or Type II activators (Panel B). Dashed arrows indicate suppression of activation obtained by mixing APTES Type 0 activator with either Type I (Panel A) or Type II (Panel B) activators. Error bars represent uncertainty in apparent αFXIIa concentration calculated by propagation of error from calibration curves. Solid lines are a guide to the eye. Note difference in ordinate scaling between (A) and (B).

Figure 5. Surface-area titration of FXII with hydrophilic clean glass activator (Type II, Table II; s=6, Table 1) in neat-buffer solution (solid symbols) compared to activation in supernatant derived from autoactivation of FXII with the APTES activator (Type 0, open circles, Type 0; s=5, Table 1). Amidolytic fragments within the Type 0 activation fluid (Fig. 2) suppress production of procoagulant protein fragments (dashed arrow). Error bars represent uncertainty in apparent αFXIIa concentration calculated by propagation of error from calibration curves. Solid lines are a guide to the eye.

Figure 6. Schematic diagram of the structure FXII (Panel A) adapted from references [26-28]. Panel B shows a schematic representation of FXII proteolytic cleavage sites by α FXIIa and kallikrein adapted from [5]. Thick arrows indicate dominant cleavage sites. Thin arrows indicate minor cleavage sites.

Table 1

Water Wettability of Treated Glass Procoagulants

Activator Type	Number Designation (s)	Adsorbent Treatment	Advancing Contact Angle Range (Degrees)
I	1	Nyebar	109-118
I	2	OTS	106-109
0	3	PTES	93-97
0	4	VTES	85-90
0	5	APTES	42-58
II	6	Clean Glass	0