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ISOLATION AND CHARACTERIZATION OF THE VITAMIN K DEPENDENT DOMAIN OF HUMAN PROTHROMBIN

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Summary : Chymotryptic cleavage of human prothrombin produces two fragments, prothrombin (des 1-44) previously characterized and peptide 1-41. This peptide has been purified by barium citrate adsorption and Sephadex G100 chromatography. It contains the 10 γ-carboxyglutamic residues of prothrombin. Added to a prothrombin activation mixture containing FXa, phospholipid and Ca^{++}, peptide 1-41 inhibits thrombin generation with the same potency as prothrombin fragment 1. Ca^{++} produces a 20 % quenching of the intrinsic fluorescence of the peptide. So do Mn^{++} and Mg^{++} although to a lesser extent. Phospholipid enhances the Ca^{++} induced quenching by a factor of 1.7 and shifts the midpoint of the transition from 0.34 to 0.46 mM Ca^{++}. The major difference with titration curves obtained with prothrombin F1 is the absence of cooperativity. Hence peptide 1-44 has retained some of the prothrombin properties to interact with Ca^{++} and phospholipid and represents the vitamin K dependent domain of the molecule. The presence of the remaining part of F1 (residues 44-155) however is necessary for the expression of cooperativity.

INTRODUCTION

The rate of prothrombin activation by factor Xa (FXa) is greatly enhanced by calcium, phospholipid and a proteic "cofactor", factor Va (FVa). Ca^{++} binding to prothrombin has been extensively studied (for a review see ref. 1). It is considered to be dependent on the presence of 10 γ carboxyglutamic acid residues (Gla) localized within the 40 N-terminal residues of the molecule (2-4). These residues result from a vitamin K-dependent post translational modification of an inactive precursor. Prothrombin isolated after antivitamin K treatment (4) or thermically decarboxylated (6) has indeed no ability to bind Ca^{++} and to interact with phospholipid. Tryptic peptide 12-44 contains 8 of the 10 Gla residues and interacts with divalent cations (7-8). Prothrombin undergoes a conformational transition upon Ca^{++} binding as shown by different approaches (9-12). This transition is essentially located in the

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N-terminal portion of the molecule, prothrombin fragment 1 (F1 : residues 1-155). We reported previously that α chymotrypsin cleaves human prothrombin in two products, one of which is prothrombin (des 1-44) (13). This product has lost the vitamin K dependent region. It is still able to generate thrombin, but its activation is no longer enhanced by Ca^{++} and phospholipid. A similar observation has been made for bovine FXa (14).

This paper describes the characterization of the other product of chymotryptic cleavage of human prothrombin, peptide 1-41. It contains the 10 Gla residues and constitutes the vitamin K dependent domain of prothrombin.

MATERIALS AND METHODS

Prothrombin. Human prothrombin was purified from vitamin K dependent factors concentrate (PPSB) obtained from the Centre National de Transfusion Sanguine, Orsay, France, by a procedure adapted from Butkowski *et al.* (15). The last step of purification is a DEAE cellulose chromatography in citrate tri Na buffer 0.05 M pH 6 using a NaCl gradient from 0.05 to 0.45 M.

Chymotryptic cleavage. Purified prothrombin (6 mg/ml) was hydrolysed with α chymotrypsin (Boehringer, Manheim), in a 0.02 M NH_4HCO_3 pH 8.6 buffer at 37°C. TLCK treated chymotrypsin was added at 0 and 2 hrs (E/S = 1/250 final). Digestion was stopped after 4 hrs by addition of diisopropyl fluorosphosphate 10 mM final.

Purification of the chymotryptic products. Chymotryptic products were separated by barium citrate adsorption. Prothrombin (des 1-44) remained in the supernatant (13). Peptide 1-41 and non hydrolysed prothrombin, adsorbed quantitatively on the barium citrate precipitate, were eluted in 1 M Na_2SO_4 and separated on Sephadex G100 in a 0.02 M ammonium bicarbonate buffer pH 8.6.

Prothrombin fragment 1 (F1) was prepared according to Mann (16). Homogeneity of the purified products was ascertained by SDS gel electrophoresis (17).

Amino acid analyses were performed on a Chromaspeck J 180 amino acid analyser (Rank Hilger) using a 75 min. program.

Prothrombin activation and thrombin amidolytic activity measurement. F1 and peptide 1-41 were added to the activation mixture containing prothrombin (0.1 mg/ml), RVV activated bovine FX (0.07U/ml), Ca^{++} (10 mM) and rabbit brain cephalin (4 µg/ml), in molar ratios to prothrombin varying from 0.2/1 to 60/1. Generated thrombin activity was measured on the chromogenic substrate S 2238 (Kabi Diagnostica).

Fluorescence measurements. Fluorescence spectra were obtained on an Aminco Bowman 4-8203 spectrofluorometer. 1 ml of protein solution was used for each measurement containing 0.5 to 1.5 nmoles of F1 or peptide 1-41 in a 50 mM Tris buffer pH 7.4. Emission fluorescence intensity was recorded from 200 to 600 nm (λ exc. = 286 nm). Titrations were obtained by addition of small volumes (1 to 20 µl) of concentrated solutions of $CaCl_2$, $MgCl_2$ and $MnCl_2$ and the spectra recorded after a 5 min. equilibration at 20°C. Rabbit brain cephalin (Sigma) was used at a final concentration of 200 µg/ml.

RESULTS

Products of chymotryptic cleavage of human prothrombin were separated by barium citrate adsorption. One of them (apparent Mr : 69 000) remains in the supernatant. It has been previously shown to be prothrombin (des 1-44) (13). The adsorbed products, chromatographed on Sephadex G100, separated in two molecular species : unhydrolysed prothrombin and a peptide with an apparent Mr of 5 000. Amino acid analysis of this peptide shows that it contains the 41 N-terminal residues of prothrombin and can therefore be referred to as peptide 1-41. Presence of Trp 41 as the C-terminal residue was ascertained from the UV spectrum. Its presence is very dependent upon the conditions of hydrolysis, since a longer incubation with chymotrypsin, or the use of a higher E/S ratio gives rise only to peptide 1-40, the C-terminal residue of which is Phe 40. It is therefore likely that our preparation of peptide 1-41 is contaminated with a certain amount of peptide 1-40. Both peptides contain the 10 Gla residues of prothrombin. Their amino acid sequence is given in figure 1.

Since peptide 1-41 contains the vitamin K dependent region of prothrombin thought to be responsible for the Ca^{++} dependent interaction with phospholipid, its ability to interfere with prothrombin activation by FXa in the presence of Ca^{++} and phospholipid was tested. Addition of the peptide to the activation mixture drastically inhibits the rate of prothrombin activation (Fig.2). This behaviour is identical to that of prothrombin fragment 1 (F1) tested in comparison. Added amounts of peptide 1-41 were calculated from the amino acid analysis data.

Peptide 1-41 presents an intrinsic fluorescence due to the presence of Tyr 24 and mainly of Trp 41 with a maximum intensity at 345 nm (λ excitation : 286 nm). As already reported for prothrombin and F1, Ca^{++} induces a quenching of this intrinsic fluorescence allowing direct measurement of the interaction of peptide 1-41 with divalent cations. Figure 3 shows the titration curves obtained with Ca^{++}, Mn^{++} and Mg^{++} ions. Ca^{++} is the most effective in inducing

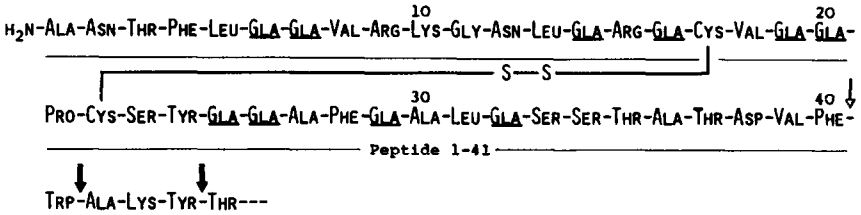

Figure 1. N-terminal amino acid sequence of human prothrombin. Arrows indicate the sites of chymotryptic cleavage. The 10 γ-carboxyglutamic residues (Gla) are underlined.

fluorescence quenching, (22 % quenching at 2 mM Ca^{++}), followed by Mn^{++} and Mg^{++}. Interaction of peptide 1-41 with Ca^{++} has also been tested in the presence of phospholipid. Figure 4 shows the titration curves in the absence (Fig. 4A) and presence (Fig. 4B) of phospholipid, in comparison to those obtained for F1. Differences between the two products are first the maximum

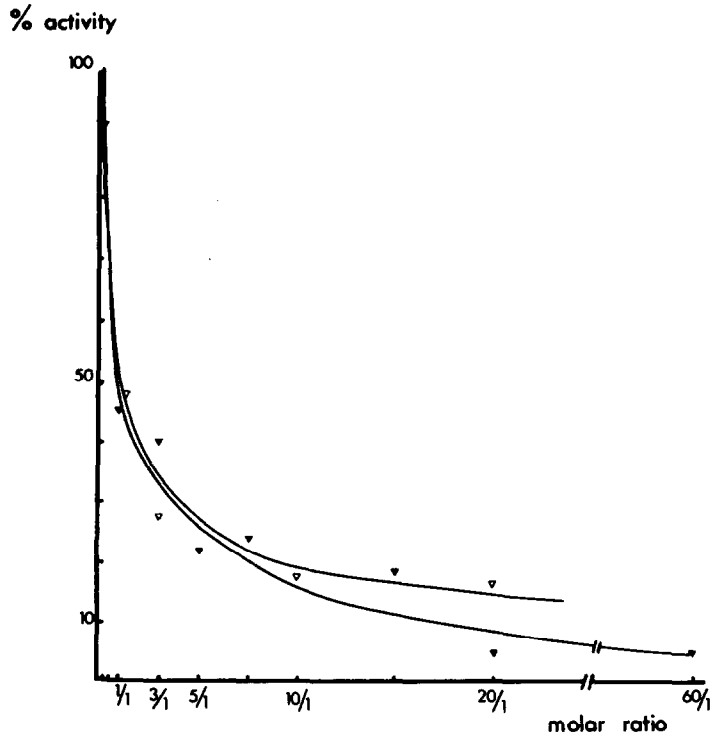

Figure 2. Inhibition of prothrombin activation by peptide 1-4 (▼) and fragment 1 (▽) in a mixture containing factor Xa, phospholipid and Ca^{++}. Percentage of the maximum generated thrombin activity is plotted versus different molar ratios of peptide 1-41 or fragment 1 to prothrombin.

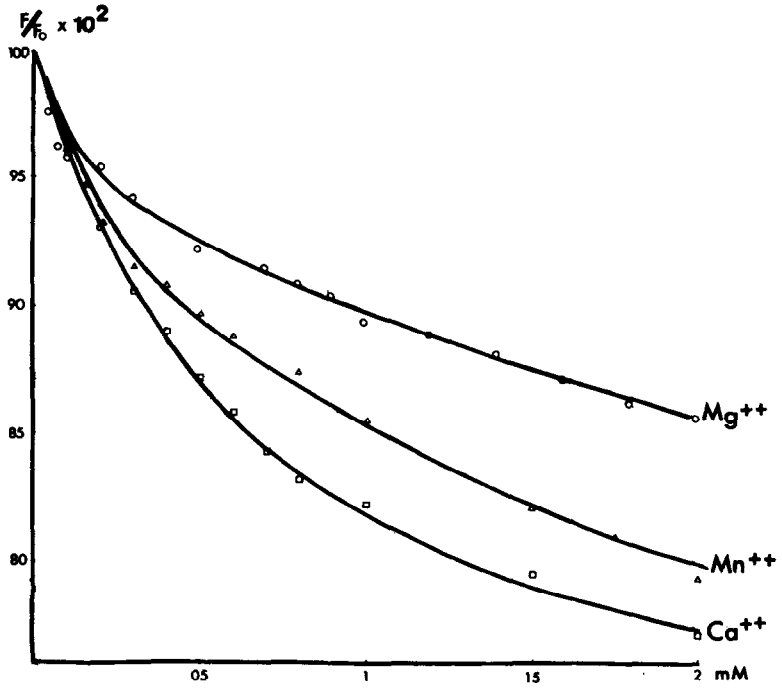

Figure 3. Effect of Ca^{++} (□), Mn^{++} (△) and Mg^{++} (○) on the intrinsic fluorescence of peptide 1-41. The ratio F/Fo, where F is the observed fluorescence and Fo the value in absence of divalent cation, is plotted versus cation concentrations (mM).

quenching, more pronounced in the case of F1, second the shape of the curve no longer sigmoidal for the peptide. Phospholipid increases the maximum quenching for peptide 1-41 by a factor of 1.7 and shifts the midpoind of the transition (Tm) from 0.34 to 0.46 mM Ca^{++}. In the case of F1, the maximum quenching is not appreciably affected but the transition is sharpened and the Tm shifted from 0.44 to 0.47 mM.

DISCUSSION

We previously reported the existence on the N-terminal part of human prothrombin of a region particularly exposed to proteolysis (13). From the results obtained with chymotrypsin, it is clear that this region extends at least from Phe 40 to Tyr 44. Chymotrypsin cleaves the molecule only in that region and gives rise to two products, peptide 1-41 and prothrombin (des 1-44).

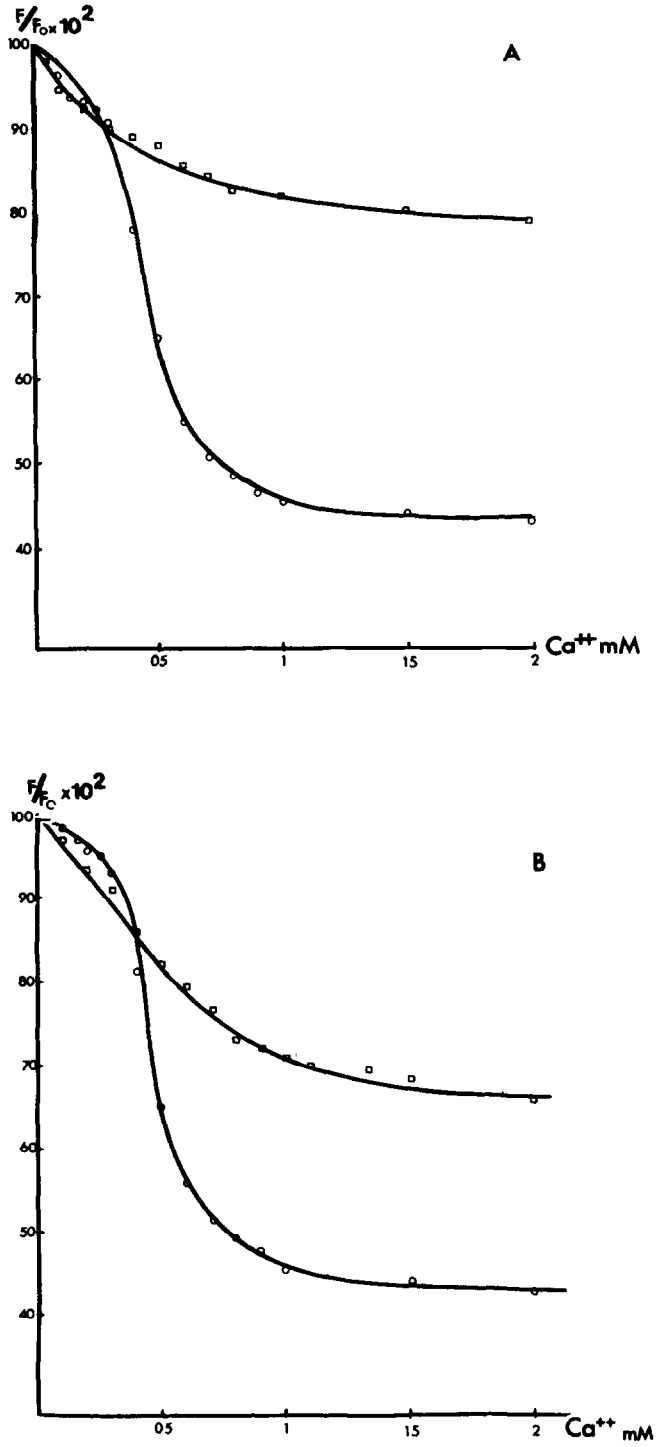

Figure 4. Ca^{++} titration curves by fluorescence quenshing of peptide 1-41 (□) and prothrombin fragment 1 (○) in the absence (A) and the presence of phospholipid.

Interaction of prothrombin with Ca^{++} (or other divalent cations), with or without phospholipid, has been studied on native prothrombin, F1 and tryptic peptide 12-44 (1,7-11,19). Peptide 1-41 presents the advantage of being a simpler model than prothrombin or F1 but still to retain the 10 Gla residues and the 17-22 disulfide bridge. It is as potent as F1 to inhibit prothrombin activation in the presence of Ca^{++} and phospholipid. This shows that peptide 1-41 competes with prothrombin for phospholipid binding. It has therefore retained some functionality and can be viewed as a functional domain in itself.

Fluorescence quenching has been used to monitor conformational transition occurring upon Ca^{++} binding to prothrombin and F1 (9,10,18). Ca^{++} also induces a quenching of the intrinsic fluorescence of peptide 1-41. Bovine peptide 12-44 has been reported to precipitate in the presence of Ca^{++}(7). Similar precipitation of peptide 1-41 however is unlikely to occur in the range of concentration of peptide and Ca^{++} used in this study. First, occurence of turbidity has not been observed. Second, Mn^{++} is much more powerful in precipitating peptide 12-44 (mid-point of precipitation, Tm = 2.5 mM) than Ca^{++} (Tm = 26 mM). In our study, Mn^{++} induces a fluorescence quenching to a lesser extent than Ca^{++}, and the two kinetics are very similar. Furthermore, Mg^{++} do not precipitate peptide 12-44 but induces a fluorescence quenching of peptide 1-41. Fluorescence quenching therefore must indicate a Ca^{++} dependent conformational transition of peptide 1-41. Fluorescence quenching of prothrombin and F1 has been suggested to reflect a change in the environment of Trp 41 (18). Since it is the only Trp residue in peptide 1-41 and although the kinetics of transition are different from those of F1, our data seem to corroborate this hypothesis.

These observations raise the question of the functional role of the remaining part of F1 (F1 des 1-44 = residues 45 to 155). Comparing this fragment with prothrombin fragment 2, the domain of interaction with FVa (16), shows 24 % of identical residues and identical location of the disulfide bridges (1). A major difference between peptide 1-41 and prothrombin or F1, is the lack of

cooperativity for Ca^{++} interaction. It is therefore likely that the cooperativity observed for Ca^{++} binding to prothrombin or F1 requires an additional process to be coupled to ion binding, process in which F1 (des 1-44) must be involved. The absence of chymotryptic cleavage at 10 mM Ca^{++} (13,14) further suggests that peptide 1-41 is not the only part of the molecule involved in the conformational transition of prothrombin induced by Ca^{++} ions.

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