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A Comparison of Blood Factor XII Autoactivation in Buffer, Protein Cocktail, Serum, and Plasma Solutions

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Abstract

Activation of blood plasma coagulation *in vitro* by contact with material surfaces is demonstrably dependent on plasma-volume-to-activator-surface-area ratio. The only plausible explanation consistent with current understanding of coagulation-cascade biochemistry is that procoagulant stimulus arising from the activation complex of the intrinsic pathway is dependent on activator surface area. And yet, it is herein shown that activation of the blood zymogen factor XII (Hageman factor, FXII) dissolved in buffer, protein cocktail, heat-denatured serum, and FXI deficient plasma does not exhibit activator surface-area dependence. Instead, a highly-variable burst of procoagulant-enzyme yield is measured that exhibits no measurable kinetics, sensitivity to mixing, or solution-temperature dependence. Thus, FXII activation in both buffer and protein-containing solutions does not exhibit characteristics of a biochemical reaction but rather appears to be a “mechanochemical” reaction induced by FXII molecule interactions with hydrophilic activator particles that do not formally adsorb blood proteins from solution. Results of this study strongly suggest that activator surface-area dependence observed in contact activation of plasma coagulation does not solely arise at the FXII activation step of the intrinsic pathway.

Keywords

Autoactivation; FXII; plasma coagulation

1. Introduction

Decades of hematological research by many superb investigators following Oscar Ratnoff’s key discovery that contact of blood or blood plasma with glass and other “hydrophilic anionic” surfaces induced coagulation culminated in a descriptive mechanism of the plasma coagulation cascade (see, as notable examples of the era, refs. [1, 2] and citations of the review [3] that generally support this section). According to this mechanism, contact

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activation of plasma involved assembly of proteins comprising an “activation complex” on activating surfaces mediated by specific chemical interactions between complex proteins and the surface. This activation complex was thought to consist of FXII (Hageman factor), prekallikrein (PK), high-molecular-weight kininogen (HMWK), and possibly FXI. When brought together on an activating surface, this activation complex produced the procoagulant enzyme FXIIa. In turn, FXIIa potentiated subsequent steps of the intrinsic pathway of plasma coagulation. The intrinsic pathway consists of a series of linked zymogen-enzyme conversions that ultimately lead to thrombin (FIIa) hydrolysis of fibrinogen to fibrin fragments. These fibrin fragments oligomerize into a fine mesh that causes blood or blood plasma to undergo a phase transition from liquid to gel (coagulate or clot).

It was subsequently found, however, that FXII undergoes activation into procoagulant enzyme(s) in purified buffer solutions when brought into contact with either hydrophobic or hydrophilic activating particles [4–8]. This “autoactivation” reaction demonstrates that assembly of an activation complex is not a biochemical necessity to producing procoagulant enzyme(s) and that autoactivation is not specific to anionic hydrophilic surfaces as previously thought (herein we subscribe to 65° advancing contact angle as the boundary between hydrophilic and hydrophobic materials; see ref. [9] for a recent review related to protein adsorption). Indeed, our work strongly suggests that assembly of activation-complex proteins does not occur in plasma and that products of autoactivation (specifically FXIIa) are amplified by interaction with PK and HMWK in homogeneous plasma solution through the so-called reciprocal loop in a manner that does not require mediation by activating surfaces [3].

FXII autoactivation is a curious surface-catalyzed reaction of unknown mechanism that is widely implicated as a cause of the poor hemocompatibility of cardiovascular biomaterials [3]. We have found that FXII autoactivation in buffer produces an ensemble of as-yet unidentified proteins represented herein by $FXII_{act}$ in the descriptive chemical formula

$FXII \xrightarrow[\text{surface}]{\text{activator}} FXII_{act}$ [7]. Some of these proteins exhibit amidolytic activity (cleavage of amino acid bonds in s-2302 chromogen) but not procoagulant activity (protease activity inducing clotting of blood plasma). The relative proportions of these enzymes produced in buffer solution 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 [7] or surface chemistry [8]; at least in buffer solution. Curiously, autoactivation yield of procoagulant enzymes was found not to measurably scale with activator surface area. Instead, a highly-variable burst of procoagulant-enzyme yield is produced that exhibits no measurable kinetics using the functional assays employed in this work.

FXII autoactivation in plasma can be studied *in vitro* using so-called activator surface-area titration (SAT) of plasma coagulation. A SAT curve plotting plasma coagulation time (CT) against activator surface area is characteristically smooth and reproducibly asymptotic, leading to a lower-CT plateau that depends on activator surface energy (water wettability) [3, 10, 11]. Among different activator surface energies tested, hydrophilic surfaces such as glass and oxidized polymers are observed to be the most efficient activators with the highest “catalytic potential” and lowest CT plateau [11]. The rate-limiting step that controls SAT CT has not been clearly identified but a working assumption has been that the extent of FXII autoactivation controls the amount of procoagulant enzyme(s) produced (putatively FXIIa). In turn, the amount of procoagulant enzyme produced controls the intensity of procoagulant stimulus that propagates down the intrinsic cascade [10], ultimately leading to observed CT dependent on the intensity of surface activation. Indeed, the only plausible explanation

consistent with current understanding of intrinsic-pathway biochemistry is that procoagulant stimulus arising from the activation complex is dependent on activator surface area.

Thus, it is inferred that FXII autoactivation in plasma is surface-area dependent. But this inference is in direct conflict with the pronounced lack of surface-area dependence of FXII autoactivation in buffer [7]. The conundrum that arises is that some part of the plasma activation process *must be surface area dependent* in order to explain observed SAT surface-area dependence. If this part of the activation process is not FXII autoactivation, then how exactly does surface-area dependent SAT arise?

Herein we explore some of the more subtle characteristics of FXII autoactivation by measuring autoactivation in buffer, protein-cocktail, heat-denatured serum, and FXI deficient plasma under different experimental conditions of mixing and temperature. We compare autoactivation yield of procoagulant enzyme(s) in buffer and plasma to discover if autoactivation in these two very different fluid phases is fundamentally different.

2. Methods and Materials

2.1 Preparation and Characterization of Particulate Activators

Test activator surfaces used in this work were 425–600 μm diameter glass particles (Sigma Aldrich) with $5 \times 10^{-3} \text{ m}^2/\text{g}$ nominal specific area (based on 512.5 μm mean diameter and 168 $\mu\text{g}/\text{particle}$). The surface area measured by the Brunauer-Emmett-Teller (BET) method (Micromeritics ASAP 2000 using liquid nitrogen as the probe gas) was $(8.5 \pm 0.1) \times 10^{-3} \text{ m}^2/\text{g}$. 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.

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 $^\circ\text{C}$) 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. Glass surfaces treated in this manner were found to be fully water wettable and designated “clean glass” or “hydrophilic glass”.

Phosphate buffered saline (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 to confirm perfect wettability of glass coverslip witness samples (see references [12, 13] for a comparison of goniometric techniques and discussion of experimental errors).

2.2 Plasma, Heat-denatured serum, Protein Cocktail, and Coagulation Proteins

2.2.1 Plasma—Citrate human platelet-poor plasma (PPP) was prepared from unexpired blood-plasma obtained from the M.S. Hershey Medical Center Blood Bank, and prepared as described previously [14, 15]. This work was performed with three lots of PPP (designated Lot I, II and III respectively) aliquoted into 15 ml polypropylene tubes (Falcon, Becton Dickinson) and frozen at -20°C until use. Whereas results with stored PPP were qualitatively consistent, minor quantitative variations arose within the lots over about six months of experimentation. Variations in stability of coagulation factors during storage may be responsible for these differences [16–18]. Variation in coagulation properties of plasma was corrected for by use of FXIIa titration curves (Section 2.3). FXI depleted platelet-poor

plasma (FXIdPPP) was obtained from Haematologic Technologies (Essex Junction, VT). FXI was obtained from Haematologic Technologies (Essex Junction, VT).

2.2.2 Heat-denatured serum—Heat-denatured serum was prepared by adding 100 mg of hydrophilic glass (Section 2.1) to 9 mL of plasma (Lot II) and 1 mL of 0.1 M CaCl_2 , and mixing for 1 hr on a rotating hematology mixer. Serum was separated from the coagulated mass after centrifugation at 500 x g (Eppendorf centrifuge 5702). The resulting serum phase was heated in a water bath at 90-95 °C for 1 hr, separated from a small clotted mass by centrifugation, and frozen overnight at -10 °C. Thawed serum was centrifuged again before use in FXII activation studies (Section 2.4) and tested in the plasma coagulation assay (Section 2.3) for procoagulant activity. Heat-denatured serum so prepared was found not to measurably decrease CT of test plasma ($\text{CT} = 27.2 \pm 0.5$ min, $N=3$ for serum compared to 26.0 ± 1.0 min blank without added serum, $N = 3$). Serum prepared using the above protocol but without heat denaturation was found to cause plasma to clot promptly with $\text{CT} = 1.4 \pm 0.2$ min, $N = 3$.

2.2.3 Protein Cocktail—An arbitrarily-designed mixture of proteins not directly involved in plasma coagulation was prepared in PBS consisting of bovine albumin at 12 mg/ml, bovine IgG at 2 mg/ml, and bovine IgM at 1.1 mg/ml; yielding a total of 15.1 mg/ml protein (used as received from Sigma Aldrich, St. Louis, MO). This protein concentrate was used full strength (100%) or diluted to 25% and 50% in PBS as desired.

2.2.4 Coagulation Proteins—Human FXII (4 lots designated Lots A, B, C, D) and αFXIIa (2 lots designated Lots 1, 2) were used as received from Haematologic Technologies (Essex Junction, VT) and Enzyme Research Laboratories (South Bend, IN) respectively. Activity of both FXII and αFXIIa was specified by the vendor in either mg/mL or traditional units of plasma equivalent units per mL (PEU/mL) [19, 20]. Vendor provided conversion factor was 75.07 PEU/mg for αFXIIa Lot 1, and 75.13 PEU/mg for αFXIIa Lot 2.) Neat-buffer solutions of FXII and αFXIIa solutions were prepared in PBS (Sigma; 0.14M NaCl, 3mM KCL prepared from powder in 18M Ω de-ionized water, pH 7.4).

2.3 Coagulation Time Assay and Apparent FXIIa Yield

2.3.1 Plasma Coagulation—Plasma coagulation time (CT) was used as the traditional hematology method to quantify procoagulant activity resulting from addition of procoagulant enzymes or surface activation. Protocol for the CT assay has been described in detail elsewhere [4, 11, 21-23]. Briefly, procoagulants (enzymes such as FXIIa, solutions containing unknown enzymes, or activator surfaces) were added to 50% recalcified citrated plasma in proportions specific to each assay described below. Procoagulants caused plasma to coagulate at a CT in proportion to activation dose. CT was detected by a distinct change in fluid-like rheology to gel, allowing determination of CT endpoint to within 10 sec or so [23]. This simple, yet highly sensitive, recalcification-time assay eliminated extraneous contributions to coagulation associated with many modern instrumented tests (*e.g.* activating surfaces of stirrers and tubing) and yielded smooth dose-response curves. Strictly speaking, this CT assay actually measured net procoagulant activity of the test materials and, as such, neither discriminated among various activated proteins that might be in test solutions nor accounted for differential activity of these proteins.

2.3.2 FXIIa Titration of Plasma—Net procoagulant activity (stimulus dose) was optionally measured in terms of “apparent αFXIIa concentration” by appealing to a “ αFXIIa titration calibration curve” that related CT to exogenous αFXIIa concentration expressed either in PEU/mL or mg/ml [19, 20]. In this way CT achieved by any plasma-

coagulation activator (enzymic or surface) could be converted to the equivalent purified α FXIIa that must be added to the 50% plasma test system to achieve that CT.

Briefly, α FXIIa titrations were carried out by equilibrating 500 μ l of thawed PPP in 12 x 75mm polystyrene tubes (VWR), mixing with serially increasing FXIIa concentration in PBS, serum (Section 2.2.2), or protein cocktails (Section 2.2.3), 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.). Measured CT was observed to be exquisitely sensitive to FXIIa with a minimum quantifiable concentration of $\sim 9 \times 10^{-4}$ PEU/ml. α FXIIa titration curves in PPP provided linear-like calibration curves when scaled on a logarithmic concentration axis over the dynamic range of α FXIIa activity of interest in this work (see refs. [22, 24] for a detailed description). Linear calibration curves were fit to $CT \log_{10} [FXIIa] + c$ where m and c are adjustable parameters of the linear regression. Three additional calibrations were conducted each month of plasma lot in use, to account for variations in m and c not exceeding 43% and 10%, respectively. A typical plasma calibration (Lot I) yielded $m = -10.38 \pm 0.42$, $c = 6.50 \pm 0.50$, $R^2 = 0.96$ from triplicate measurements. These calibration curves were used to quantify procoagulant activity of test solutions from FXII and plasma autoactivation experiments, reported herein as apparent FXIIa concentration in PEU/mL.

α FXIIa titrations in reconstituted FXI deficient platelet poor plasma (rFXIdPPP), were carried out by equilibrating 500 μ l of thawed FXIdPPP reconstituted with FXI at nominal physiological concentration (5 μ g/mL, [3]) in 12 x 75mm polystyrene tubes (VWR), mixing with serially increasing FXIIa concentrations 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.). Measured CT was found to be effectively insensitive to FXIIa concentration below 0.05 PEU/ml. FXIdPPP was thus found to be an insensitive assay vehicle for estimating FXII activation in plasma (see further Section 4.43).

Heat-denatured serum (Section 2.2) was tested in the plasma coagulation system by mixing 400 μ L as-prepared serum with 500 μ L plasma and 100 μ L CaCl_2 solution.

2.4 Autoactivation of FXII in Buffer and Protein Solutions

Autoactivation of FXII in buffer and protein solutions by hydrophilic glass-particle activators was the primary phenomena studied in this work. Surface-area dependence of autoactivation was measured using the surface-area-titration (SAT) method in which 500 μ l of 30 μ g/mL FXII (nominal physiological concentration [3]) in PBS, 100% denatured serum (Section 2.2.2), protein cocktail solutions (Section 2.2.3), or FXIdPPP (Section 2.2.3) was brought into contact with varying particulate activator mass between 0 and 1000 ± 0.1 mg (0.50×10^{-4} m² range, based on a nominal specific surface area 5×10^{-3} m²/g).

Activator particles were weighed into test tubes using a laboratory electronic balance, mixed with FXII solution, and incubated over 30 min timed contact in either “static” mode (FXII in solution initially mixed with activator particles and subsequently allowed to stand undisturbed until analysis) or “dynamic” mode (FXII in solution continuously mixed with activator particles) at ambient laboratory temperature. Continuous mixing was carried out with either a shaking-platform mixer or a rotating hematology mixer (Roto-shake Genie, Scientific Industries Inc.) at two speeds (6 rotations/min and 11 rotations/min). Autoactivation of FXII in protein solutions was performed only in static mode.

Procoagulant activity of FXII autoactivation products was measured as apparent FXIIa yield using the CT assay (Section 2.3). Specifically, 100 μl of autoactivation supernate was mixed with 500 μl of thawed normal plasma in 12 x 75mm polystyrene tubes (VWR), mixed 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 (“dynamic mode”, 6 rotations/min). Apparent FXIIa yield was calculated by appealing to appropriate FXIIa titration-calibration curve (Section 2.3). Plasma Lot I and FXII Lot A and B were used for autoactivation of FXII in PBS whereas Plasma Lot II and FXII Lot C were used for autoactivation experiments performed with 100% denatured serum and protein cocktails.

2.5 Evaluation of Activator-Particle Poisoning

An initial FXII (Lot A) autoactivation in PBS was performed as described in Section 2.4. Supernatant was then removed by careful pipette-aspiration, and gentle wicking away of any remaining fluid with Kimwipe towels. Particles were then incubated for 30 minutes with a fresh solution of 500 μl of 30 $\mu\text{g/ml}$ FXII in buffer at ambient temperature. Apparent FXIIa generated by secondary activation in “static” mode, was measured using CT (Plasma Lot I) assays as described in Section 2.3.

A specially-designed static autoactivation assay permitted a constant-composition FXII solution to be exposed to serially-increasing clean-glass-particle activator surface without changing original autoactivation fluid composition for second exposure to fresh activator surface. The purpose of this alternative protocol was to avoid the change in solution volume that necessarily accompanied removal of an aliquot of supernate for measurement of apparent FXIIa yield. For this purpose, 6 identical tubes were set up initially containing 10 mg ($0.5 \times 10^{-4} \text{ m}^2$) of hydrophilic glass activator. After incubation with 500 μl of 30 $\mu\text{g/ml}$ FXII in PBS for 15 minutes, tube 1 was sampled for apparent FXIIa concentration and discarded. An additional 200 mg ($10 \times 10^{-4} \text{ m}^2$) of glass activator was then added to each remaining tube, mixed by hand, and incubated for 15 minutes. The second tube was as sampled for apparent FXIIa concentration and discarded. An additional 200 mg of glass activator was then added to each remaining tube, mixed by hand, incubated for 15 minutes. Tube 3 was sampled and discarded. This process was repeated for the remaining 3 tubes, measuring autoactivation yield dependence on activator surface area at constant test-solution composition.

2.6 Temperature-Dependent Autoactivation of FXII in Buffer Solution

Test solutions of FXII (Lot D) in neat-buffer solutions were prepared at 30 $\mu\text{g/mL}$ [25]) in 2 ml microtubes (VWR) and placed in a water bath to bring the solution to the trial temperature ($20^\circ\text{C} < T < 50^\circ\text{C}$). Following equilibration to test temperature, 200 μL of solution was incubated with 100 mg ($5 \times 10^{-4} \text{ m}^2$) of clean-glass activator in “static” mode. Apparent FXIIa concentration produced by timed contact (2 to 30 min) was quantified using the CT assay (using plasma Lot III) described in Section 2.3.

2.7 Surface Area Titration and FXIIa Titration of Blood Plasma

Procoagulant catalytic potential to activate whole plasma coagulation was assayed using the surface-area titration method reported previously [11, 14, 15, 21, 23, 24, 26–28]. Briefly, 500 μl of thawed plasma equilibrated with ambient temperature was transferred into 12x75 mm polystyrene tubes (VWR) containing sufficient weight of clean glass activator to deliver surface area within the $(0 \text{ A } 50) \times 10^{-4} \text{ m}^2$ range, based on a nominal specific surface area $5 \times 10^{-3} \text{ m}^2/\text{g}$ and diluted with sufficient PBS to bring the final total liquid volume to 900 μl . Plasma was recalcified with 100 μl of 0.1 M CaCl_2 , and tubes were immediately capped with parafilm and mounted on the table of a slowly-turning hematology mixer

(“dynamic” mode, 6 rotations/min). Coagulation time after recalcification was noted by a distinct change in fluid-like rheology to gel formation as described in Section 2.3.

SAT data was fit to theory described in ref. [23] designed to extract the K_{act} parameter that measured procoagulant catalytic potential relative to an internal standard (K_{act} has units of mL/m² [21], not m⁻² as reported in [23]). A rate constant required by theory (designated as either k_p in ref. [21] or k_2 in ref. [23]) that measured the rate of fibrin polymerization (for a particular lot of plasma) was determined by a two-parameter fit (k_p and K_{act}) to the glass standard as described in refs. [21, 23]), yielding $k_p = 0.29 \pm 0.02 \text{ min}^{-1}$ and $K_{act} = 34.4 \pm 12.6 \text{ mL/m}^2$ (best fit value $\pm$ standard error of the fit, $R^2 = 94.6\%$). Holding $k_p = 0.29 \pm 0.02$ constant and performing a single-parameter fit to K_{act} yielded $K_{act} = 32.3 \pm 4.7$ for plasma Lot I. Thus, K_{act} measured activation potential at an arbitrary k_p value and should not to be interpreted as a quantitative characteristic of procoagulants studied herein. Likewise k_p should not be regarded as representative of human plasma in general (see ref. [23] for more discussion).

Surface-area dependence of autoactivation of endogenous FXII in FXIdPPP was measured using the basic SAT protocol by which 500 μL of FXIdPPP was mixed with varying activator mass between 0 and $1000 \pm 0.1 \text{ mg}$ ($(0 \text{ A } 50) \times 10^{-4} \text{ m}^2$ range, based on a nominal specific surface area $5 \times 10^{-3} \text{ m}^2/\text{g}$). Activator particles were weighed into test tubes using a laboratory electronic balance, mixed with FXIdPPP, and incubated over 30 min timed contact in “static” mode. Procoagulant activity products of endogenous FXII activation in plasma was measured in terms of apparent FXIIa yield using the CT assay (Section 2.3).

3. Results

FXII (30 $\mu\text{g/mL}$ nominal physiological concentration [3] in all cases) autoactivation yield in buffer and protein solutions was measured and compared to surface-area titration of plasma (plasma SAT). Autoactivation experiments were performed both in “static” mode (FXII in solution initially mixed with activator particles and subsequently allowed to stand undisturbed until analysis), optionally at different incubation temperatures, and “dynamic” mode (FXII in solution continuously mixed with activator particles) at ambient laboratory temperature or optionally at different solution temperatures. Yield of procoagulant protein (enzymes that cause coagulation of a test plasma solution) was quantified as the equivalent concentration of exogenous αFXIIa that must be added to plasma to achieve the same plasma coagulation time (CT) obtained by adding an aliquot of autoactivation fluid to plasma, bearing in mind volumetric dilutions. This “apparent FXIIa solution concentration” in plasma-equivalent units/mL (PEU/mL) was measured using FXIIa titration curves performed in dynamic mode (as further described in Section 2.3.2 and Appendix A). It is important to stress that apparent FXII subsumes only those proteins with procoagulant properties in plasma and does not necessarily constitute total FXII conversion, which can include proteins with only amidolytic properties (cleavage of amino acid bonds in s-2302 chromogen) or no detectable enzymic activity (see ref. [7] for more discussion of FXII autoactivation products produced in buffer). FXIIa titration of reconstituted FXI depleted platelet poor plasma (rFXIdPPP, Section 2.3) was performed in the same manner as FXIIa titration of normal plasma.

Contact activation of plasma coagulation was performed in dynamic mode and herein referred to as a plasma surface-area titration (plasma SAT) using hydrophilic glass-particle activator (Section 2.3). Apparent yield of endogenous FXIIa obtained in plasma SAT was computed using data of an exogenous FXIIa titration of plasma that converted CT observed at different activator surface area to apparent FXIIa solution concentration in the manner

described above for FXII autoactivation in buffer. Apparent FXIIa yield was compared to that obtained by FXII autoactivation in buffer. FXII autoactivation in FXI-depleted plasma (FXIdPPP) was performed in the same manner as the normal plasma SAT except that no CT was measured because the plasma coagulation cascade in FXIdPPP was dysfunctional, unable to propagate the FXIIa procoagulant stimulus down the intrinsic pathway to FIIa hydrolysis of fibrinogen (FI). In this case, yield of protein(s) with procoagulant properties was quantified by adding activated FXIdPPP solution to normal plasma, measuring CT, and converting observed CT to apparent FXIIa solution concentration as mentioned above.

3.1 Surface Area Titration (SAT) of Human Blood Plasma

Fig. 1 (left ordinate) is a typical plasma SAT curve (Section 2.3, Lot I) obtained with hydrophilic glass activator particles showing a diminishing-returns trend wherein small increases in activator surface area caused rapid decrease in CT, followed by decreasing rate-of-change in CT with increasing surface area to a lower asymptotic plateau. These results corroborated that obtained in numerous previous studies (see review [3]). The “catalytic potential” of a particulate activator to activate plasma coagulation has been quantified by the K_{act} parameter which has been found to be characteristic of activator surface chemistry/energy [10, 11]. The line through the CT data of Fig. 1 (left ordinate) is the statistical best fit of the model described in ref. [11] that yielded $K_{act} = 32.3 \pm 4.7 \text{ m}^2/\text{mL}$, which was somewhat higher than the 19.2 ± 1.9 [8] and 21.1 ± 2.1 [10] values obtained for clean-glass particle activation of different lots of human plasma studied in previous work (different lots of prepared plasma are unique test vehicles not to be quantitatively compared).

3.2 Activated Factor Titrations of Human Blood Plasma

Fig. 2A is an FXIIa (Lot 1) titration of plasma (Lot I) obtained by adding PBS solutions of FXIIa solutions at different concentrations to plasma (Section 2.3). Increasing exogenous FXIIa concentration led to monotonically decreasing CT to a lower-CT plateau. This trend in FXIIa concentration appeared linear-like on a log concentration scale (inset) which served as a convenient calibration curve relating CT to the amount of exogenous FXIIa that must be added to plasma to achieve a particular CT.

Fig. 2B is an FXIIa (Lot 1) titration of FXI reconstituted FXIdPPP (rFXIdPPP) performed as above for normal plasma. Blank coagulation time of rFXIdPPP was nearly 4x lower than normal plasma and data for two trials were not as reproducible as normal plasma (Fig. 2A). The weak dependence of CT on FXIIa concentration was not linear-like on a log FXIIa scale (Fig. 2B inset).

FXIIa (Lot 2) titration of plasma (Lot II) with FXIIa dissolved in PBS, 100% protein-cocktail (Section 2.2.3), and 100% heat-denatured serum (Section 2.2.2) were similar to Fig. 2A in general appearance (not shown). Data corresponding to PBS solution was more reproducible than that of FXIIa dissolved in serum and had measurably sharper dependence on FXIIa concentration than titrations in either protein-cocktail or denatured serum ($m = -4.93 \pm 0.36$, $c = 5.37 \pm 0.62$, $R^2 = 0.95$; see Section 2.3.2). FXIIa titrations in either protein-cocktail or denatured serum could not be reliably distinguished ($m = -7.47 \pm 0.78$, $c = 7.49 \pm 0.75$, $R^2 = 0.94$ for protein-cocktail, and $m = -8.48 \pm 1.02$, $c = 9.02 \pm 0.53$, $R^2 = 0.90$ for denatured serum).

3.3 FXII Autoactivation in Buffer

Fig. 3A, B compiles results of autoactivation experiments designed to test the hypothesis that clean-glass activator particles were “poisoned” upon first activation of FXII in buffer solution (Lot A). Fig. 3A compares apparent FXIIa yield obtained upon first static activation with varying activator surface area to a second static activation obtained after removing the

initial FXII solution with autoactivation products and carrying out the second activation with fresh FXII solution (Section 2.5).

This straightforward protocol did not test first and second activations against the same initial FXII solution composition due to supernate sampling after the first activation. To avoid this, a specially-designed assay that permitted a constant-composition FXII solution (Lot B) to be exposed to serially-increasing clean-glass-particle activator surface area (Section 2.5) was used to collect data of Fig. 3B. No surface area dependence was detected using either protocol and results were highly variable in response to unknown experimental variables. We applied the term “stochastic” to these highly-variable results in the sense that autoactivation in buffer appeared to exhibit an element of chance or probability. The average yield of the second protocol was approximately 2x that obtained by the first protocol, possibly because these two assays used two of different lots of FXII with presumably different protein activity (Lots A, B). In general we observed lot-to-lot variability in FXII preparations and stochastic autoactivation within any particular FXII lot.

Fig. 4A compiles results of four static autoactivation experiments (Lots A and B) that expanded the activator surface-area range of Fig. 3A. Apparent FXII conversion fell within a band ranging from a low of 2% to a high near 8%, with no discernible dependence on activator surface area. Continuous mixing on a shaking-platform mixer did not increase yield (not shown). Likewise, aggressive mixing at 6 revolutions/min and 11 revolutions/min obtained on a rotating hematology mixer did not produce a statistically-significant increase autoactivation yield, as shown in Fig. 4B. Results collected in Fig. 4 reproduced the stochastic nature of FXII autoactivation compiled in Fig. 3.

Fig. 5 compares static FXII autoactivation yield at four incubation temperatures ranging from 20° to 50° C as a function of activator surface area (Section 2.6). There was no statistical dependence of FXIIa yield on either temperature or activator surface area. Results collected in Fig. 5 reproduced the stochastic nature of FXII autoactivation observed in Fig. 3 and 4.

3.4 FXII Autoactivation in Protein Solutions

FXII autoactivation (Plasma Lot II, FXII Lot C) in serially-increasing concentration of protein cocktail (0, 25, 50, and 100%; Section 2.2.3) and in heat-denatured serum (section 2.2.2) were qualitatively the same as observed in buffer (not shown), except that autoactivation yield with this particular lot of FXII was, on average, 4x lower than Lot A and Lot B shown in Figs. 3-5. The general experience of this and prior work [7] was that autoactivation yield performed with different lots of FXII under identical experimental conditions were quantitatively different but exhibited similar stochasticity. Interestingly, average FXII autoactivation yield in heat-denatured serum (9.16 ± 2.91) was observed to be nearly 10x greater than in comparable PBS controls (1.00 ± 0.22) with no discernible dependence on activator surface area.

Fig. 6 compiles results of triplicate activation experiments designed to measure autoactivation of endogenous FXII in FXIdPPP with increasing activator surface area. Apparent concentration of FXIIa in PEU/mL exhibited no discernible dependence on activator surface area and was stochastic in nature, especially at low surface area. In this sense, autoactivation of endogenous FXII in FXIdPPP was similar to that observed in buffer (Section 3.1) but FXIIa concentrations were significantly above blank levels (no added activator) for only some of the trials at different activator surface area.

4. Discussion

This work is motivated by a need to understand the mechanism of contact activation of blood plasma for the purpose of engineering advanced cardiovascular materials with improved hemocompatibility. Critical to achieving this end is an understanding of the mechanism of contact activation of FXII, a most mysterious surface-catalyzed reaction which is thought to potentiate the intrinsic pathway of plasma coagulation leading to clot formation (see further Section 1). Part of our program has focused on whole plasma as a test system, seeking to quantify and rate the “catalytic potential” of a material to induce coagulation per-unit-area of surface, hoping to identify classes of surface chemistry/energy with minimal catalytic potential [3, 6, 11]. We further have sought to measure how procoagulant stimulus at the contact-activation step propagates down the intrinsic pathway and the role that blood proteins other than those of the coagulation cascade might play in mediating or moderating coagulation [6, 10]. Another part of our program has focused on FXII autoactivation in buffer solution, seeking to understand details of autoactivation in the simplest possible solution phase [7]. In this work, we attempt to bring together these different approaches into a combined interpretation by answering two central questions.

The first of these questions asks if FXII autoactivation in buffer or protein solutions that exclude all known proteins of the coagulation cascade exhibits dependence on activator surface area that can account for activator surface-area dependence observed in activator surface-area titration of plasma coagulation (plasma SAT). That is to say, we seek to know if autoactivation of endogenous FXII in plasma is fundamentally different than FXII autoactivation in buffer or protein solutions emulating the protein content of plasma. Similar surface-area dependence would suggest that surface-area dependent SAT arises at the autoactivation step of the intrinsic pathway of plasma coagulation. Demonstrably different surface-area dependence would suggest that activator surface-area dependence occurs in steps other than FXII autoactivation. This latter finding would further suggest that activator surfaces play a mediating role in plasma coagulation more complex than represented by the

descriptive chemical formula $FXII \xrightarrow[\text{surface}]{\text{activator}} FXII_{act}$.

The second question asks if the yield of procoagulant enzymes (enzymes that cause coagulation of a test plasma solution) produced by autoactivation of FXII in buffer and protein solutions by hydrophilic glass particles can account for the decrease in plasma coagulation time (CT) observed in plasma SAT. A rough activity balance taking into account reciprocal-loop amplification in plasma would suggest that autoactivation of endogenous FXII can account for observed CT reduction. Failure to strike a reasonable activity balance suggests that other steps of the intrinsic pathway contribute to the observed CT reduction in a plasma SAT.

Although results discussed below are necessarily indirect and inferential, the experimental approach provides a perspective on autoactivation that is difficult-or-impossible to obtain in any other way. And results reveal heretofore unknown enigmatic features of surface activation of blood plasma coagulation. Discussion is organized as follows. First, critical characteristics of plasma SAT are discussed in the context of a considerable literature background [3]. Second, details of FXII autoactivation in buffer, synthetic protein cocktails, heat-denatured serum, and FXI depleted plasma (FXIdPPP) by hydrophilic glass activator particles are disclosed. These observations lead to summary conclusions regarding procoagulant yield and sensitivity to experimental variables including activator surface area, mixing, and temperature. The focus of work herein is necessarily on hydrophilic, fully-water-wettable glass activator particles because hydrophobic counterparts do not activate plasma coagulation (but do autoactivate FXII in buffer [3]). Hence a comparison of

autoactivation of FXII in buffer [4–7] and plasma [3, 10, 11, 29] by hydrophobic activators by these methods is not possible. Viability of FXIdPPP as a test vehicle will be discussed by comparison to normal plasma. Finally, autoactivation yield of procoagulant enzymes in plasma will be estimated by reference to FXIIa titration and compared to that directly measured in buffer. Conclusions will draw observations together in answer to the central questions discussed above.

4.1 Surface-Area Titration (SAT) of Human Blood Plasma

Current understanding [3] has it that autoactivation of endogenous FXII in plasma produces procoagulant enzymes (putatively FXIIa) that can be amplified by the so-called reciprocal loop. A proportional relationship between activator surface area and FXIIa activity has been inferred from experimental SAT curves like Fig. 1 (left-hand ordinate, see further Section 1). But this proportional relationship has not been proven by recovery and measurement of FXIIa from plasma activated by contact with varying activator surface area. Indeed, such an assay would be difficult-or-impossible to actualize because of the complexity of the plasma/serum proteome [9, 30, 31] and the multiplicity of proteolytic enzymes in activated plasma.

4.1.1 Contact Activation Intensity—Plasma activation intensity depends on activator “catalytic potential” K_{act} as well as surface area. Mathematical models of plasma coagulation we have used to estimate K_{act} values do not explicitly link activator surface area or surface chemistry/energy with FXIIa production [10, 11, 32], relying instead on the black-box assumption that greater activator potential (as measured by activator surface area or surface energy) ultimately produces higher thrombin (FIIa) concentrations, which in turn cause more rapid production of fibrin and commensurately reduced CT. Trends in SAT curves obtained using activators with incrementally-decreasing surface energy (increasing hydrophobicity) are similar to that shown in Fig. 1 (left-hand ordinate), forming nested SAT curves when collected on the same axes, with the lower-CT plateaus systematically increasing with increasing activator hydrophobicity, up to the non-activated blank value (not shown, see ref. [11] for examples). It is further found that K_{act} scales nearly exponentially with activator surface energy (water wettability) with values near zero for hydrophobic activators and much higher values for hydrophilic activators [10, 11].

These observations lead to the conclusion that plasma SAT is a self-limiting process whereby the lower-CT asymptote is characteristic of activator K_{act} . That is to say, a SAT curve represents a convolution of procoagulant stimuli measured by K_{act} and surface area, not surface area alone. In fact, Zhuo *et al.* have shown that response of the plasma coagulation cascade in terms of FIIa production is exponential in K_{act} and only modestly linear in activator surface area, with but 1.7x increase in endogenous FIIa production corresponding to 10x increase in hydrophilic-glass activator surface area (see Fig. 3 and Table 2 of ref. [10]).

4.1.2 Asymptotic Reduction of Plasma Coagulation Time—The asymptotic trends observed in plasma SAT to a lower-CT plateau that depend on activator surface energy (procoagulant catalytic potential) is unusual in that more procoagulant stimulus (activator surface area) does not lead to continuously lower CT. It is also unanticipated behavior for a series of linked zymogen-enzyme conversions that have been aptly described as an enzyme amplifier system in early literature [33–35]. Ordinarily, enzymes in a mixture with substrate continuously produce product. Applied to the coagulation cascade *in vitro*, it might be thus anticipated that procoagulant enzyme concentration would rapidly build, causing FIIa concentrations to spike. And indeed bolus production of FIIa in proportion to activation stimulus is both inferred [10] and experimentally observed [3]. Yet plasma SAT CT are much higher than the minimum time required for plasma to coagulate. For example, high

surface area of hydrophilic glass reduces CT ~ 10 min (Fig. 1, left-hand ordinate) whereas the lowest possible CT associated with FIIa titration of plasma is < 1 min in similar test systems (a.k.a. thrombin time) [10].

Our interpretation is that CT is controlled by the rate at which FIIa hydrolyzes plasma fibrinogen (FI). Accordingly, a fixed amount of FI must be converted to fibrin before the fibrin-oligomerization reaction reaches sufficient density to cause a phase transition from liquid to solid [11, 36]. The time required to reach this density of oligomerized fibrin thus depends on FIIa concentration, which in turn depends on the procoagulant stimulus propagated down the intrinsic pathway. However, this does not explain details of this procoagulant propagation, which steps along the intrinsic pathway are rate limiting, and why maximal contact activation does not lead to thrombin time.

4.1.3 Summary Conclusions on Surface-Area Titration (SAT) of Plasma—The proportional relationship between activator surface area and autoactivation of endogenous FXII in plasma inferred from a plasma SAT is the result of a convolution of activator surface area and catalytic potential K_{act} . This co-dependence complicates the authentic dependence of plasma SAT on surface area. The activation response of the plasma coagulation cascade in terms of FIIa production is exponential in K_{act} and only weakly linear in activator surface area FXIIa activity, suggesting that the apparent surface-area dependence of a plasma SAT arises from time-dependent processing of procoagulant stimulus through the intrinsic pathway of coagulation that controls CT, as further confirmed in the following sections.

4.2 Activated Factor Titration of Human Blood Plasma

Direct addition of activated coagulation factors to plasma can bypass selected portions of the intrinsic pathway of coagulation. An example already mentioned in the previous section is FIIa titration that bypasses the entire intrinsic pathway by direct hydrolysis of FI. Another example used in this work is exogenous FXIIa titration of plasma that bypasses autoactivation of endogenous FXII. According to current understanding of the coagulation cascade, exogenous FXIIa can be amplified by the reciprocal-loop using endogenous FXII as a source, but this process does not involve direct autoactivation of endogenous FXII. Thus, FXIIa titration of plasma simulates the effect of serially-increasing autoactivation of endogenous FXII.

4.2.1 Titration of Plasma with Exogenous FXIIa Dissolved in Buffer—Fig. 2A,B are FXIIa titrations of normal plasma and FXI reconstituted FXIdPPP (rFXIdPPP), respectively. FXIIa titration of normal plasma exhibits the same trend of monotonically decreasing CT to a 10 min lower CT asymptote as observed in plasma SAT using hydrophilic glass particles (Fig. 1 left-hand ordinate). This similarity is striking because it suggests, but does not prove, that surface-area dependence in plasma SAT is directly linked to FXIIa concentration produced by autoactivation. The asymptotic decrease to a lower-CT plateau observed in both cases is also unusual in that more procoagulant stimulus (FXIIa or activator surface area) does not lead to continuously lower CT to thrombin time, as discussed above. Apparently, the asymptotic trend arises from time-dependent processing of procoagulant stimulus (exogenous FXIIa) through the intrinsic pathway.

Detailed examination of Fig. 2A shows that the initial linear-like decrease in CT terminates at only ~ 0.01 PEU/mL, or about 0.13 $\mu\text{g/mL}$ (using 75.07 PEU/mg for FXIIa Lot A). It is of interest to assume, for the sake of argument, that the linear-like decrease in CT observed in a plasma SAT corresponds to this amount of endogenous FXIIa produced from 15 $\mu\text{g/mL}$ endogenous FXII (50% diluted nominal physiological concentration). This assumption

implies that < 1% autoactivation yield in plasma already begins to saturate the intrinsic pathway of coagulation, with additional autoactivation yield unable to significantly reduce CT due to unidentified procoagulant-stimulus-processing delays along the intrinsic pathway (see further Section 5).

4.2.2 Titration of Plasma with Exogenous FXIIa Dissolved in Protein Solutions

—We find that FXIIa titration of plasma using exogenous FXIIa solutions dissolved in protein cocktail and heat-denatured serum measurably affects the functional relationship between CT and FXIIa concentration compared to FXIIa dissolved in buffer. FXIIa titration curves from both protein cocktail and denatured serum exhibited a shallower asymptotic decrease in CT than titration from buffer (not shown, see Section 3.4). Protein cocktail and denatured serum titrations were not resolvable. Given that volumetrics were constant among experiments, the difference between FXIIa titration from buffer and protein-containing solutions cannot be attributed to a simple dilution effect. We conclude that exogenous protein added to plasma along with exogenous FXIIa changes the activity of one or more of the enzymes involved in coagulation. Possibly, for example, exogenous protein undergoes limited proteolysis by enzymes such as FXIIa affecting the stoichiometric relationship between enzyme and primary substrate.

We have previously shown that FXII autoactivation in protein cocktails enhances procoagulant catalytic properties of hydrophilic activators and reduced catalytic properties of hydrophobic activators [4]. These findings taken together with the above suggest that the enhancement of hydrophilic activator catalytic potential in protein cocktail may not be entirely a surface-mediated effect, as suggested by Zhuo *et al.* [4].

FXIIa titration of rFXIdPPP (Fig. 2B) is highly scattered compared to FXIIa of normal plasma and is distinctly non-linear on a logarithmic FXIIa concentration scale (inset, compare to Fig. 2A inset). Comparison of normal plasma FXIIa titration to rFXIdPPP titration strongly suggests that FXIdPPP is not a reliable test vehicle, possibly due to plasma processing steps involved in commercial production of FXIdPPP.

4.2.3 Summary Conclusions on FXIIa Titration of Plasma—The trend in CT observed in plasma SAT and FXIIa titration is the result of a convolution of procoagulant stimulus intensity and procoagulant-stimulus-processing limitations imposed by the intrinsic pathway of coagulation. Relatively low procoagulant stimulus (low activator surface area or dilute FXIIa exogenous bolus) is promptly processed, leading to proportional linear-like reduction in CT. Delays in processing relatively higher procoagulant stimulus through the intrinsic pathway to FIIa production lead to asymptotic CT reduction to a lower-CT asymptote for both of these very different methods of activating plasma coagulation. Thus, the linear-like initial rate-of-change in CT with increasing surface area or exogenous FXIIa concentration (from plasma SAT or FXIIa titration, respectively) most probably represents unlimited processing of increasing procoagulant stimulus. Insofar as this linear range is a very small portion of each of these methods of activating plasma coagulation, it can be concluded that asymptotic titration plasma SAT curves are dominated by processing of procoagulant stimulus rather than generation of that procoagulant stimulus.

4.3 FXII Autoactivation in Buffer

Autoactivation of FXII in buffer solution is the simplest example of surface-mediated conversion of the zymogen FXII into active enzymes represented by the descriptive

chemical formula ($FXII \xrightarrow[\text{surface}]{\text{activator}} FXII_{act}$). We have shown that autoactivation in buffer occurs by contact with activator surface chemistries incrementally sampling the observable

water wetting range, giving rise to an ensemble of activated fragments represented by the symbol FXII_{act} that varies with activator surface chemistry/energy [6, 7]. This work focuses on the yield of procoagulant enzymes produced by contact with hydrophilic glass particle activators measured by apparent FXIIa yield, as already mentioned above.

The relationship between activation intensity and apparent FXIIa yield is complex. We have observed that the total yield of enzymatically-active proteins produced by FXII autoactivation in buffer solution in the continuous presence of hydrophilic particulate activators is a low percentage of the total FXII available in solution, even when activator surface area is relatively high [7]. Discontinuous autoactivation in the continuous presence of activator in buffer suspension strongly suggests one of three possibilities: (i) particulate activator does not remain catalytically active throughout the autoactivation experiment; (ii) autoactivation in buffer is not a facile reaction, possibly because (iii) autoactivation self-terminates through unknown biochemistry [7]. The following subsections examine each of these possibilities.

4.3.1 Activator Particle Poisoning—We have shown previously that activator surfaces are not poisoned by activation of plasma coagulation and remain active in multiple activations [10, 29]. Fig. 3A extends these observations to FXII autoactivation in buffer by showing that the amount of FXIIa produced by first-and-second activations with the same hydrophilic particles were not statistically different (mean $\pm$ standard deviation band superimposed over data). Furthermore, there was no clear dependence in apparent FXIIa yield with either reaction time or particulate activator surface area, leading to an apparent FXII conversion to FXIIa $< 5\%$.

Fig. 3B expands this latter observation using a specially-designed assay that permitted a constant FXII solution concentration to be exposed to serially-increasing clean-glass-particle activator surface area. Apparent FXIIa yield had no discernible functional relationship with increasing glass-particle surface area and apparent FXII conversion was less than 10%. This overall yield was approximately twice that obtained using the simpler protocol of Fig. 3A. The exact reason for this difference in FXIIa yield is not clear from data at hand, but it is observed that autoactivation yield can vary approximately 2x within and among similar experiments, generally falling within the 2-10% range (see further below), and that quantitatively different autoactivation results are obtained with different preparation lots of FXII. All taken together, we conclude that hydrophilic particulate activators remain catalytically active after participating in an initial FXII autoactivation in buffer and poisoning can be ruled out as a cause of low conversion of FXII by autoactivation in the continuous presence of activator surface.

4.3.2 Effect of Mixing and Temperature on FXII Autoactivation—Comparison of four sequentially-performed static autoactivation experiments compiled in Fig. 4A leads to the conclusion that autoactivation in buffer at ambient temperature is a stochastic process (*i.e.* a process with an element of random chance, see further Section 3.3), with apparent FXII conversion falling in a band ranging from a low of 2% to a high near 8%, with no discernible dependence on activator surface area. In general we observed lot-to-lot variability in FXII preparations and stochastic autoactivation within any particular FXII lot. Neither continuous mixing on a shaking-platform mixer (not shown) nor aggressive mixing obtained on a rotating hematology increased autoactivation yield (Fig. 4B). Fig. 5 shows that there was no discernible dependence of static FXII autoactivation on solution temperature ranging 20° to 50° C.

4.3.3 Self-termination of FXII Autoactivation and Autohydrolysis—Unknown self-termination biochemistry has been repeatedly invoked to explain unusual autoactivation

kinetics [4] and resistance of FXII to prolonged hydrolysis by FXIIa or kallikrein (see ref. [7] for a brief review). We have recently shown that FXII autoactivation in buffer by a particular category of so-called “Type 0” silanized-glass activator particles generated proteins that were capable of suppressing autoactivation by “Type II” hydrophilic-glass particulate activators studied herein [7]. This latter observation raises the unproven possibility that suppression proteins are initially co-produced with procoagulant proteins by Type II activation of FXII in buffer and these suppression proteins “turn off” subsequent autoactivation; again by unknown and possibly surface-mediated chemistry [4]. A simpler alternative explanation, consistent with Section 4.3.1 above is that the stochastic nature of autoactivation leads to very slow kinetics after an initial autoactivation burst in a way that has the appearance of a self-limiting reaction but is not, in fact, self-limiting.

We have also observed that chromogenic and plasma-coagulation assays for FXIIa in buffer solution give very different results and attributed this difference to a facile autohydrolysis

reaction $FXII + FXII_a \xrightarrow[\text{surface}]{\text{activator}} 2FXII_a$ that occurs in buffer but not in plasma (where ref. [37] interpreted autoactivation and autohydrolysis as involving only FXII and FXIIa). However, Figs. 3–5 and previous work [7] show that there is no continuous rise in apparent FXIIa over time of continuous contact with activator particles, suggesting that autohydrolysis of the kind described in the above chemical equation and [37] is not, in fact, an important reaction. The discovery that autoactivation in buffer produces an ensemble of activated proteins with either-or-both amidolytic (cleaves s-2302 chromogen) and procoagulant properties suggests that autohydrolysis studied in [37] is possibly due to a reaction more like

$FXII + FXII_{act} \xrightarrow[\text{surface}]{\text{activator}} \text{amidolytic proteins}$, rather than procoagulant proteins such as FXIIa, which accounts for the dissimilarity between chromogenic and plasma coagulation assays for FXIIa noted in [37]. Until-and-unless a complete inventory of activated proteins arising from FXII autoactivation in buffer becomes available, more speculation is probably unwarranted. But it seems reasonable to suggest that production of a mixture of protein products represented by $FXII_{act}$ contributes to the observed stochastic nature of FXII autoactivation in buffer.

4.3.4 Summary Conclusions on Autoactivation of FXII in Buffer—FXII

autoactivation in buffer solution by hydrophilic glass-particle activators converts less than 10% of the total FXII available into procoagulant enzyme(s) as measured by apparent FXIIa, even under conditions of extreme mixing. Previous work shows that some of the FXII available in solution is converted to proteins with amidolytic but not procoagulant activity, and there is the possibility that some solution FXII is converted to proteins exhibiting autoactivation-suppression properties [7]. Thus, the absolute per-cent FXII conversion remains unknown, but the summed apparent $FXII_{act}$ yield of both amidolytic and procoagulant enzymes is under 10% of the FXII available in solution [7].

The apparent discontinuous activation of FXII in buffer by the continuous presence of hydrophilic activator particles is not due to activator-surface poisoning and there is no clear dependence of apparent FXII yield on reaction time, particle surface area, solution mixing, or solution temperature. FXII autoactivation in buffer solution leads to a “stochastic” burst of procoagulant enzyme(s) and appears to terminate in the continuous presence of activator particles; either because the biochemistry is not facile, there is an unknown autoactivation-suppression mechanism at work, or the reaction is random in nature controlled by random “collisions” between reactants and activator particles leading to imperceptibly slow autoactivation kinetics following the initial burst.

Although FXII autoactivation in buffer is definitively a surface-mediated reaction [7], it does not exhibit measurable dependence on activator surface area over the $(2-100) \times 10^{-4} \text{ m}^2/\text{mL}$ range explored in this work. The stochastic and self-limiting nature of the reaction together with no temperature dependence strongly suggests that autoactivation involves little-or-no reproducible biochemistry with observable Arrhenius-like kinetics over the experimental timeframes explored ($1 \leq t \leq 30 \text{ min}$). We are thus led to speculate that FXII autoactivation in buffer by hydrophilic glass-particle activators is mechanochemical [38, 39] rather than purely biochemical in nature, arising from random “collisions” of one-or-more of the reactive constituents (FXII, FXIIa, and possibly other proteins arising from FXII autoactivation [7]) with activator particle surfaces (the mechanical aspect of mechanochemical); where the term surface here subsumes both the physical activator surface as well as hydration layers (interphase) that might separate the hydrophilic physical surface from bulk solution (see ref. [9] for a review relevant to protein-surface interactions). These collisions do not constitute protein adsorption because FXII does not formally adsorb to hydrophilic surfaces as it does to hydrophobic surfaces [3, 9], but instead represent transient interactions that somehow cause scission(s) at a number of possible locations along the polypeptide chain (the chemical aspect of mechanochemical, see ref. [7] for a brief review of known FXII cleavage sites). The random/probabilistic nature of these collisions and scissions accounts for both low overall autoactivation yield and insensitivity to both surface area and solution mixing; either or both of the latter two experimental variables failing to significantly increase the low probability of collisions that lead to autoactivation.

It is to be emphasized that the above speculation is arrived at by process of elimination; if autoactivation is not biochemical in nature, then this implies autoactivation is a physical reaction. Furthermore, this speculation is inferred from experimental outcomes that neither directly test for mechanochemical reaction characteristics nor explain how reaction products of autoactivation are created by polypeptide chain scissions. Finally, it is worthwhile noting that FXII autoactivation is occasionally attributed to autohydrolysis precipitated by trace FXIIa contaminant in FXII preparations that is “amplified” in the presence of a surface, as

represented by the chemical formula $FXII + FXII_a \xrightarrow[\text{surface}]{\text{activator}} 2FXII_a$. We discount this possibility on the basis that FXII preparations used in our work do not precipitate coagulation of plasma (see, for example, ref. [5]) and there is no continuous rise in apparent FXIIa over time of continuous contact with activator particles, as already mentioned in Section 4.3.3.

4.4 FXII Autoactivation in Protein Solutions

We have shown that blood proteins other than those known to be active participants of the plasma coagulation cascade moderate both FXII autoactivation [5] and plasma coagulation itself [6]. In the former case, synthetic protein cocktails in buffer enhance the observed yield of procoagulant enzymes produced by autoactivation of FXII by hydrophilic activator particles and reduce yield produced by hydrophobic activator particles. In the later case, we have shown that the catalytic potential of hydrophobic activators to induce plasma coagulation is due to a so-called “adsorption-dilution effect” wherein adsorption of proteins from the plasma milieu blocks contact of FXII with surfaces, effectively rendering hydrophobic activators inert. Quite apparently, surface interactions with proteins of the plasma proteome cannot be ignored in the construction of an overall mechanism of contact activation of plasma coagulation [3, 6].

FXII autoactivation in protein cocktails of arbitrary composition that exclude proteins of the coagulation cascade represent an incremental increase in biochemical complexity over FXII autoactivation in buffer. We advance this biochemical complexity significantly by using heat-denatured serum shown to exhibit no measurable procoagulant activity, fully

recognizing the protein composition of the serum milieu is itself undefined. In principle, FXII autoactivation in FXIdPPP with a dysfunctional coagulation cascade most closely emulates plasma. Autoactivation in FXIdPPP can be measured using the same basic experimental plan applied in measuring FXII autoactivation in buffer. The basic idea is that procoagulant enzymes produced by autoactivation of endogenous FXII collect in FXIdPPP since the intrinsic cascade terminates at the $FXIIa + FXI \rightarrow FXIa$ step. In practice, FXIdPPP proved not to be a reliable test system (Section 4.4.3), possibly due to unknown artifacts introduced by the plasma processing steps that remove FXI. Nevertheless, FXII autoactivation in FXIdPPP provides some insights that are worth comparing to FXII autoactivation in buffer, protein cocktail, and serum.

4.4.1 FXII Autoactivation in Protein Cocktail—We found that FXII autoactivation with increasing activator surface area in serially-increasing concentration of protein cocktail (0%, 0.25%, 0.5%, and 100%, Section 3.4) was similar to autoactivation in buffer in that there was no discernible surface area dependence and a stochastic burst of procoagulant enzyme was produced (not shown).

4.4.2 FXII Autoactivation in Heat-denatured Serum—We found that FXII autoactivation with increasing activator surface area in heat-denatured serum was similar to autoactivation in buffer in that there was no discernible surface area dependence and a stochastic burst of procoagulant enzyme was produced (not shown). However, it is worth noting that the blank (no surface) level of FXIIa detected in serum was approximately 5x higher than observed in buffer for the same lot of FXII (Lot C), even though serum itself exhibited no procoagulant activity in plasma (Section 2.2.2). We attribute this to the added-protein effect on FXIIa titration curves mentioned in Section 3.4 that enhances calculated apparent FXIIa yields compared to buffer. We cannot discount the possibility that exogenous FXII added to serum is spontaneously converted to procoagulant enzyme(s) upon addition through unknown biochemistry. Whatever the source of increased autoactivation yields in serum, there was no discernible surface-area dependence observed in FXII autoactivation in heat-denatured serum.

4.4.3 Autoactivation of Endogenous FXII in FXIdPPP—Fig. 6 suggests that FXII autoactivation in FXIdPPP is stochastic and is not a function of activator surface area, as noted in buffer and heat-denatured serum. Although results of FXII autoactivation in FXIdPPP must be skeptically viewed and interpreted with caution in light of FXIIa titration of rFXIdPPP (Fig. 2B, Section 4.2.2), it can be at least concluded that the stochastic aspect of FXII autoactivation in FXIdPPP was *not inconsistent* with autoactivation in buffer.

4.4.4 Summary Conclusions on FXII Autoactivation in Protein Solutions—FXII autoactivation exhibits no discernible surface area dependence in protein cocktail, heat-denatured serum, or FXIdPPP. In each case, there appears to be a stochastic burst of procoagulant enzyme(s) and the autoactivation reaction terminates in the continuous presence of activator particles. Addition of protein increases the complexity of the autoactivation experiment and interpretation thereof by participating in some way reactants. FXIIa titration curves in protein solutions are different than FXIIa titration in buffer and FXIdPPP appears to be an unreliable surrogate for plasma with a dysfunctional coagulation system. Nevertheless, in spite of these experimental obstacles, we find no evidence that FXII autoactivation in protein solution exhibits surface area dependence.

5. Comparison of Autoactivation in Buffer and Plasma

The similarity of plasma SAT (Fig. 1) and FXIIa titration (Fig. 2A) leads to the hypothesis that FXIIa titration can be used to estimate the endogenous FXIIa produced by

autoactivation of endogenous FXII in plasma SAT. The computational strategy outlined in Appendix A applies data of an exogenous FXIIa titration (Fig. 2A) as a calibration curve that relates CT to exogenous FXIIa concentration in plasma. Using this relationship, CT observed in a plasma-SAT CT at various activator surface areas were converted to apparent endogenous FXIIa concentrations produced by autoactivation of endogenous FXII. In view of the discussion of the preceding Sections 4.1-4.2, this approach must be viewed as a lower-bound estimate of endogenous FXIIa that is more accurate at higher CT (low surface area) than at asymptotic CT (high surface area) where CT is presumed to be influenced by procoagulant-stimulus-processing delays. Furthermore, the technical veracity of the computational method relies on the proposition that reciprocal-loop amplification of FXIIa in plasma is a constant multiplier of FXIIa produced by either autoactivation of endogenous FXII in a plasma SAT or exogenous FXIIa added to plasma in FXIIa titration, as further discussed in Appendix A.

5.1 Autoactivation Yield in Plasma

The right-hand ordinate of Fig. 1 plots results of converting plasma-SAT CT to apparent endogenous (open and closed triangles correspond to open and closed squares on the left-hand CT ordinate). These estimates of endogenous FXIIa indicate that autoactivation rises sharply with increasing activator surface area (left-hand ordinate). At higher activator surface area, increase in endogenous FXIIa concentration follows a linear-like rise with a slope of approximately 10 PEU/m². The initial autoactivation burst estimated from the first few surface-area data points up-to-and-including the bend of the SAT curve is an order-of-magnitude higher at about 100 PEU/m². In this latter regard, autoactivation of endogenous FXII in plasma is like FXII autoactivation in buffer (Figs. 3–5) because the rise in FXIIa with activator surface area from null autoactivation at zero surface area to 10×10^{-2} PEU/mL at less than 10×10^{-4} m²/mL corresponds to a similar 100 PEU/m² procoagulant enzyme burst. Given the procoagulant-stimulus-processing limitations of the coagulation cascade, it is not possible to interpret surface-area dependence of endogenous FXIIa estimates lying between these extremes as surface-area dependent autoactivation without further information regarding rate-limiting steps.

5.2 Summary Conclusions on Autoactivation Yield in Buffer and Plasma

We conclude that the yield of procoagulant enzymes obtained by FXII autoactivation in buffer can reasonably account for the extent of endogenous FXII autoactivation anticipated in plasma as estimated from FXIIa titration above. The initial FXIIa burst in plasma is similar to that in buffer and the total conversion of available FXII in plasma is similar to that measured in buffer. Although it cannot be definitively concluded that autoactivation yield in buffer is the same as in plasma, estimates suggest that it is feasible that the extent of autoactivation observed in buffer is similar to that occurring in plasma.

6.0 Conclusions

This work sought to answer two fundamental questions that ask (i) if FXII autoactivation in buffer or protein solutions exhibit dependence on activator surface area that can account for the activator-surface-area dependence observed in surface-area titration of plasma coagulation (plasma SAT) and (ii) if the yield of procoagulant enzymes produced by autoactivation of FXII in buffer and protein solutions by hydrophilic glass particles can account for the decrease in plasma coagulation time observed in plasma SAT. The answer to the first question is no. Lack of surface-area dependence of FXII autoactivation in buffer and protein solutions strongly suggests that surface-area-dependent activation of plasma coagulation arises in steps of the intrinsic pathway other than FXII autoactivation. This finding further suggests that activator surfaces play a mediating role in plasma coagulation

more complex than represented by the simple descriptive chemical formula

$FXII \xrightarrow[\text{surface}]{\text{activator}} FXII_{act}$. The answer to the second question seems to be yes, but the apparent autoactivation yield in buffer and plasma is low, creating considerable uncertainty about the diagnostic value of estimates employed herein. We conclude that FXII autoactivation by hydrophilic glass-particle activators is mechanochemical rather than purely biochemical in nature, arising from random “collisions” of one-or-more of the reactive constituents with activator particle surfaces. The random/probabilistic nature of these collisions and scissions accounts for both low overall autoactivation yield and insensitivity to activator surface area, solution mixing, and solution temperature.

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Appendix A: Autoactivation Yield in Plasma Estimated from FXIIa Titration

FXIIa titrations of plasma used in this work were linear-like on a $\log [FXIIa]_{ex}$ scale over the coagulation time (CT) range of interest (see Fig. 2A), where $[FXIIa]_{ex}$ the exogenous FXIIa titrant concentration expressed in PEU/mL is prepared by serial dilutions of purified FXIIa solutions of known concentration. Linear regression through the linear-like range yields:

$$CT = m \log [FXIIa]_{ex} + b \quad (1)$$

The linear-regression parameters m and b correspond to the slope and intercept of the linear-like range of the exogenous FXIIa titration curve, respectively. Eq. (1) is actually an approximation that implicitly assumes the contribution of reciprocal-loop amplification of the exogenous bolus is small relative to $[FXIIa]_{ex}$. An explicit accounting of reciprocal-loop amplification requires Eq. (1) to be re-written as:

$$CT = m \log [FXIIa]_{tot} + b \quad (2)$$

where $[FXIIa]_{tot} = ([FXIIa]_{ex} + \alpha_{RL} [FXIIa]_{ex})$ and α_{RL} is the reciprocal-loop amplification factor. The slope and intercept are the same as in Eq. (1) because the observed CT actually depends on the sum of exogenous and endogenous FXIIa contributions. Based on the work of Chatterjee *et al* [40], α_{RL} is assumed to be a constant factor over the linear-like range of a FXIIa titration.

Eq. (2) can be used to determine an unknown amount of FXIIa in a test solution added to plasma if two additional assumptions are made. The first assumption is that an unknown FXIIa concentration $[FXIIa]_{uk}$ in a test solution is amplified by the same reciprocal-loop amplification factor α_{RL} as in FXIIa titration of plasma. Such test solutions might be obtained by autoactivation of exogenous FXII in PBS solution or autoactivation of endogenous FXII in plasma by surface-area titration (SAT). Again taking reciprocal-loop

amplification explicitly into account, $[FXIIa]_{tot} = ([FXIIa]_{uk} + \alpha_{RL} [FXIIa]_{uk})$, where $[FXIIa]_{tot}$ is the total unknown concentration of FXIIa causing plasma to coagulate at a particular measured CT.

The second assumption is that a particular plasma CT occurs at a unique total FXIIa concentration independent of how that FXIIa was generated in plasma. In other words, a particular CT caused by activation of endogenous FXII in a plasma SAT is due to exactly the same total FXIIa concentration as that causing the same CT in an FXIIa titration or by addition of a solution containing an unknown FXIIa concentration to plasma. With these underlying assumptions in mind, the mass balance of Eq. (3) holds for a particular CT caused by an unknown FXIIa concentration and exogenous FXIIa titration:

Thus, unknown FXIIa concentrations in plasma can be determined by equating equivalent CT obtained by FXIIa titration of that plasma.

Figure 1.

Surface-area titration of human-blood plasma. Coagulation time (CT, open and closed squares representing two different trials) decreases asymptotically with increasing clean-glass particle activator surface area (left-hand ordinate) from 41 min (see blank annotation, no activator particles) to about 10 min. The smooth curve through the data corresponds to fit of the data to a mathematical model of plasma coagulation (see Section 3.1). Endogenous FXIIa (right-hand ordinate, open and closed triangles) estimated from FXIIa titration corresponds to the two SAT trials (open and closed squares, see Section 5.1). Error bars represent uncertainty in apparent FXIIa concentration calculated by propagation of error from calibration curves.

Figure 2.

FXIIa titration of human plasma. Panel A shows a smooth asymptotic decrease in normal plasma coagulation time (CT, open and closed triangles representing two different trials) as a function of exogenous FXIIa from 41 min (see blank annotation, no FXIIa added) to about 10 min, approximating a linear relationship in a logarithmic FXIIa concentration scaling (inset). Line through the data of the main panel is a guide to the eye whereas line through inset data is the result of linear least squares fitting. Notice that FXIIa titration with linear FXIIa concentration scaling resembles the SAT of Fig. 1 in that both titrations asymptotically reach a 10 min lower-CT plateau, suggesting that CT is controlled by the same intrinsic-pathway procoagulant stimulus processing biochemistry. Panel B compiles two trials of FXIIa titration of FXI reconstituted FXI depleted platelet poor plasma (rFXIdPPP) on the same scaling as Panel A. Although the high blank value (> 50 min, see blank annotation, no FXIIa added) suggested that rFXIdPPP did not contain procoagulant enzymes, CT at lowest exogenous FXIIa concentrations was much lower than with normal plasma (Panel A) and was relatively insensitive to increasing FXIIa concentration (see also inset). Results suggest that FXIdPPP is an unreliable test vehicle.

Figure 3.

Surface-area dependence of FXII autoactivation in buffer solution. Panel A compares FXIIa yield (ordinates) of first (closed circles) and second (open circles) FXII activation in buffer by contact with increasing surface area of clean-glass particles (abscissa). Right-hand ordinate converts measured apparent FXIIa activity in plasma equivalent units (PEU/mL, left-hand ordinate) to percent FXII conversion by autoactivation. Error bars represent uncertainty in apparent FXIIa concentration calculated by propagation of error from FXIIa-titration calibration curves. Horizontal line and grey band represent mean and standard deviation of the entire data set across the surface-area range tested. Notice that there was no discernible dependence on activator surface area and that, on average, less than 3% of FXII was converted by static contact with glass particles. Panel B used a specially-designed protocol to activate FXII through successively-increasing activator surface area (same presentation style as in Panel A). Notice that there was no discernible dependence on activator surface area and that, on average, less than 7% of FXII was converted by static contact with glass particles, even when surface area is increased three fold compared to Panel A. Average FXII conversion was approximately 2x that observed in Panel A most probably because two different lots of FXII were used.

Figure 4.

Surface-area dependence of FXII autoactivation in buffer solution. Panel A compiles results of four replicate experiments (different circle symbols) showing that autoactivation of FXII in buffer solution is stochastic (axes same as in Fig. 3). Error bars represent uncertainty in apparent FXIIa concentration calculated by propagation of error from FXIIa-titration calibration curves. Horizontal line and grey band represent mean and standard deviation of the entire data set across the surface-area range tested. Notice that there was no discernible dependence on activator surface area and that, on average, less than 5% of FXII was converted by static contact with glass particles (compare to Fig. 3). Panel B used three different speeds of a rotating hematology mixer to test effect of particle mixing with FXII solution (open circles = 0 revolutions/min; closed circles = 6 revolutions/min; half-filled circles = 11 revolutions/min). Error bars represent uncertainty in apparent FXIIa concentration calculated by propagation of error from FXIIa-titration calibration curves. Horizontal line and grey band represent mean and standard deviation of the entire data set across the surface-area range tested. Notice that there was no discernible dependence on either activator surface area or stirring speed and that, on average, less than 5% of FXII was converted by mixed contact with glass particles. Compare to Fig. 3.

Figure 5.

Temperature dependence of static FXII autoactivation in buffer solution. Autoactivation with $25 \times 10^{-4} \text{ m}^2/\text{mL}$ clean-glass particles at $20^\circ - 50^\circ \text{ C}$ (closed circles = 20° , half-filled circles = 37° , hour-glass filled circles = 45° , open circles = 50°). Error bars represent uncertainty in apparent FXIIa concentration calculated by propagation of error from FXIIa-titration calibration curves. Horizontal line and grey band represent mean and standard deviation of the entire data set across the surface-area range tested. Notice that there was no discernible dependence on temperature and that, on average, less than 5% of FXII was converted at any temperature. Compare to Figs. 3–4.

Figure 6.

Surface-area titration (SAT) of FXI depleted platelet poor plasma (FXIPPP) with a dysfunctional coagulation cascade compiling results of three replicate experiments (different hexagon symbols) suggesting that autoactivation of FXII in FXIdPPP solution is stochastic and not a function of activator surface area. Error bars represent uncertainty in apparent FXIIa concentration calculated by propagation of error from FXIIa-titration calibration curves. Horizontal dashed line represents mean and standard deviation of three blank measurements with no added surface area.