SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
(PhD)

THE ASSOCIATION OF FACTOR XIII SUBUNITS AND THE INTERACTION OF ACTIVATED FACTOR XIII WITH ITS PEPTIDE SUBSTRATE

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	Raymund Machovich MD, PhD, DSc
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The Examination takes place at the library of the Department of Pediatrics, Faculty of Medicine, University of Debrecen at 11:00 am, 11th September, 2014.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 13:00, 11th September, 2014.

INTRODUCTION AND REVIEW OF LITERATURE

Blood coagulation factor XIII (FXIII) is a pro-transglutaminase that is converted into an active form (FXIIIa; protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) by the concerted action of thrombin and Ca^{2+} in the final phase of clotting cascade. Transglutaminases cross-link peptide chains by ε(γ-glutamyl)lysyl bonds, and the main task of FXIIIa is to cross-link fibrin α-, and γ-chains and covalently attach α_2-plasmin inhibitor to fibrin. This mechanism is essential for maintaining hemostasis; it protects newly formed fibrin from the shear stress of circulating blood and from the degradation by the fibrinolytic machinery. Plasma FXIII (pFXIII) is a heterotetramer ($FXIIIA_2B_2$) consisting of two potentially active A subunits (FXIII-A_2) and two carrier/inhibitory B subunits (FXIII-B_2). FXIII-A consists of four structural domains (β-sandwich domain, catalytic core domain and two β-barrel domains) and an N-terminal activation peptide. FXIII-B is a mosaic protein consisting of ten so-called sushi domains, each hold together by a pair of disulfide bonds. It is a glycoprotein containing approximately 8.5% carbohydrate. FXIII-A_2, also exists in the cytoplasm of certain cells, particularly of platelets and monocytes/macrophages.

FXIII-A is synthesized by cells of bone marrow origin, while FXIII-B is produced by hepatocytes. The two subunits form complex in the plasma, FXIII-B is in excess, approximately 50% of it exists in free form, very likely as dimer. Complex formation between the two subunits is essential for maintaining normal hemostasis. The main role of FXIII-B is to prolong the lifespan of FXIII-A in the circulation. In FXIII-B deficient patients FXIII-A_2 concentration in the plasma is significantly decreased and due to the low FXIII-A_2 level, these patients present moderate bleeding diathesis. This role of FXIII-B may be related to the prevention of a slow progressive activation of FXIII-A_2, which may occur in plasmatic conditions in the absence of FXIII-B. Considering the importance of complex formation between the two FXIII subunits, it is surprising that the biochemistry of their interactions is only superficially explored. The epitopes involved in the interaction of the two subunits have not been specified. Although it is generally accepted that FXIII-A in the circulation is “fully” complexed, it has not been proven experimentally. So far two values, 4×10^{-7} M and 8×10^{-8} M, have been reported for the apparent equilibrium dissociation constant (K_d). However, these values were determined in ELISA-type binding assays using relatively high ($>10^{-8}$ M) FXIII-B subunit concentration as receptor. If total receptor

concentrations [Rt] >> Kd, the binding constant could be significantly overestimated. Indeed, using the above two binding constants and the mean plasma concentrations of FXIII-A and FXIII-B subunits 87% or 55% of plasma FXIII-A_2 should be in free form, which is evidently not the case. Most recently the administration of recombinant FXIII-A_2 (rFXIII-A_2) has been introduced as substitution therapy in FXIII-A deficiency. The fact that the long-term survival of rFXIII-A_2 in the circulation depends on its complex formation with the available free FXIII-B_2 underlines the importance of exploring the mechanism of FXIII-A_2B_2 complex formation.

There is a single major Ca^{2+} binding site per FXIII-A monomer that binds one Ca atom. The results of X-ray structural studies of FXIII-A_2 co-crystallized with Ca^{2+}, Sr^{2+} and Yb^{3+} ions also indicate a single main cation binding site per subunit. A K_d of 0.1 mM was determined by equilibrium dialysis and intrinsic fluorescence studies, while a K_d of 0.51 mM was obtained by ^{43}Ca NMR titration. These reports also suggest the existence of additional low affinity Ca^{2+} binding sites. X-ray crystallography and Ca^{2+} binding to mutant FXIII-A_2 identified that the carboxylate group of Asp438, Glu485 and Glu490 side chains, as well as the backbone carbonyl O atom of the Ala457 residue forms the pocket for Ca^{2+} binding. The main chain oxygen from Ala457 is the main amino acid residue that binds Ca^{2+} directly. The other residues are not close enough for direct interaction; they might participate in Ca^{2+} binding through water molecules.

The binding of Ca ions to the high affinity Ca^{2+} binding sites on the A subunits is sufficient to dissociate the subunits and to activate the released A' dimer. In vitro cFXIII could be activated by thrombin and Ca^{2+} the same way as pFXIII, excluding the dissociation of FXIII-B. At extremely high Ca^{2+} concentrations ($\geq$ 100 mM) the A_2B_2 complex dissociates without prior proteolytic cleavage and the non-truncated FXIII-A_2 becomes transformed into an active TG (FXIII-A_2°). The occupancy of low-affinity Ca^{2+} binding sites might play a role in this process. The non-proteolytic activation of cFXIII is fundamentally different from that of pFXIII. In this case a low Ca^{2+} concentration is sufficient to induce a slow progressive activation of cFXIII in physiological conditions; the rate of activation is greatly enhanced by increasing the ionic strength. Complex formation with FXIII-B fully abrogates this activation process. The non-proteolytic pathway seems to be the physiological activation mechanism of cFXIII in platelets and monocytes.

Activation and function of FXIII

Thrombin cleaves off the activation peptide from the N-terminal end of FXIII-A, and then, in the presence of Ca^{2+}, FXIII-B subunits dissociate and the truncated FXIII-A dimer assumes an active conformation (FXIII-A_2*), in which the active site Cys314 becomes available to react with appropriate substrates. In the presence of Ca^{2+}, an innate crosslinking activity of zymogen FXIII against physiologic substrates, such as fibrinogen, fibrin, and α_2 plasmin inhibitor (α_2PI), has also been demonstrated.

FXIIIa catalyzes a modified double displacement acyl transfer reaction. In the first step, the acyl donor, a peptide-bound glutamine, forms a binary complex with the enzyme via a thioester bond involving the active site cysteine, and ammonia is released. Then, in the presence of an appropriate primary amine substrate, the acyl group is transferred from the acyl enzyme intermediate to the acyl-acceptor amine, and the amine becomes covalently linked to the γ-glutamyl residue through an 'isopeptide' bond. In a physiologic setting, the substrate primary amine is the ε-amino group of a peptide-bound lysine residue, and the end-result of the reaction catalyzed by FXIIIa is the crosslinking of peptide chains through an ε(γ-glutamyl)lysyl residue.

Substrates for FXIIIa

Fibrin α-chains and γ-chains, α_2PI and fibronectin are the major physiologic substrates of FXIIIa . Crosslinking of fibrin chains and covalent attachment of α_2PI to fibrin mechanically strengthen the fibrin clot and protect it from prompt proteolytic breakdown by the fibrinolytic system. α_2PI is secreted by hepatocytes as a protein of 464 amino acids, starting with a methionine residue (M1-α_2PI). A plasma protease removes a dodecapeptide from the N-terminal end of some of the molecules, and about 70% of α_2PI circulates as a 12 amino acid shorter isoform with an N-terminal asparagine (N1-α_2PI). Only the truncated isoform is an efficient acyl donor substrate in the crosslinking reaction catalyzed by FXIIIa. In addition, several other protein substrates that donate glutamine residues to the FXIIIa-catalyzed transglutaminase reaction have been reported. In a few cases the acyl-donor glutamine and its surroundings were identified in the primary structure of the protein. As there is no obvious consensus sequence around the substrate glutamine, it seems important to

shed light on the structural requirements of the binding of FXIIIa to its individual substrate peptides. Studies with synthetic oligopeptides corresponding to the sequence around the reactive glutamine in β-casein and N1-α_2PI have provided information on the mechanism of FXIIIa binding to these substrates.

The methods of determining FXIII activity

There are two reasons why FXIII is determined in clinical practice and clinical research: 1) the diagnosis of FXIII deficiencies and monitoring FXIII substitution of deficient patients; 2) elevated FXIII level might represent a risk factor of thrombotic diseases in certain conditions.

The methods suitable for determining FXIII activity are based on two principles: 1) measurement of labeled amines incorporated by FXIIIa into a glutamine residue of a substrate protein, 2) monitoring ammonia released from a peptide bound glutamine residue by FXIIIa using NAD(P)H dependent glutamate dehydrogenase indicator reaction. The incorporation assays are sensitive, but cumbersome and time-consuming, they are difficult to standardize and cannot be automated. The ammonia release assays are less sensitive, but quick, well standardized, and can be automated; this type of assay is recommended for the screening of FXIII deficiency. The traditional clot solubility assay should not be used for this purpose.

Ammonia release assays

The first FXIII assay utilizing the measurement of ammonia released by FXIIIa from its Gln donor protein substrate was published in 1969 (51) . In this case ammonia formed in recalcified clotted plasma was estimated. Further early methods determined ammonia formed in plasma or plasma supplemented with fibrinogen by ammonia selective electrode or a modified Berthelot reaction. These end-point methods were impractical and required a high volume of plasma, they did not gain popularity. A new principle was introduced by using glutamate dehydrogenase (GlDH) indicator reaction for monitoring the production of ammonia. This principle made it possible to design the first true kinetic FXIII assay. In the assay, fibrinogen was removed by bentonite treatment. FXIII was activated by thrombin and Ca^{2+} during the lag phase of the reaction. FXIIIa cross-linked the amine substrate (ethylamine) to the single substrate Gln residue in acetylated dephosphorylated β -

casein and ammonia was released. The ammonia release was continuously monitored by glutamate dehydrogenase-mediated indicator reaction, in which NADPH became converted into NADP and the decrease in absorbance at 340 nm was measured. The above principle was used in the development of two kinetic ammonia release FXIII assays. In the new modified assays fibrin formation was prevented by peptides that inhibited fibrin polymerization, the protein substrate was replaced by Gln containing oligopeptides and glycine ethylesther was used as amine substrate. Both modified assays are now commercially available as Berichrom FXIII (Siemens, Marburg, Germany), REA-chrom FXIII (Reanal-ker, Budapest, Hungary) and TECHNOCHROM FXIII (Technoclone, Vienna, Austria) tests. These ammonia release assays are quick (only 10 min), one-step, true kinetic tests with good reproducibility and can be easily automated. Neither assay is influenced by the FXIII-A Val34Leu polymorphism. They are widely used as screening tests for FXIII deficiency. We compared the Berichrom and TECHNOCHROM assays (the Rea-chrom assay is identical with the TECHNOCHROM assay). The two kits use different Gln containing substrate oligopeptides; in the Berichrom assay the decapeptide possesses an amino acid sequence similar to that of β-casein around the substrate Gln residue, the sequence of the dodecapeptide used in the TECHNOCHROM (and Rea-chrom) kit is identical with the N-terminal sequence of N1-isoform of α_2PI. In the indicator reaction NADH and NADPH are used in the Berichrom and TECHNOCHROM assays, respectively.

THE AIM OF THE STUDY

In the present study several aspects of the interaction between the FXIII subunits and its glutamine containing substrates were investigated. The aim of the study was:

1. To determine the K_d for the interaction of FXIII subunits with surface plasmon resonance (SPR) technique and an ELISA-type binding assay.

2. To calculate and experimentally determine the concentration of free FXIII-A_2 in the plasma and other body fluids (cerebrospinal fluid and tears). To test if free FXIII-A present in the plasma was functional.

3. As from a physiologic point of view, the crosslinking of N1-α_2PI to fibrin by FXIIIa is of great importance, our further aim was to investigate the molecular mechanism of the interaction between FXIIIa and N1-α_2PI(1–12), a dodecapeptide with a sequence that corresponds to the N-terminal part of N1-α_2PI. Within this part of the study we determined the kinetic parameters for N1-α_2PI(1–12) and for its truncated or synthetic mutants by a spectrophotometric FXIIIa assay and investigated the interaction of N1-α_2PI(1–12) with FXIII-A_2* by proton nuclear magnetic resonance (NMR) and saturating transfer difference (STD) NMR.

MATERIALS AND METHODS

Materials and methods used in the study of interaction of FXIII subunits

Materials

Human thrombin, hirudin, 3,3',5,5'-tetramethylbenzidine (TMB) substrate, bovine serum albumin (BSA) fraction V, horse radish peroxidase (HRPO) type VI, biotinamido-hexanoic acid hydrazide (BACH), goat anti-mouse IgG (Fc specific), and trypsin (proteomics grade) were purchased from Sigma Aldrich (St. Louis, MO). Bis(sulfosuccinimidyl)suberate (BS3) was a product of Pierce (Rockford, IL). Batroxobin moojeni was purchased from Pentapharm (Basel, Switzerland). Sheep anti-human FXIII-A and rabbit anti-human FXIII-B polyclonal antibodies were obtained from Affinity Biologicals (Ancaster, Canada) and Calbiochem (La Jolla, CA), respectively. Vectastain ABC reagent was the product of Vector (Burlingame, CA). Horseradish peroxidase (HRPO) conjugated anti-rabbit IgG was purchased from Jackson Immunoresearch Laboratories (Westgrove, PA). Streptavidin coated micro-plates were from Thermo Fisher Scientific (Vantaa, Finland). Monoclonal anti-FXIII-A and anti-FXIII-B antibodies were produced in BALB/c mice by my colleague, Éva Katona. IgG was separated from ascites fluid by affinity chromatography on Protein G Sepharose column (GE Healthcare, Uppsala, Sweden). For the binding assay anti-FXIII-B IgG was biotinylated at the carbohydrate moiety using BACH reagent. CNBr-activated Sepharose 4B gel and CM5 sensor chips were the products of GE Healthcare.

FXIII preparations and FXIII antigen determinations

Highly purified human pFXIII and pFXIII-B_2 were prepared from pooled human plasma. cFXIII was prepared from human placenta, recombinant FXIII-A_2 (rFXIII-A_2) was a kind gift from Dr. Eva H.N. Olsen, (Novo Nordisk, Måløv, Denmark). Recombinant FXIII-B_2 (rFXIII-B_2) produced in insect cells was from Zedira (Darmstadt, Germany). FXIII-A_2B_2 and FXIII-A_2 antigen concentrations were determined as described earlier.

Surface plasmon resonance investigations

Surface plasmon resonance (SPR) is a phenomenon that allows real-time, label-free detection of biomolecular interactions. In Biacore instruments from GE Healthcare, target molecules, most frequently proteins, are immobilized on a prepared gold sensor surface and a sample containing a potential interacting partner in solution is injected over the covered surface of flow cells. During the course of the interaction, polarized light is directed toward the sensor surface and the angle of minimum intensity of reflected light is detected. This angle changes as molecules bind and dissociate and the interaction profile is thus recorded in real time. For most applications, a dextran matrix enables molecules to be immobilized on the sensor surface. As light does not penetrate the sample, interactions can be followed in colored, turbid, or opaque samples. No labels are required and the detection is instantaneous.

Surface plasmon resonance investigations were performed on Biacore X instrument (GE Healthcare). 100 μg/mL pFXIII-B_2 or rFXIII-B_2 was immobilized to flow cell 2 (FC2) of CM5 sensor chip according to the amine coupling protocol. BSA was used to cover the control flow cell 1 (FC1). Various concentrations of cFXIII-A_2 or rFXIII-A_2 in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 3 mM EDTA, 0,005 % v/v surfactant were used as analytes. The flow-rate through the cells was 10 μL/min. The solution used for regeneration contained 50 U/mL thrombin, 250 mM $CaCl_2$, 10 mM HEPES, 150 mM NaCl, and 0,005 % v/v surfactant (pH: 7.4). In this set-up, after cleaving off the activation peptide by thrombin, the bound FXIII-A_2 dissociates from FXIII-B_2 linked to the sensor chip. Association rate constant (ka), dissociation rate constant (kd) and equilibrium dissociation constant (Kd) were calculated by Biaevaluation software (GE Healthcare).

ELISA-type binding assay

Various concentrations of cFXIII-A_2 or rFXIII-A_2 were incubated with 0.25 nM FXIII-B_2 for one hour at 23 °C. In the mean time 100 μL biotinylated monoclonal anti-FXIII-B antibody (1 μg/mL) was added to the wells of a streptavidin-coated micro-plate. After 30 min incubation the non-bound biotinylated antibody was removed by washing. Then, 100 μL of the incubation mixture containing the formed complex was transferred to the wells and incubated for one hour. After extensive washing the amount of formed complex was determined by using HRPO-labeled anti-

FXIII-A antibody and TMB substrate. The concentration of formed complex was determined by comparison with a standard curve of FXIII-A_2B_2. The K_d was calculated by GraphPad (San Diego, CA) Prism Software, Version 5.0.

Calculation and measurement of free FXIII-A in body fluids

The formula which was used for the calculations was published in 2004 by Wilkinson KD et al. For plasma [total FXIII-B_2] and [total FXIII-A_2] were represented by the mean of total FXIII-B_2 and total FXIII-A_2 concentrations determined in the plasma of 35 healthy volunteers. Mean and median concentrations reported earlier were used for the calculation concerning cerebrospinal fluid (CSF) and tears, respectively. K_d used in the formula was the mean of K_d values determined by SPR and ELISA type binding assay (4.17×10^{-10} M).

The measurement of free FXIII-A_2 concentration in the plasma was performed on samples immuno-depleted of FXIII-A_2 present in complex. Citrated plasma samples from 11 healthy volunteers were depleted of FXIII-A_2B_2 and free FXIII-B by immuno-absorption using monoclonal anti-FXIII-B antibody covalently coupled to Sepharose 4B. The complete removal of complexed FXIII-A_2 was verified by the absence of FXIII-A_2B_2 in immuno-depleted plasma samples. In the case of CSF and tears the difference between the measured total molar concentrations of FXIII-A_2 and FXIII-A_2 in complex was considered as free FXIII-A_2 concentration.

Fibrin cross-linking by free FXIII-A_2 remaining in the plasma after the removal of FXIII-A_2B_2 complex

The free FXIII-A_2 that remained in the plasma after immuno-depletion was investigated for fibrin cross-linking activity. Plasma samples were clotted by 2 U/mL human thrombin and 10 mM $CaCl_2$ in the absence and presence of 2 mM iodoacetamide (a FXIIIa inhibitor) at 37 °C for 60 min. After extensive washing with saline, the recovered clots were solubilized in reducing SDS PAGE sample buffer and the dissolved clots were analyzed by SDS PAGE. In certain experiments thrombin was replaced by batroxobin (11 U/mL), a thrombin-like snake venom protease that clots fibrinogen by releasing fibrinopeptide A from the Aα-chain of fibrinogen, but doesn't activate FXIII. In this case thrombin formed in the plasma, as a result of the activation of coagulation cascade by Ca^{2+}, was inhibited by 10 U/mL hirudin.

Materials and methods used in the study of interaction of FXIIIa and its substrates

Proteins and reagents

Highly purified human plasma FXIII and bovine thrombin were prepared in our laboratory. Beef liver glutamate dehydrogenase and NADPH were purchased from Roche (Meylan, France). ADP, α-ketoglutarate, glycine-ethyl ester, dithiothreitol, HEPES and iodoacetamide were produced by Sigma (St. Louis, MO). *N,N'*-dicyclohexylcarbodiimide, 1-hydroxybenzotriazole hydrate, Fmoc-O-t-butyl-L-serine, Fmoc-O-t-butyl-L-threonine, Na-Fmoc-Ne-Boc-L-lysine, Fmoc-L-alanine and Wang resin were from Fluka, Fmoc-L-proline, Fmoc-L-valine, Fmoc-L-leucine and Fmoc-L-glutamic acid 5-t-butyl ester were from Reanal, and Na-Fmoc-Nd-trityl-Lglutamine and Na-Fmoc-Nc-trityl-L-asparagine were from Novabiochem (Darmstadt, Germany). The dodecapeptide N1-a2PI(1–12) and its synthetic mutants (Table 2) were prepared by the solid-phase method of Merrifield. The purity of the peptides was estimated to be >95% by analytical reverse-phase high-performance liquid chromatography. The molecular mass of the peptides was assessed with a matrix assisted laser desorption/ionization time-of-flight Voyager DE STR mass spectrometer (Applied Biosystems, Foster City, CA).

Kinetic FXIIIa assays

The activation of purified plasma FXIII and the measurement of FXIIIa activity was carried out essentially by the one-step UV kinetic assay. In this assay, FXIII becomes fully activated by thrombin during the 5-min lag phase of the reaction, and the amount of ammonia released from the glutamine-containing peptide substrate by FXIIIa is monitored in a glutamate dehydrogenase reaction measuring the decrease of NADPH absorbance at 340 nm. The transformation of NADPH to NADP is a linear function of transglutaminase activity from 5 to 10 min. The assay mixture contained 7 nM FXIII, 20 U/mL bovine thrombin, 10 mM $CaCl_2$, 0.1 mM dithiothreitol, 5 mM glycine-ethyl ester, 0.35 mM NADPH, 20 U/mL glutamate dehydrogenase, 0.6 mM ADP, 7 mM α-ketoglutarate, 5.4 mg/mL bovine serum albumin and various amounts of peptide substrates in 60 mM HEPES buffer (pH 7.7). Each assay was run at least in duplicate, the values were averaged, and k_{cat}, K_m and

k_{cat}/K_m values were calculated using GRAFIT version 6 data analysis software (Erithacus Software, Horley, UK).

NMR studies

NMR experiments were carried out on a Bruker Avance DRX 500 spectrometer equipped with a 5-mm BBI actively shielded z-gradient probe. In a water (H_2O/D_2O, 9:1 v/v) gradient, enhanced two-dimensional (2D) total correlation spectroscopy (TOCSY) and nuclear Overhauser enhancement spectroscopy (NOESY) spectra were recorded with a combination of water flip-back sequence, radiation damping suppression using weak bipolar gradients during t_1, and an excitation sculpting module for further reduction of the residual water signal. Five hundred-megahertz 1H-NMR spectra of 2 mM N1-α_2PI(1–12) or N1-α_2PI(1–12;2E) in 500 μL of 90% H_2O/10%D_2O with 22 mM borate buffer were investigated in the absence or presence of 10 μM FXIIIa at 310 K (pH 7.4) or 290 K (pH 5.5). In these experiments, FXIII was activated by 40 U/mL thrombin and 10 mM $CaCl_2$ for 20 min; as further increases in activation time did not result in increased FXIII activity, we considered FXIII to be fully activated.

Saturating transfer difference (STD) 1H-NMR spectra were recorded on samples of 10 μM FXIIIa and 20 mM N1-α_2PI(1–12) peptide at pH 7.4 and 310 K, applying 2-s on-resonance saturation of the protein methyl resonances at 0 p.p.m. In the reference experiment, the off-resonance frequency was set to -15 000 Hz. Selective saturation of protein signals was achieved with a Gaussian pulse cascade (40 Gaussian pulses of 50 ms each, with a delay of 1 ms between each pulse). STD ^{1}H-NMR spectra were recorded at 30-min intervals. A complete ^{1}H-NMR assignment of peptide N1-α_2PI(1–12) was obtained using the classic assignment strategy of Wüthrich, based on 2D TOCSY and NOESY experiments.

Measurement of substrate–product conversion by tandem mass spectrometry

The conversion of N1-α_2PI(1–12) substrate into N1-α_2PI(1–12;2E) by FXIIIa was assessed by tandem mass spectrometry on API 2000 equipment (MDS SCIEX, Concord, Canada) operated in multiple reaction monitoring mode under optimized conditions. The experimental conditions were identical to those described for STD NMR investigation. The tranglutaminase reaction was stopped at predefined intervals

by the addition of 1 mM iodoacetamide, and the b2 fragment (m/z = 243.2) containing the first two amino acids of N1-α_2PI (1–12) that formed during fragmentation was measured.

RESULTS

The interaction of FXIII subunits

Equilibrium dissociation constant (K_d) and kinetic parameters for FXIII subunit interaction

The K_d for the interaction of FXIII-A_2 and FXIII-B_2 was determined by SPR and an ELISA-type binding assay. The representative SPR sensorgrams and the binding curve demonstrate the binding of rFXIII-A_2 to plasma derived FXIII-B_2. The fitting curves nicely followed the sensorgrams. The same experiments were also carried out with cFXIII-A_2 prepared from human placenta. The K_d values obtained by both techniques were similarly in the range of 10^{-10} indicating tight binding. The dissociation rate constants, approximately 2 x 10^{-4} sec^{-1}, suggest slow dissociation. As expected, there was hardly any difference between results with rFXIII-A_2 and cFXIII-A_2. The binding of rFXIII-A_2 to rFXIII-B_2 was also tested by SPR. rFXIII-B_2 was produced in insect cells, in which the pattern of N-glycosylation is different from mammalian cells. The difference in N-glycan residues on FXIII-B_2 resulting in a protein with lower molecular mass did not influence significantly its interaction with FXIII-A_2.

Free FXIII-A_2 in the plasma and other body fluids

Using the mean of K_d values measured by the two techniques and the mean total FXIII-A_2 and FXIII-B_2 concentration the amount of complexed FXIII-A_2 and free FXIII-A_2 in the plasma was calculated. According to the calculation approximately 1% of the total FXIII-A_2 should exist in free form, non-associated to FXIII-B_2. This surprising finding was tested experimentally. Eleven human plasma samples were fully depleted from FXIII-A_2B_2, and also from free FXIII-B_2 by immuno-absorption using a monoclonal anti-FXIII-B antibody, that equally reacted with both the free and complexed forms of FXIII-B_2. The complete removal of FXIII-A_2B_2 was proven by a highly sensitive ELISA, the detection limit of which is 0.003 nM that corresponds to 0.004% of average plasma FXIII-A_2B_2 concentration. In the immuno-depleted plasma samples 1.06±0.49 nM free FXIII-A_2 was measured, i.e., 1.38±0.55% of plasma FXIII-A_2 existed in free form.

To test the validity of the established K_d, calculated and measured free FXIII-

A_2 were also compared in cerebrospinal fluids and tears, in which FXIII subunit concentrations were at least three magnitudes lower than in the plasma. In such condition one would expect a much higher proportion of free FXIII-A_2. Indeed, both the calculated and measured values were above 80%, and they showed fair agreement.

Free FXIII-A_2 in the plasma is functionally intact

To answer the questions if free FXIII-A_2 present in the plasma is functionally intact, plasma immuno-depleted of FXIII-A_2B_2 was treated by thrombin and Ca^{2+} and the formed clot was analyzed for fibrin cross-linking. Fibrin formed in non-depleted plasma showed complete dimerization of its γ-chains and the formation of highly cross-linked α-chain polymers (α_p). Fibrin chain cross-linking was prevented by the FXIIIa inhibitor, iodoacetamide. In the immuno-depleted plasma the transglutaminase-dependent formation of γ-chain dimers (γ-γ) was well detectable demonstrating that free plasma FXIII-A_2 can be transformed into an active transglutaminase. As opposed to non-depleted plasma, no α_p was detected on the top of concentrating gel. This is not surprising; γ-chain dimerization is a highly sensitive indicator of FXIIIa activity, while α-chain polymerization requires much higher FXIIIa concentration and it is a much slower process than γ-chain cross-linking. Fibrin cross-linking pattern in immuno-depleted plasma well corresponds to the finding that only approximately 1% of plasma FXIII-A_2 is in free form. The next question was if free FXIII-A_2 was in non-activated or in active form. To address this question fibrin clot formation was induced by batroxobin moojeni and Ca^{2+} in immuno-depleted plasma. Batroxobin moojeni cleaves off fibrinopeptide A but not fibrinopeptide B from fibrin and fails to activate FXIII. The activation of FXIII by thrombin generated in the plasma in the presence of Ca^{2+}, was prevented by hirudin. In such a set-up no fibrin chain cross-linking occurred, indicating that free FXIII-A_2 in the plasma was in non-activated form.

The interaction of FXIIIa and its substrates

The effect of synthetic mutations in the N-terminal part of N1-α_2PI(1–12) on the kinetic constants

As has been reported, replacement of the acyl-donor Gln2 by asparagine in N1-α_2PI(1–12) completely abrogated the transglutaminase activity of FXIIIa; that is, the Gln4 could not serve as an acyl-donor site. The increased K_m value for N1-α_2PI(1–12; 4N) without significant change in the k_{cat} value suggests that the side-chain of Gln4 participates in the enzyme–substrate interaction, but does not influence the rate of catalytic turnover of bound substrate. Drastic changes in the kinetic parameters after removal of the N-terminal Asn1 or its replacement by an alanine clearly demonstrated the importance of the Asn1 in the transglutaminase reaction. In the case of N1-α_2PI(2–12), a nine-fold higher K_m together with the concomitant decrease in the catalytic rate constant resulted in a tremendous (30-fold) decrease in the catalytic efficiency. The fact that Asn1A replacement also leads to a seven-fold increase in the K_m value suggests that the asparagine side-chain, rather than the presence of an amino acid linked N-terminally to Gln2, is of primary importance in promoting the binding of the substrate to FXIIIa.

Demonstration of the interaction of amino acid side-chains in the vicinity of Gln2 with FXIIIa by proton NMR

At pH 5.5 and 290 K, the amide signals of N1-α_2PI(1–12) are sharp and well resolved, but they become broadened when the pH is raised to 7.4 and the temperature to 310 K, indicating an accelerated chemical exchange with water protons at higher pH. The free peptide does not seem to exhibit any folded conformation with characteristic hydrogen bonded or solvent-shielded amide protons or other throughspace interactions. Comparison of the 1H-NMR spectrum of the free peptide with the spectrum of peptide in complex with FXIIIa recorded under the same conditions (pH 7.4, T = 310 K) reveals that the most striking difference is observed in the amide region of the spectra. In the presence of FXIIIa, this region of the spectrum resembles that of the spectrum obtained with the free peptide at pH 5.5 and 290 K. Studying the interaction of N1-α_2PI(1–12) with FXIIIa at physiologic pH, we had to consider that the thioester formed between the glutamine side-chain and the active-

site thiol hydrolyzes and the Gln2 becomes transformed into a Glu2. At pH 7.4, the product, N1-α_2PI(1–12; 2E), had the same broadened amide signals as the substrate. This finding indicated that changes in the amide proton spectra in the presence of FXIIIa are not due to substrate-to-product conversion, but to the interaction of N1-α_2PI(1–12) with FXIIIa. The well-resolved amide proton signals indicate that NH hydrogens become hydrogen-bonded or solvent-shielded in the peptide–protein complex. It was also observed that some of the side-chain proton resonances of the N-terminus of peptide N1-α_2PI(1–12), namely Asn1-β and Gln2,4-γ, were also markedly shifted in the complex, that is, in the presence of FXIIIa. These chemical shift changes reflect the presence of a new chemical environment upon binding to FXIIIa. The findings, together with the kinetic data, underline the importance of Asn1 and Gln4 side-chains in supporting the binding of substrate to the enzyme.

The effect of synthetic mutations in the C-terminal part of N1-α_2PI(1–12) on the kinetic constants

The data demonstrate that the C-terminal part of the peptide, namely residues 7–12, plays an essential role in binding of the substrate to the active enzyme and performance of the catalytic activity. Serial truncation of the dodecapeptide results in the gradual loss of enzymatic activity, up to its complete loss in the case of peptides containing fewer than seven residues. The removal of the C-terminal lysine resulted in an almost three-fold increase of the K_m without a significant change in the k_{cat}. This finding suggests that the Lys12 residue contributes to the binding of the dodecapeptide but is not important for the enzyme activity. One-by-one removal of the next two leucine residues results in a moderate and gradual increase in the K_m and a sharper decrease in catalytic efficiency. To distinguish between the role of the two leucine side-chains, either or both of them were replaced by alanine residue(s). Both individual replacements increased the K_m without significantly influencing the k_{cat}. The more drastic increase in K_m in the case of the N1-α_2PI(1–12; 10A) variant suggests that the terminal methyl group of Leu10 is more important in the enzyme–substrate interaction than that of Leu11. This conclusion is supported by the finding that the N1-α_2PI(1–12; 10,11A) variant, in which both leucine residues were replaced by alanine, had a K_m similar to that of N1-α_2PI(1–12; 10A). Further truncation, that is, the removal of Thr9, did not influence the K_m significantly, but decreased the k_{cat}.

To investigate the function of this threonine, containing a polar -OH group and a terminal methyl group, it was substituted in the non-truncated peptide by a serine containing the polar group without a terminal -CH3 or by alanine, a short-chain apolar amino acid with a methyl group. It was interesting to see that both replacements decreased the K_m, and the catalytic efficiency of the N1-α_2PI(1–12; 9S) variant was even higher than that of the non-modified dodecapeptide.

Investigation of the interaction between FXIIIa and N1-α_2PI(1–12) by STD NMR

STD spectroscopy has been used in the past few years to assess ligand–protein receptor interactions, and it may reveal which parts of a ligand contact the receptor. In the STD experiment, the receptor protein is selectively excited in a spectral region that is devoid of ligand peaks. Then, a saturation time of 2.0–5.0 s follows, during which magnetization is transferred from the receptor protons to the adjacent protons in the bound ligand. In the STD spectrum, the signals come from these adjacent ligand protons, and their intensities are proportional to the intimacy of the contact. The STD 1H-NMR study on the FXIIIa–N1-α_2PI(1–12) complex detected definite STD peaks that correspond to the methyl groups of C-terminal leucine residues. The resolving power of the measurement could not distinguish between Leu10 and Leu11. Additional weaker STD peaks corresponded to the methyl group of Thr9 and the leucine methylene group. The spectrum represents the recording made in the first half-hour, during which the concentration of the substrate, as measured by tandem mass spectrometry, decreased from 20 to 2 mM. The latter concentration is still four-fold higher than the K_m value, and keeps the enzyme saturated with the substrate. Then, in parallel with further transformation of N1-α_2PI(1–12) into N1-α_2PI(1–12; 2E), the signals gradually weakened, and after 60 min they disappeared. In accordance with the kinetic data, the results suggest that intermolecular hydrophobic interactions between the C-terminal residues of N1-α_2PI(1–12) and hydrophobic patches of FXIIIa play an important role in binding.

DISCUSSION

Unequivocal results obtained by two different techniques showed that the K_d for the association of the two types of FXIII subunit is in the range of 10^{-10} M, which is magnitudes lower than the values published in earlier reports. The estimated K_d is in the range of high affinity antigen-antibody interaction and suggests very tight binding between FXIII-A_2 and FXIII-B_2. It has been generally accepted that FXIII-A_2 in the plasma is fully complexed with FXIII-B. However, calculations based on the estimated K_d and the FXIII subunit concentrations present in the plasma suggested that, in spite of the tight binding, a minor fraction of FXIII-A_2, (approximately 1%), exists in free non-complexed form. The presence of free FXIII-A_2 in plasma was also confirmed experimentally. In tears and cerebrospinal fluid at FXIII subunit concentrations minimum three magnitudes lower than in the plasma the proportion of free FXIII-A_2 was much higher. The established K_d was also validated by the fair agreement between calculated and measured free FXIII-A_2 in the three body fluids. It was also shown that the measured free FXIII-A_2 is neither a degraded nor an activated form of FXIII; it is functionally active, when activated by thrombin and Ca^{2+}, it can cross-link fibrin. At this stage one can only offer speculations on the physiological significance of free FXIII-A_2 in the plasma. FXIII-A_2 in the absence of FXIII-B_2 does not need the removal of activation peptide for activation. Non-cleaved FXIII-A_2 goes through a slow progressive activation at Ca^{2+} and NaCl concentrations present in the plasma and fibrinogen might promote this non-proteolytic activation. Years ago a low-grade “constitutive” fibrin and fibrinogen cross-linking activity of non-activated plasma FXIII has been described. One wonders if this constitutive activity could be associated with the free fraction of FXIII-A_2. This presumption seems to be contradicted by the finding that in immuno-depleted plasma, containing only free FXIII-A_2, fibrin formed by batroxobin was not cross-linked. However, any non-proteolytically activated free FXIII-A_2 might bind to cellular elements or to fibrin(ogen) fibrils and might be cleared from the plasma. It is also possible that in newly formed fibrin this more easily activated fraction of plasma FXIII contributes to the rapid cross-linking of fibrin γ-chains which needs only a minute amount of FXIIIa.

FXIII-B is N-glycosylated at Asn142 and Asn525, which reside in the 3rd and 9^{th} sushi domains, respectively. Neither the removal of carbohydrate residues from

FXIII-B by neuraminidase or N-glycosidase F nor the different carbohydrate side-chain on rFXIII-B expressed in insect cells prevented the formation of A_2B_2 complex. In the present experiments the K_d for the binding of plasma derived FXIII-B_2 to rFXIII-A_2 did not differ significantly from the K_d for the interaction of rFXIII-B_2 and rFXIII-A_2. These findings strongly suggest that the binding epitope(s) responsible for the interaction with FXIII-A_2 must reside in the protein part.

N1-α_2PI is an excellent glutamine, but not lysine, donor substrate for FXIIIa. During coagulation, it is quickly crosslinked to the α-chain of fibrin, and the fibrin α-chain–N1-α_2PI heterodimers then become incorporated into high molecular mass α-chain polymers. N1-α_2PI crosslinked to fibrin retains its full inhibitory capacity against plasmin. It is now clear that the single primary acyl-donor glutamyl residue in N1-α_2PI is located at the position penultimate to the N-terminus (Gln2). With the use of recombinant Q2A N1-α_2PI mutants, three further glutamine donor sites were found in the molecule at positions 21, 419 and 447. However, their kinetic efficiency was much lower than that of Gln2. The presence of propeptide in M1-α_2PI makes it a poor substrate for FXIIIa, and the addition of only the last three C-terminal amino acids of the propeptide to the N-terminal end of N1-α_2PI was sufficient to prevent its crosslinking to fibrin.

In this study, we also investigated the molecular mechanism of the interaction of FXIIIa with N1-α_2PI(1–12), which is an excellent substrate of FXIIIa and competitively inhibits the crosslinking of N1-α_2PI to fibrin. The kinetic parameters for N1-α_2PI(1–12) agreed well with those found in our earlier study, and the K_m value was also identical to that reported for N1-α_2PI(1–15), an oligopeptide with a sequence that corresponds to the first 15 N-terminal residues in N1-α_2PI. The lower k_{cat} value and catalytic efficiency obtained for N1-α_2PI(1–15) might be due to the use of frozen and re-thawed FXIIIa preparations or, perhaps, to the inhibitory effect of the last three amino acids.

As opposed to earlier NMR studies carried out at pH 6.5, we studied the interaction of FXIIIa with N1-α_2PI(1–12) at physiologic pH. The interaction is clearly demonstrated by striking changes in the amide region of the spectra of N1-α_2PI(1–12). The sharp amide signals of peptide N1-α_2PI(1–12) in the presence of FXIIIa, as opposed to the broadened signals in its absence, suggest that the amide protons

became solvent shielded in the complex, and the exchange with water became hindered.

In the first part of the study, we investigated the role of the N-terminal amino acids of N1-α_2PI(1–12) in its interaction with FXIIIa. As expected, the Q2N change completely abrogated the enzymatic activity, and an earlier report on the supportive role of Gln4 was confirmed. It is to be noted that two other natural substrates of FXIIIa have glutamine two amino acids C-terminal from the reactive glutamine, and screening for FXIIIa substrates from a phage-displayed peptide library also identified several substrates with glutamine in this position. The dramatic changes in the kinetic parameters after the substitution of Asn1 by alanine or its removal demonstrated for the first time the importance of Asn1 in the interaction of the N1-α_2PI N-terminal peptide with FXIIIa. The low field shift of Asn1-β proton signals, as detected by proton NMR, also supports the importance of this residue and suggests interaction with polar groups surrounding the substrate-binding cavity. It is interesting that 24 of 28 preferred substrate sequences selected from the phage-displayed library for FXIIIa also contain only a single amino acid preceding the reactive glutamine, and most of them have hydrophilic characteristics. In summary, Asn1 has a major role in binding N1-α_2PI(1–12) to FXIIIa and, together with the Gln4 side-chain, and perhaps also the Glu3 side-chain, it is involved in directing the reactive glutamine to the active site in the substrate-binding cavity.

On the basis of indirect evidence provided by 1D proton NMR line broadening and 2D transferred NOESY spectra of N1-α_2PI(1–15), Marinescu et al. suggested an interaction between the C-terminal part of the substrate peptide and FXIIIa. In our study, the existence of a second substrate-binding site, important for the transglutaminase reaction, was clearly proven by kinetic experiments with C-terminally truncated/substituted substrates and STD NMR. The gradual decrease of catalytic efficiency in parallel with the decreasing number of amino acid residues, and the absence of detectable transglutaminase activity below seven residues, demonstrated the importance of the C-terminal part of N1-α_2PI(1–12) in its binding to FXIIIa. The removal of Lys12 increased the K_m about 2.5-fold, which suggests that its long, positively charged, side-chain promotes the binding of N1-α_2PI(1–12) to FXIIIa. STD NMR experiments demonstrated the involvement of hydrophobic interactions in the binding of the C-terminal part of N1-α_2PI(1–12), and identified the

methyl, and perhaps also the methylene, groups of one or two leucines as the main source of hydrophobic interaction. Kinetic experiments with the synthetic mutants pointed to the importance of Leu10. STD NMR also suggested a hydrophobic interaction between the methyl group of Thr9 and FXIIIa. However, removal of the methyl group by T9S substitution did not change the k_{cat}, and the K_m even somewhat decreased; that is, the methyl group of Thr9, even if it can interact with certain hydrophobic amino acid side-chains, has little importance for the enzyme–substrate interaction.

In summary, on the basis of data available in the literature on FXIIIa acyl-donor peptide/protein substrates and of the results presented above, it seems likely that, in addition to binding to the reactive site, several peptide substrates interact with a secondary binding site on FXIIIa. Such a secondary binding site could be important in promoting the binding of substrate peptide to FXIIIa and in improving the catalytic efficiency of the transglutaminase reaction. The absence of significant consensus in the primary sequence among different efficient glutamine substrates suggests that distinct secondary binding sites exist for different substrates, and mapping of such binding sites could significantly contribute to our knowledge of the interaction of FXIIIa with its substrates and on its function as a transglutaminase.

SUMMARY

Coagulation factor XIII (FXIII) is a heterotetramer consisting of two catalytic A subunits (FXIII-A_2) and two protective/inhibitory B subunits (FXIII-B_2). FXIII-B, a mosaic protein consisting of ten sushi domains, significantly prolongs the lifespan of catalytic subunits in the circulation and prevents their slow progressive activation in plasmatic conditions. Considering the importance of complex formation between the two FXIII subunits, it is surprising that the biochemistry of their interactions is only superficially explored. So far two values have been reported for the equilibrium dissociation constant (K_d) but calculations based on these values are contradictory to experimental data.

Activated factor XIII (FXIIIa), a dimer of the Ca^{2+} activated truncated A-subunits (FXIII-A_2*), is a transglutaminase that crosslinks primary amines to peptide-bound glutamine residues. Because in the few natural substrates of FXIII-A_2* no consensus sequence could be identified around the reactive glutamine, studying the interaction between individual substrates and FXIII-A_2* is of primary importance. Most of the α_2 plasmin inhibitor (α_2PI) molecules become truncated by a plasma protease, and the truncated isoform (N1-α_2PI) is an important substrate of FXIII-A_2*. The crosslinking of N1-α_2PI to fibrin plays a major role in protecting newly formed fibrin from fibrinolysis. The N-terminal dodecapeptide N1-α_2PI(1–12) of this molecule is an excellent glutamine donor peptide substrate of FXIIIa.

In this study the biochemistry of the interaction between the two FXIII subunits and the interaction of FXIII-A_2* with N1-α_2PI(1–12) were investigated. Using surface plasmon resonance technique and an ELISA-type binding assay the K_d for the interaction of FXIII subunits was established in the range of 10^{-10} M. Based on the measured K_d, it was calculated that in plasma approximately 1% of FXIII-A_2 should be in free form. This value was confirmed experimentally by measuring FXIII-A_2 in plasma samples immuno-depleted of FXIII-A_2B_2 and free FXIII-B_2. Free plasma FXIII-A_2 became functionally active, when activated by thrombin and Ca^{2+}, it cross-linked fibrin. In cerebrospinal fluid and tears with much lower FXIII subunit concentrations higher than 80% of FXIII-A_2 existed in free form.

Kinetic parameters for N1-α_2PI(1–12) and for its truncated or synthetic mutants were determined by a spectrophotometric FXIIIa assay. The interaction of N1-α_2PI(1–12) with FXIII-A_2* was investigated by proton nuclear magnetic

resonance (NMR) and saturating transfer difference (STD) NMR. Kinetic experiments with peptides in which the Asn1 residue was either truncated or replaced by alanine and proton NMR analysis of the FXIIIA_2*–N1-α_2PI(1–12) complex demonstrated that Asn1 plays an important role in effective enzyme–substrate interaction. Experiments with C-terminally truncated peptides proved that amino acids 7–12 are essential for the interaction of N1-α_2PI(1–12) with the enzyme, and suggested the existence of a secondary binding site on FXIII-A_2*. Hydrophobic residues, particularly Leu10, and the C-terminal Lys12 seemed to be especially important in this respect. The direct interaction between hydrophobic C-terminal amino acid side-chains and FXIII-A_2* was demonstrated by STD NMR.

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List of publications related to the dissertation

1. Katona, É., **Pénzes, K.**, Csapó, A., Fazakas, F., Udvardy, M.L., Bagoly, Z., Orosz, Z.Z., Muszbek, L.: Interaction of factor XIII subunits.
   Blood. 123 (11), 1757-1763, 2014.
   DOI: http://dx.doi.org/10.1182/blood-2013-10-533596
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2. Katona, É., **Pénzes, K.**, Molnár, É., Muszbek, L.: Measurement of factor XIII activity in plasma.
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   DOI: http://dx.doi.org/10.1111/j.1538-7836.2009.03291.x
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List of other publications

4. Losonczy, G., Fazakas, F., Pfliegler, G., Komáromi, I., Balázs, E., **Pénzes, K.**, Berta, A.: Three novel germ-line VHL mutations in Hungarian von Hippel-Lindau patients, including a nonsense mutation in a fifteen-year-old boy with renal cell carcinoma.
BMC Med. Genet. 14 (3), 1-8, 2013.
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