Tb(III)-ion-binding-induced conformational changes in platelet Factor XIII

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Ca(II) ions are crucial during proteolytic conversion of Factor XIII zymogen into the active enzyme Factor XIIIa. Factor XIII proteolysed by thrombin or trypsin in the presence of 5 mM-EDTA resulted in rapid inactivation of transglutaminase activity. Factor XIIIa formed by thrombin or trypsin in the presence of 40 μ M-Tb(III) ions, however, was indistinguishable from Factor XIIIa formed in the presence of 2–5 mM-Ca(II) ions with respect to molecular mass and transglutaminase activity. Thrombin treatment of Factor XIII in the presence of 1–5 μ M-Tb(III) ions resulted in three fragments (76 kDa, 51 kDa and 19 kDa) with simultaneous loss of transglutaminase activity. Tb(III) ions at concentrations greater than 40 μ M made platelet Factor XIII resistant to proteolysis by either thrombin or trypsin. Other lanthanide(III) ions [Ln(III) ions] tested [Ce(III), La(III) and Gd(III) ions] functioned similarly to Tb(III) ions during proteolytic activation of Factor XIII. Ln(III) ions (10–100 μ M) were unable to replace the Ca(II) ions required for transglutaminase activity of Factor XIIIa. Tb(III) ions also inhibited in a non-competitive manner the transglutaminase activity of Factor XIIIa (K_i 71 μ M) even when measured in the presence of 200-fold molar excess of Ca(II) ions. Factor XIII selectively bound to a Tb(III)-chelate affinity column, and could not be eluted by 100 mM-CaCl₂. Binding of Tb(III) ions to Factor XIII was demonstrated by fluorescence emission due to Forster energy transfer. A 10⁴-fold molar excess of CaCl₂, but not of NaCl, partially quenched Tb(III) fluorescence. Low concentrations (5–20 μ M) of Tb(III) ions also inhibited the binding of Factor XIII to des-A-fibrinogen by about 43 %, whereas higher concentrations (40–100 μ M) promoted binding. Conformational changes in Factor XIII consequent to the binding of Tb(III) ions could be responsible for the observed effects on protein structure and function.

INTRODUCTION

Blood coagulation Factor XIIIa (FXIIIa) is a transglutaminase (EC 2.3.2.13) that occurs as a zymogen in plasma and platelets [1]. Platelet Factor XIII (FXIII) is composed of two identical 80 kDa polypeptides, designated a-chains [1]. The activation of platelet FXIII occurs through a thrombin cleavage of the Arg³⁷-Gly³⁸ peptide bond near the N-terminus of the a-chains [2]. Thrombin and trypsin have been reported both to activate and to inactivate FXIII [3–7]. It was reported that Ca(II) and several other bivalent metal ions played a crucial role during the proteolytic activation of FXIII and in preservation of the transglutaminase activity of FXIIIa [7,8]. In the absence of metal ions (5 mM-EDTA), trypsin and thrombin treatment resulted in rapid inactivation of FXIII [6,7].

The ionic and spectroscopic properties of tervalent lanthanide ions [Ln(III) ions], especially Tb(III) and Eu(III) ions, make them valuable tools to probe the conformational changes in Ca(II)-ion-binding proteins [9–13]. Ln(III) ions have also been demonstrated to substitute for Ca(II) ions in a variety of protein functions [9–13].

However, there are only two reports dealing with the interactions of Ln(III) ions with plasma and platelet FXIII [14,15]. The first paper, by Sarasua *et al.* [14], reported significant differences in the binding of Tb(III) or Eu(III) ions by plasma and platelet FXIII. During

these studies they mentioned briefly that Ln(III) ions could partially replace Ca(II) ions during thrombin activation of FXIII [14]. It was not clear whether the FXIII studied was from platelets or plasma, since few details regarding the effect of Ln(III) ions on thrombin activation of FXIII were provided [14]. The second paper reported that Ca(II) ions essential for transglutaminase activity of plasma FXIIIa could partially be replaced by La(III) or Tb(III) ions [15].

We found that Tb(III) ions effectively replaced Ca(II) ions during FXIII proteolytic activation both by thrombin and by trypsin. We also found that Tb(III) ions strongly inhibited the transglutaminase activity of FXIIIa. Interactions of Ca(II) and Tb(III) ions with FXIII and consequent conformational changes in the protein were demonstrated by using fluorescence spectroscopy and Tb(III)-chelate affinity chromatography and by an effect of Tb(III) ions on FXIII binding to des-A-fibrinogen. Such conformational changes could be responsible for the observed effects of Tb(III) ions on protein structure and enzymic activity.

MATERIALS AND METHODS

Chemicals

Chloride salts of La(III), Tb(III), Ce(III) and Gd(III) ions were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All salts were of the highest

Abbreviations used: FXIII, Factor XIII; FXIIIa, Factor XIIIa; Ln(III) ions, lanthanide(III) ions.

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purity available (99.9% or greater). Na₂EDTA was purchased from Mallinckrodt (Paris, KY, U.S.A.). Water was made 'metal-free' by passage through a Chelex-100 column [16]. All solutions were made in 'metal-free' water.

Proteinases

L-Tosylphenylalanylchloromethane- ('TPCK'-) treated trypsin (23 kDa) was purchased from Worthington Corp. (Freehold, NJ, U.S.A.). Purified α -thrombin (7776 NIH units/ml; 69.2 μ M) was generously given by Dr. John Fenton, II (New York State Department of Health, Albany, NY, U.S.A.). Reptilase (Atroxin) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Protein purification and radioiodination

Platelet FXIII was purified from outdated human platelets (American Red Cross, Charlotte, NC, U.S.A.) [17]. Purified FXIII a-chains were radioiodinated as described previously [17]. Fibrinogen was purified as described previously [17] and was 98% clottable.

Activation and assay of FXIII

Activation of FXIII (0.24 mg/ml) by thrombin (100 units/ml) in the presence of 5 mM-CaCl₂ for 30 min at 37 °C was conducted as described previously [7]. Complete activation of FXIII (80 kDa) was confirmed by densitometric quantification of thrombin-cleaved a-chains (76 kDa) [7] and also by the expression of maximal transglutaminase activity by the thrombin-cleaved a-chains (= 100% activity). Transglutaminase activity was assayed in the presence of 10 mM-CaCl₂ [7] and both in the presence and in the absence of various concentrations of Ln(III) ions. Enzyme kinetic data were analysed by Dixon plots [18]. FXIIIa (activated FXIII) activity was expressed as μ mol of putrescine incorporated into casein in 60 min, after extrapolation of activity to 1 mg of FXIIIa protein.

Proteolysis of FXIII in the presence of Ln(III) ions

Factor XIII was activated by thrombin in the presence of different concentrations of Ln(III) ions replacing Ca(II) ions as described above. Factor XIII (40 μ g/ml) was also proteolysed by trypsin (0.4 μ g/ml) for 30 min at 37 °C [7] in the absence of metal ions (5 mM-EDTA) or in the presence of either 5 mM-CaCl₂ or various concentrations of Ln(III) ions. Control FXIIIa assays were performed simultaneously. Factor XIIIa in these experiments was diluted so that the final concentrations of Ln(III) ions during transglutaminase activity measurements were non-inhibitory (less than 10 μ M). Transglutaminase activity of 'Factor XIIIa' activated by thrombin or trypsin in the presence of Ln(III) ions was then related as percentage of control FXIIIa activity generated after FXIII activation by thrombin in the presence of 5 mM-CaCl₂ [7].

Immunoblotting

The proteolytic cleavage patterns of FXIII after thrombin or trypsin treatment in the presence or in the absence of metal ions were revealed by subjecting the FXIII digests to SDS/polyacrylamide-gel electrophoresis and immunoblotting as described previously [7]. The antigen bands were quantified by laser scanning densitometry [7]. Formation of FXIIIa (76 kDa) from

FXIII (80 kDa) as a result of proteolytic cleavage is expressed as percentage peak area of the 76 kDa band relative to the 80 kDa band.

Esterase activity of thrombin and trypsin

The esterase activities of thrombin and trypsin were measured with chromogenic substrates as described previously [7]. Assays were performed in the presence and in the absence of Ln(III) ions (0–100 μ M).

Fluorescence spectroscopy

Fluorescence measurements were made at ambient temperature with an Aminco-Bowman spectrofluorimeter (American Instrument Co., Silver Spring, MD, U.S.A.). Samples were excited at 290 nm, and emission intensity was quantified by monitoring the uncorrected fluorescence signal at 10 nm intervals. All spectra, except in experiments to determine the effect of ionic strength on Tb(III) emission, were taken in 150 mM-NaCl/50 mM-Tris/acetate buffer, pH 7.0, in quartz cuvettes with 1 cm light-path. Titrations of fluorescence intensity were performed by sequential additions of 5 μ l portions of titrant (CaCl₂ or NaCl in buffer) to the fluorescent-solution cuvette, mixing and then recording the fluorescence after an interval of 5 min to allow for equilibration. The total volume of a titrant added during titration experiments never exceeded 5% of the original volume of the sample (1 ml). Protein samples analysed by SDS/polyacrylamide-gel electrophoresis after taking fluorescence measurements revealed no degradation during fluorescence spectroscopy.

Tb(III)-chelate affinity chromatography of FXIII

A 0.8 ml column of chelating Sepharose 6B resin (Pharmacia, Uppsala, Sweden) was washed with 150 mM-NaCl/50 mM-Tris/acetate buffer, pH 7.0. The resin was then charged by passing 2 ml of a 5 mg/ml solution of TbCl₃ (approx. 13 mM) in buffer. Then 1 ml of ¹²⁵I-FXIII suitably diluted in buffer was passed through the column. The column was then washed sequentially with buffer, 100 mM-CaCl₂ in buffer and 50 mM-EDTA in buffer. Fractions (200 μ l in volume) were collected and radioactivity was measured in a γ -radiation counter. Only fractions containing at least 2.5% or more of the total radioactivity applied to the column were used to calculate recovery of material from the column. Individual fractions containing sufficient radioactivity were subjected to SDS/polyacrylamide-gel electrophoresis and immunoblotting. After detection of the antigen bands, the nitrocellulose membrane was exposed to a Kodak X-Omat XAR-5 film for 12–18 h at –70 °C. The X-ray film was developed and the bands were aligned with the FXIII antigen appearing on the immunoblots. Band intensities on the X-ray film were quantified by scanning densitometry [7]. In certain experiments ¹²⁵I-FXIII was applied to a Ca(II)-chelating Sepharose 6B column or a Mg(II)-chelating Sepharose 6B column and eluted as described above. Fractions collected from these columns and containing radioactivity were subjected to SDS/polyacrylamide-gel electrophoresis and autoradiography as described above.

Effect of Tb(III) ions on the binding of FXIII to non-cross-linked des-A-fibrinogen

The binding of FXIII to non-cross-linked des-A-fibrinogen was conducted as described previously [19]

with minor modifications. Briefly, FXIII (0.125 mg/ml) was mixed with purified fibrinogen (1.5 mg/ml) in Tris-buffered saline (130 mM-NaCl/100 mM-Tris/HCl buffer, pH 7.4). The fibrinogen was clotted by adding reptilase (0.5 μ g/ml) and incubating at 37 °C for 10 min. Incubations were carried out simultaneously in presence of 1 mM-EDTA or 1 mM- CaCl_2 or 1 mM- CaCl_2 plus various concentrations (5–100 μ M) of TbCl_3 . TbCl_3 was first added to FXIII and the mixture allowed to incubate for 5 min at room temperature before other components of the reaction mixture were added. After clotting, samples were chilled on ice for 5 min and then spun in a Beckman model Microfuge B for 5 min. The samples were then chilled once again on ice for 5 min. The supernatant was quantitatively aspirated with a Hamilton glass syringe equipped with a sharp pointed needle. The des-A-fibrinogen precipitate was washed thrice by resuspension in Tris-buffered saline followed by centrifugation. The supernatant and precipitate were solubilized by adding (final concentrations) 6 M-urea, 5% (w/v) SDS, 50 mM-Tris, 1 mM-EDTA, 1% (v/v) 2-mercaptoethanol and 0.005% Bromophenol Blue dye, and heating at 37 °C overnight. The solubilized samples were then subjected to immunoblotting as described above.

Effect of Tb(III) ions on des-A-fibrinogen formation

The effect of Tb(III) ions on des-A-fibrinogen formation was measured as follows. Increasing concentrations of TbCl_3 (0–100 μ M) were incubated with purified fibrinogen (1.5 mg/ml) plus 1 mM- CaCl_2 for 5 min at room temperature. The fibrinogen was clotted by adding reptilase (0.5 μ g/ml). The des-A-fibrinogen was pelleted by centrifugation and the supernatant assayed for non-clotted fibrinogen ($A_{1\text{cm}}^{1\text{cm}}$ at 280 nm = 15.1) [17].

RESULTS

Thrombin activation of FXIII in the presence of Ln(III) ions

The presence of Ca(II) ions during thrombin cleavage of FXIII (80 kDa) is crucial both to the formation of FXIIIa (76 kDa) and expression of transglutaminase activity [7,8]. In the absence of Ca(II) ions thrombin proteolysis of FXIII resulted in a 56 kDa 'inactivation peptide' with simultaneous loss of enzymic activity [6,7]. We therefore first investigated the effect of substituting Ln(III) ions in place of Ca(II) ions on FXIIIa formation following limited proteolysis by either thrombin or trypsin and the resulting transglutaminase activity of FXIIIa. In the presence of increasing concentrations of Tb(III) ions (up to 40 μ M) thrombin (100 units/ml) increased linearly the amount of enzymically active FXIIIa antigen formed. Tb(III) ions in the concentration range 1–5 μ M could not prevent the proteolytic inactivation of FXIII. At these concentrations the FXIII digest, composed of three peptide fragments (76 kDa, 51 kDa and 19 kDa), expressed about 30% of enzymic activity or less (Fig. 1, lanes 2 and 3). Tb(III) ions in the range 20–40 μ M were sufficient to aid optimal activation of FXIII by thrombin (Fig. 1). Under these conditions but in the absence of Tb(III) ions 2–5 mM- CaCl_2 was needed to achieve similar extents of FXIII activation by thrombin [7]. Excellent correlation was observed between the amount of FXIIIa antigen formed and its transglutaminase activity (Fig. 1) as expected [7]. Regardless

Fig. 1. Effect of Tb(III) ion concentration on thrombin cleavage of FXIII and transglutaminase activity

Purified platelet FXIII (0.24 mg/ml) was proteolysed by α -thrombin (100 units/ml) in the presence of increasing concentrations of Tb(III) ions as described in the Materials and methods section. The FXIII digest was assayed for transglutaminase activity (○) and a portion of the digest was also subjected to SDS/polyacrylamide-gel electrophoresis and immunoblotting (see inset). The FXIIIa antigen bands in immunoblots (inset) were quantified by laser scanning densitometry (●). Inset: lane 1 represents intact FXIII; lanes 2–7 represent FXIII digested by thrombin in the presence of Tb(III) ions, the concentrations of Tb(III) ions used being respectively 1 μ M, 5 μ M, 10 μ M, 20 μ M, 30 μ M and 40 μ M; the molecular masses (in kDa) of the antigen bands are indicated on the right-hand side of the inset. The specific activity of thrombin-activated FXIII in the presence of 5 mM-Ca(II) ions (= 100% control) was approx. 1.9 μ mol of putrescine incorporated into casein.

of the differences in their ionic radii [from 0.1061 nm for the La(III) to 0.0923 nm for the Tb(III) ion] all Ln(III) ions tested were nearly equal in their ability to mediate limited proteolytic activation of FXIII (Fig. 2). It must be noted that in these experiments Ln(III) ions replaced Ca(II) ions only in the thrombin cleavage of FXIII step and not during FXIIIa activity measurements.

When the concentration of Tb(III) ions was increased above 40 μ M, however, both thrombin cleavage of FXIII and the resulting FXIIIa transglutaminase activity declined (results not shown). At a concentration of 100 μ M-Tb(III) ions thrombin cleavage of FXIII was completely inhibited with no effect on the esterase activity of thrombin. The remainder of the Ln(III) ions tested [Ce(III), La(III) and Gd(III) ions] functioned similarly to Tb(III) ions during proteolytic activation of FXIII. Ln(III) ions (up to 100 μ M) have not been reported to inhibit thrombin activity [20–22]. A 50-fold molar excess of Ca(II) ions was unable to reverse the inhibition of thrombin activation of FXIII caused by 100 μ M-Tb(III) ions.

FXIII was one-half activated by 5 units of thrombin/

Fig. 2. Effect of La(III), Ce(III) and Gd(III) ions on thrombin activation of FXIII

FXIII (0.24 mg/ml) was activated by thrombin (100 units/ml) in the presence of increasing concentrations of La(III), Ce(III) and Gd(III) ions in a manner similar to that described for Tb(III) ions (see Fig. 1). Control FXIIIa activity [100% value; FXIII activated by thrombin and Ca(II) ions] in these experiments varied in the range 1.87–1.89 μ mol of putrescine incorporated into casein. As expected (see Fig. 1), there was very good correlation between transglutaminase activity and the amount of FXIIIa antigen formed (results not shown). The metal ions represented in the Figure are La(III) ions (○), Ce(III) ions (▲) and Gd(III) ions (△).

ml in the presence of 5 mM-CaCl₂ (Fig. 3). In the presence of 40 μ M-Ln(III) ions the thrombin concentration to achieve one-half activation of FXIII was increased 2–5-fold (Fig. 3). However, the thrombin concentrations required to activate platelet FXIII fully in the presence of 40 μ M-Ln(III) or 5 mM-Ca(II) ions were the same, approx. 100 NIH units/ml (Fig. 3). In the presence of 5 mM-EDTA there was less than 25% of transglutaminase activity expressed (Fig. 3).

Trypsin activation of FXIII in the presence of Ln(III) ions

We previously reported that trypsin partially activated platelet FXIII in the presence of 5 mM-CaCl₂ [7]. We next investigated if Ln(III) ions could substitute for Ca(II) ions in this step as well. Ln(III) ions indeed replaced Ca(II) ions during trypsin activation of FXIII (Fig. 4). FXIIIa activity after trypsin activation in the presence of 5 mM-CaCl₂ varied between 56% and 63% of control (= thrombin-activated FXIII). Transglutaminase activity of FXIII activated by trypsin in the presence of 5 mM-EDTA was always less than 25%, consistent with our earlier results [7]. Consistent with earlier reports [21,22], we did not observe any effect by 10–100 μ M-Ln(III) ions on the esterase activity of a 0.4 μ g/ml solution of trypsin.

Inhibition of FXIIIa transglutaminase activity by Ln(III) ions

Ln(III) ions in the concentration range 10–100 μ M were unable to replace the Ca(II) ions required for trans-

Fig. 3. Effect of Ln(III) ions on the thrombin concentration required for proteolytic activation of purified platelet FXIII

FXIII (0.24 mg/ml) was incubated with increasing concentrations of thrombin (1–100 units/ml) in the presence and in the absence of metal ions for 30 min at 37 °C and assayed for transglutaminase activity. FXIIIa activity after activation by 100 units of thrombin/ml in the presence of 5 mM-CaCl₂ in these experiments varied between 2.08 and 1.87 μ mol of putrescine incorporated into casein. Thrombin activation of FXIII was carried out in the presence of 5 mM-Ca(II) ions (●—●), 5 mM-EDTA (○—○), 40 μ M-Tb(III) ions (▲—▲), 40 μ M-La(III) ions (△—△), 40 μ M-Ce(III) ions (■—■) or 40 μ M-Gd(III) ions (□—□).

glutaminase activity of FXIIIa. This was observed regardless of whether FXIIIa generated was by thrombin or trypsin proteolysis in the presence of Ca(II) or Ln(III) ions. Furthermore, Ln(III) ions in increasing concentrations caused a progressive inhibition of transglutaminase activity of FXIIIa (Fig. 5). Dixon-plot analyses revealed that the inhibition of FXIIIa activity by Tb(III) ions was non-competitive [K_i for Tb(III) ions is 71 μ M]. The inhibition by Ln(III) ions of FXIIIa activity was not reversed by increasing the Ca(II) ion concentration to 20 mM during transglutaminase activity measurements (results not shown).

Fluorescence spectroscopy of FXIII

To demonstrate the binding of Tb(III) ions directly we studied the fluorescence emission of Tb(III) ions bound to FXIII due to Forster energy transfer [10–12]. Tb(III)-ion-binding-induced fluorescence-emission spectra exhibit major emission peaks at the following spectral regions: 485–500 nm, 540–555 nm and 580–595 nm [11]. On the basis of experiments with different concentrations of FXIII and Tb(III) ions we found that 50 μ g of FXIII/ml (approx. 0.63 μ M) and 10 μ M-Tb(III) ions gave an adequate fluorescence-emission signal to 580 nm. Under these conditions the signal from buffer blank was undetectable and buffer containing either 10 μ M-Tb(III) ions or 0.63 μ M-FXIII gave a low signal that was subtracted from the Tb(III) fluorescence in the presence of protein to yield the protein-dependent Tb(III) emission. Irradiation at 290–295 nm selected only those

Fig. 4. Effect of Ln(III) ion concentration on trypsin activation of FXIII

Purified platelet FXIII was proteolysed with trypsin in the presence of increasing concentrations of Ln(III) ions. The digests were then assayed for transglutaminase activity as described for Fig. 1. Platelet FXIII activated by thrombin in the presence of 5 mM-CaCl₂ (=100% control) was assayed simultaneously. The specific activity of thrombin and Ca(II)-ion-activated FXIII in these experiments varied between 0.89 and 0.75 μmol of putrescine incorporated into casein. Results are expressed as percentages of control FXIIIa activity. The metal ions used during trypsin activation of FXIII were Tb(III) ions (●—●), La(III) ions (○—○), Ce(III) ions (▲—▲) and Gd(III) ions (△—△). FXIII activated by trypsin in the presence of 5 mM-CaCl₂ varied between 54% and 63% of control FXIIIa activity (i.e. thrombin-plus-Ca(II)-ion-activated FXIIIa activity).

Tb(III) ions that were bound in close proximity to a tryptophan donor. FXIII a-chain is known to contain about 14 tryptophan residues [2,6] and these could participate in energy transfer. The energy transfer following excitation at 290 nm was accompanied by a small decrease (about 14%) in fluorescence signal (340–345 nm) of the tryptophan residues and a strong re-emission at 580 nm by FXIII-bound Tb(III) ions (Fig. 6). Ca(II) ions produced a concentration-dependent gradual decrease in the fluorescence emission (Fig. 7). The quenching of fluorescence emission by Ca(II) ions was not due to a simple change in the ionic strength in the medium, since NaCl up to 250 mM had no effect on FXIII–Tb(III) fluorescence emission (Fig. 7).

Tb(III)-chelate affinity chromatography of FXIII

Purified radiolabelled platelet FXIII bound to a Tb(III)-chelating Sepharose 6B column (Fig. 8). The radioactivity coming through in the wash represented less than 20% of the total activity applied to the column. By contrast, ¹²⁵I-FXIII did not bind to either a Ca(II)-chelating Sepharose 6B column or a Mg(II)-chelating Sepharose 6B column. In three separate experiments we found that more than 80% of FXIII bound to the

Fig. 5. Effect of Ln(III) ions on the transglutaminase activity of FXIIIa

Purified platelet FXIII (0.24 mg/ml) was activated by thrombin (100 units/ml) in the presence of 5 mM-CaCl₂. FXIIIa was assayed for transglutaminase activity in the presence of 10 mM-CaCl₂ and increasing concentrations of Ln(III) ions. The metal ions tested were Tb(III) ions (●—●), La(III) ions (○—○), Ce(III) ions (▲—▲) and Gd(III) ions (△—△). FXIIIa activity in the absence of Ln(III) ions varied between 2.35 and 2.85 μmol of putrescine incorporated into casein.

Fig. 6. Fluorescence-emission spectrum (uncorrected) of the FXIII–Tb(III) ion complex

FXIII (50 μg/ml; 0.625 μM) in Tris-buffered saline was irradiated at 290 nm in the presence and in the absence of 10 μM-Tb(III) ions as described in the Materials and methods section. The amplitude of the fluorescence emission is given in arbitrary units. The broken curve represents the fluorescence-emission spectrum of FXIII alone. The continuous curve represents the fluorescence emission of the FXIII–Tb(III) ion complex corrected for the emission of Tb(III) ions alone.

Fig. 7. Effect of CaCl_2 or NaCl on fluorescence emission of the FXIII-Tb(III) ion complex

The emission at 580 nm of the FXIII-Tb(III) ion complex was titrated by sequential additions of either NaCl (○—○) or CaCl_2 (●—●). Results are expressed as percentages of fluorescence emission in the absence of either titrant.

Fig. 8. Tb(III)-chelate affinity chromatography of ^{125}I -FXIII

^{125}I -FXIII was applied to a Tb(III)-chelating Sepharose 6B column. The column was washed with buffer (150 mM- NaCl /50 mM-Tris/acetate buffer, pH 7.0) and buffer containing 100 mM- CaCl_2 . Finally the bound protein was eluted with buffer containing 50 mM-EDTA. The inset shows the immunoblot/autoradiography pattern: lane 1, immunoblot pattern of FXIII; lanes 2-5 represent autoradiograms of ^{125}I -FXIII eluted from the column at 9.6 ml, 9.8 ml, 10.0 ml and 10.2 ml respectively; the remaining fractions contained insufficient radioactivity to be detected by autoradiography.

Tb(III)-chelating Sepharose 6B column. The binding of FXIII to the Tb(III)-chelating Sepharose 6B column could not be prevented by the addition of 20 mM- CaCl_2 to FXIII before application to the column (results not shown). Furthermore, washing the Tb(III)-chelating Sepharose 6B column containing bound FXIII with buffer containing 100 mM- CaCl_2 did not elute any FXIII

Fig. 9. Effect of Tb(III) ion concentration on the binding of FXIII to des-A-fibrinogen

FXIII was bound to reptilase-treated fibrinogen in the presence of 1 mM- Ca(II) ions and in the presence and in the absence of various concentrations of Tb(III) ions. Results are expressed as the averages for five experiments. In the absence of Tb(III) ions the amount of FXIII unbound was less than 5%. Maximal inhibition of binding observed in this set of experiments was 43%. For more details see the text.

from the column (Fig. 8). By SDS/polyacrylamide-gel electrophoresis followed by immunoblotting and autoradiography we identified the radioactive material eluted in the EDTA wash as FXIII (Fig. 8 inset). Radioactive material that emerged unadsorbed could not be detected by autoradiography even after exposure to X-ray film for 15 days.

Effect of Tb(III) ions on FXIII binding to des-A-fibrinogen

FXIII bound des-A-fibrinogen equally well in the presence of 1 mM- Ca(II) ions or 1 mM-EDTA (results not shown). This is in agreement with our previous results [19]. However, in the presence of Tb(III) ions the binding was biphasic. Low concentrations of Tb(III) ions (5–20 μM) inhibited the binding of FXIII to des-A-fibrinogen by about 43%, whereas higher concentrations of Tb(III) ions (40–100 μM) progressively promoted binding (Fig. 9). Ca(II) ions were present in these experiments at 10–250-fold molar excess over Tb(III) ions. Fibrinogen was 95–98% clottable at the concentrations of Tb(III) ions used in these experiments. The biphasic effect of Tb(III) ions on FXIII binding to des-A-fibrinogen (Fig. 9) was observed regardless of whether or not Ca(II) ions were present during binding.

DISCUSSION

The presence of Ca(II) ions during proteolytic activation of FXIII is crucial to both the formation of FXIIIa and expression of optimal enzymic activity [7,8]. FXIII a-chains proteolysed by thrombin or trypsin in the absence of Ca(II) ions (5 mM-EDTA) resulted in the formation of a 56 kDa or 51 kDa 'inactivation peptide' devoid of transglutaminase activity [6,7]. However,

Tb(III) and other Ln(III) ions tested effectively replaced Ca(II) ions during both trypsin and thrombin activation of FXIII and also helped to preserve the expression of transglutaminase activity of FXIIIa (Figs. 1–4). We conclude that FXIIIa generated by thrombin or trypsin cleavage in the presence of Ca(II) or Ln(III) ions (the present study), unlike the situation in the presence of EDTA [7], existed in a conformation favourable for enzyme catalysis.

Inhibition of thrombin or trypsin activation of FXIII at Tb(III) ion concentrations in the range 40–100 μM might be due to conformational changes in FXIII that resisted proteolytic attack. A 50-fold molar excess of Ca(II) ions was unable to reverse 100 μM -Tb(III)-ion-induced inhibition of thrombin activation of FXIII (see the text above). However, no inhibition of thrombin activation of FXIII was observed in the presence of higher concentrations (up to 100 mM) of Ca(II) ions (K. E. Achyuthan, A. Mary & C. S. Greenberg, unpublished work). These findings are reminiscent of the protective effect of Nd(III) ions on staphylococcal nuclease towards trypsin digestion that Furie *et al.* interpreted as evidence for conformational changes [21].

Contrary to a previous report [15], we were unable to observe measurable transglutaminase activity of FXIIIa in the presence of Ln(III) ions. The concentrations of Ln(III) ions tested by us (approx. 5–50-fold lower than those used by Lewis *et al.* [15]) strongly inhibited FXIIIa activity (Fig. 5). We are unable to explain the differences between our results and those reported by Lewis *et al.* [15]. Inhibition of transglutaminase activity could be due to Ln(III)-ion-induced protein aggregation. A 200-fold molar excess of Ca(II) ions was unable to reverse Ln(III) ion inhibition of FXIIIa activity (see the text above).

Iminodiacetate groups strongly bind Ln(III) ions [23,24]. Chelating Sepharose 6B, a resin containing iminodiacetate groups, when charged with Tb(III) ions selectively bound FXIII (Fig. 8). A 100 mM-CaCl₂ solution failed to elute the bound protein (Fig. 8). However, FXIII did not bind to Ca(II)-chelating Sepharose 6B. Interactions of Ca(II) ions with FXIII are probably weaker compared with those of Tb(III) ions. It may be possible to use this method to isolate the Ca(II)-ion-binding domains in FXIII [6] and to purify FXIII as well. The method is a novel demonstration of the use of Tb(III) ions in metal-ion-chelate affinity chromatography involving iminodiacetate-containing resins, and is different from previous reports on Ln(III)-ion-mediated binding of proteins to resins [21,25,26].

We used reptilase to clot fibrinogen in the presence of FXIII to study the binding of FXIII to des-A-fibrinogen clot [19]. Reptilase, unlike thrombin, does not convert FXIII into FXIIIa [1,19]. Thus we studied the effect of Tb(III) ions on the specific binding of FXIII (not FXIIIa) to des-A-fibrinogen. FXIII binding to des-A-fibrinogen was reported to be independent of bivalent metal ions (see the text above) [19]. However, the binding of FXIII to des-A-fibrinogen was affected in a biphasic manner by the presence of micromolar concentrations of Tb(III) ions even in the presence of 1 mM-Ca(II) ions (Fig. 9). Tb(III)-ion-induced conformational changes and/or protein aggregation could be responsible for the observed effects on FXIII binding to des-A-fibrinogen.

A direct measure of conformational changes was fluorescence emission of FXIII-bound Tb(III) ions due to Forster energy transfer (Fig. 6). The weaker

interactions of Ca(II) ions with FXIII relative to Tb(III) ions was evident from the high (10^4 -fold molar excess) concentration of Ca(II) ions required to quench Tb(III) fluorescence by 50% (Fig. 7).

The relative fluorescence stability at high ionic strengths (250 mM-NaCl) indicates that binding of Tb(III) ions to FXIII is probably due to chelation with acidic (Glu/Asp) amino acid residues and not a result of charge-charge attractions. Ln(III) ions, with 3 positive charges, bind more tightly to acidic groups (e.g. $-\text{CO}_2^-$) and may have co-ordination numbers of 8 or 9 [27,28]. In contrast, Ca(II) ions bind less tightly to these anions and exhibit a co-ordination number of only 6 [29]. Takahashi *et al.* have identified three potential Ca(II)-ion-binding domains near the C-terminal region of FXIII a-chains [6]. One or more of these domains may also bind Ln(III) ions. There may also be other clusters of acidic amino acid residues in FXIII (e.g. Asp-343 to Asp-357) capable of binding Ln(III) ions.

Whether the various Ln(III) ions occupy the same or different sites in FXIII is not known. This could be determined by performing competition binding experiments among different Ln(III) ions. Such experiments would also yield information on the relative proximity of the cation-binding sites to each other [30,31]. Finally, conformation-specific antibodies that recognize specific protein-metal ion configurations [32] could be raised and used as probes of protein structure [32,33].

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