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CHARACTERIZATION OF A PROTEOLYTICALLY MODIFIED FORM OF HUMAN PROTHROMBIN

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SUMMARY

Treatment of human prothrombin by α chymotrypsin results in the production of a single homogeneous product with an apparent M.W. of 69 000. This product fails to adsorb on barium citrate. NH_2-terminal sequence analysis shows that a single cleavage has occurred after tyrosine 44. The proteolytically modified prothrombin which can therefore be referred to as prothrombin (des 1-44), lacks the whole vitamin K dependent part of the molecule. The region of the peptide chain around tyrosine 44 must be particularly exposed to proteolytic attack and serve as a junction between the vitamin K dependent domain and the other structural domains of prothrombin. In the presence of factor Xa alone, prothrombin (des 1-44) is indistinguishable from normal prothrombin when activation is monitored by the appearance of amidolytic activity on S2238. However, activation of prothrombin (des 1-44) is no longer enhanced by the presence of Ca^{++} and phospholipid in the activation mixture.

INTRODUCTION

Prothrombin is converted into thrombin within the so called prothrombinase complex, comprising three different proteins : the substrate, prothrombin, a protease, factor Xa and a cofactor, factor Va. It is thought to take place on a small number of specific sites, including phospholipid , present on the surface of activated platelets (1). Upon activation, the NH_2-terminal half of the molecule is cleaved in two fragments : prothrombin fragment 1 and prothrombin fragment 2. The COOH-terminal half corresponds to prethrombin 2, the immediate precursor of thrombin (2, 3). Each fragment exhibits a highly specific function and therefore corresponds to a functional domain within the molecule. Prothrom-

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bin fragment 2 has been shown to be the site of interaction with factor Va, and fragment 1 is responsible for the calcium-dependent interaction with phospholipid. The vitamin K-dependent calcium binding sites on fragment 1 are related to the presence of 10 γ-carboxyglutamic acids (Gla) amongst the 40 NH_2-terminal residues of fragment 1 (4-6).

This paper describes the characterization of a form of prothrombin proteolytically modified by α chymotrypsin, prothrombin (des 1-44), in which the vitamin K-dependent region no longer exists.

MATERIALS AND METHODS

- *Purification*
Human prothrombin was purified from plasma according to Bajaj and Mann (7) except that the last step of purification was a DEAE Sephadex chromatography according to Morrison and Esnouf (8).
- *Proteolytic digestion*
Prothrombin was incubated for different lengths of time with TLCK-treated α chymotrypsin (Boehringer) in a 0.02 M $NH_4H\ CO_3$ pH 8.6 buffer, at 37 °C. The enzyme/substrate ratio was 1/1000.
- *Electrophoresis*
Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed according to Laemmli (9). The concentration of polyacrylamide was 12 % and the acrylamide/bis acrylamide ratio 35/1 .
- *Adsorption of the hydrolysis products on barium citrate*
It was tested in the following manner : 0.28 ml of 1 M $BaCl_2$ per ml of solution were added dropwise at 4 °C to the protein solution (0.14 mg/ml in 0.02 M tri Na citrate ; 9 ‰ NaCl) and the mixture was left to stand 15 min. It was then centrifuged and the precipitate was dissolved in 0.02 M EDTA pH 7.4. Precipitate and supernatant were analysed by SDS gel electrophoresis.
- *Activation by bovine F Xa* was performed at 37° C in 0.025 M Tris 0.15 M NaCl, pH 7.4 at a protein concentration of 0.1 mg/ml. Russels Viper venom-activated bovine F Xa was obtained from Sigma and utilized at a concentration of 5U/mg of prothrombin. Calcium was used at a final concentration of 2 mM and phospholipid (crude human brain cephalin preparation) at a final concentration of 40 μg/mg of prothrombin.
- *Thrombin amidolytic activity* was tested using the synthetic substrate H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide Bichlorhydrate(S-2238) Kabi Diagnostica, according to Svendsen et al.(10).
- *N-terminal amino acid sequences* were determined by the automated method of Edman and Begg (11) with a Beckman Model 890 C automated protein sequencer using the 0.1 M Quadrol program. Amino acid phenylthiohydantoin derivatives were identified by high performance liquid chromatography (12, 13).

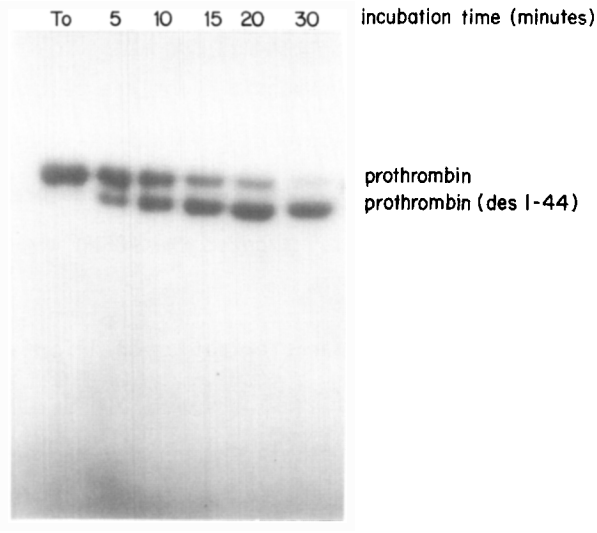

Figure 1 : Digestion of human prothrombin by α chymotrypsin. Analysis of the products by SDS polyacrylamide gel electrophoresis.

RESULTS

Digestion of human prothrombin by α chymotrypsin gives rise to a single product with an apparent molecular weight of 69 000 (fig.1) showing no amidolytic activity on the synthetic substrate S-2238. This pattern is not modified by reduction indicating that the product of proteolysis corresponds to a single chain polypeptide. Prolonged incubation of prothrombin with α chymotrypsin (up to 6 hrs) or with higher enzyme/substrate ratios (up to 1/100) results in no other changes in the cleavage pattern.

A similar product of proteolysis with an identical behavior in SDS-electrophoresis is observed upon treatment of human prothrombin by elastase, thermolysin, subtilisin and pronase in conjunction with other degradation products (results not shown). The site of chymotryptic cleavage must therefore lie on a particularly exposed region at the surface of the molecule. It is to be noted that Ca^{++} at a final concentration of 10 mM almost totally prevents cleavage by α chymotrypsin.

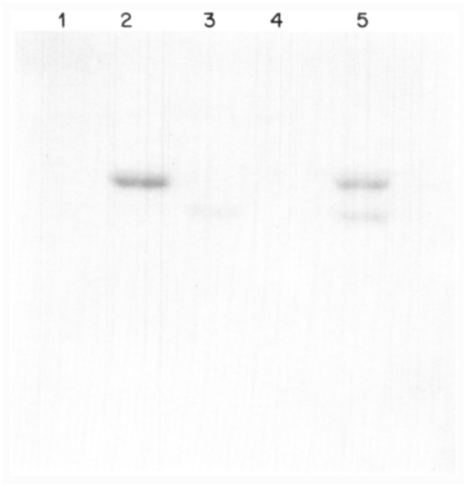

Figure 2 : Adsorption of prothrombin and prothrombin (des 1-44) on barium citrate.
Precipitates and supernatants were analysed by SDS polyacrylamide gel electrophoresis. Normal prothrombin supernatant (1), and precipitate (2). Prothrombin (des 1-44) supernatant (3) and precipitate (4).(2)+(3) (5).

Ability of the product of chymotrypsin digestion to adsorb onto barium citrate has been tested. Fig. 2 shows that this product does not adsorb and remains in the supernatant, whereas prothrombin is found in the precipitate. Since this property has been related to the γ-carboxyglutamic acid residues (Gla), proteolytically modified prothrombin must have lost a part or the totality of the Gla containing region.

The NH_2-terminal aminoacid sequence of this product has been determined for the first seven residues and shown to correspond to residues 45 to 51 of human prothrombin. Chymotryptic cleavage therefore has taken place at the tyrosine 44 - threonine 45 bond, and the product of proteolysis can be referred to as prothrombin (des 1-44).

Since prothrombin (des 1-44) lacks the whole Gla region which is considered to be responsible for the calcium-dependent interaction with phospholipid, its activation by factor Xa was tested in the presence or absence of Ca^{++} and phospholipid and monitored

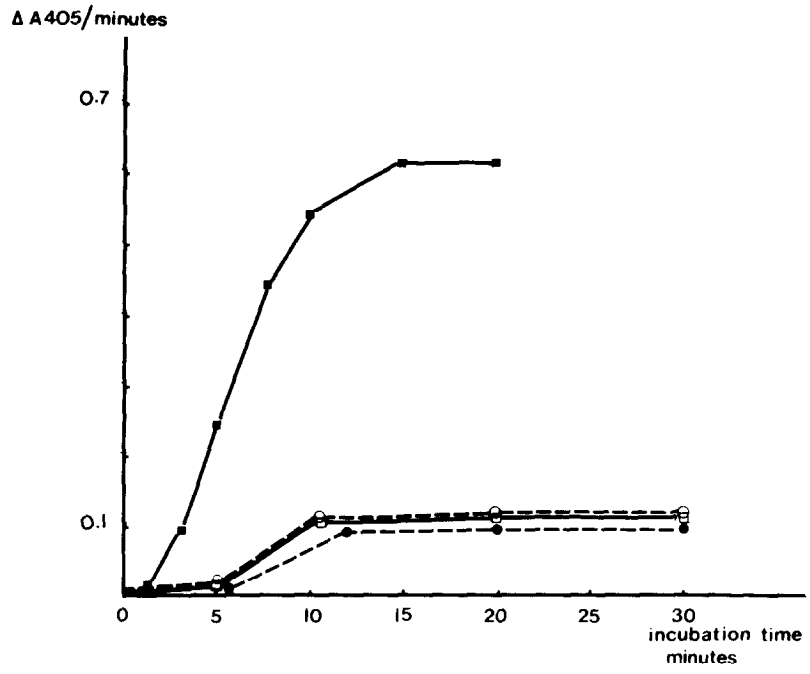

Figure 3 : Generation of thrombin activity was monitored by the appearance of amidolytic activity on S2238.
Prothrombin□-□ and prothrombin (des 1-44)o-o in the presence of factor Xa alone. Prothrombin ■-■ and prothrombin (des 1-44) ●-● in presence of factor Xa plus phospholipid and calcium.

by the appearance of amidolytic activity on S2238 (fig. 3). No difference in thrombin generation is observed between prothrombin and prothrombin (des 1-44) when activation is performed in the presence of factor Xa alone. In the presence of Ca^{++} and phospholipid however activation is no longer enhanced in the case of prothrombin (des 1-44). In fact activation is even slightly but reproducibly reduced, indicating that factor Xa bound to phospholipid is less active on prothrombin (des 1-44) than factor Xa in solution. These observations are consistent with the absence of binding of prothrombin (des 1-44) to phospholipid.

DISCUSSION

Evidence has now accumulated for the division of the prothrombin molecule in three different structural domains corres-

ponding to the functional domains. Among several arguments is the observation of a high degree of internal sequence homology between two different functional domains prothrombin, fragments 1 and 2. This suggests the existence of a certain degree of similarity in the three-dimensional structure of the two fragments and has led to the hypothesis of a gene duplication during the evolution of the prothrombin molecule (14-16). The NH_2-terminal portion of fragment 1 however (50 residues, containing the 10 Gla residues) has no equivalent in fragment 2, and, conversely, fragment 2 possesses 13 additional residues on the COOH-terminal. Prothrombin (des 1-44) lacks the NH_2-terminal vitamin K-dependent region which must be attached to the remaining part of fragment 1 by a portion of the peptide chain particularly exposed to proteolysis by α chymotrypsin and some other proteolytic enzymes showing specificity for apolar residues.

A similar observation has been recently reported by Morita and Jackson for bovine factor Xa (17). Treatment by α chymotrypsin results in a single cleavage at tryptophane 41 on the light chain of factor Xa and the resulting product therefore also lacks the vitamin K-dependent region. In fact a very high degree of homology exists among the NH_2-terminal portions of all the vitamin-K dependent factors. 29 residues are identical in the first 44 amino acids of bovine factor Xa light chain and bovine or human prothrombin.

This vitamin K-dependent region contains a disulfide bridge (cysteine 17 - cysteine 22) and might constitute a small structural domain in itself.

The isolated vitamin K-dependent domain and prothrombin (des 1-44) will most certainly be extremely useful products in the study of the calcium-dependent interaction of prothrombin

with phospholipid, and of the function of the remaining portion of fragment 1.

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