PURIFICATION AND PARTIAL CHARACTERIZATION OF A NEW VARIANT OF HUMAN PROTHROMBIN: PROTHROMBIN METZ

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1. Introduction

Ten cases of human prothrombin variants have been reported up to now. One of the last was prothrombin Metz [1] in a French family. The mother is heterozygous for the abnormal prothrombin and the father heterozygous for true hypoprothrombinemia. The 4 possible genetic combinations are realized among the 8 children. Three of them, presenting a mild bleeding tendency, are double heterozygotes for prothrombin Metz and the hypoprothrombinemia and therefore bear only the abnormal molecule. This case constitutes the third observation of such an association [2,3].

At the present time 3 of the prothrombin variants have been fully purified and characterized. In the cases of prothrombin Barcelona [4–6] and prothrombin Madrid [7] the molecular defect has been shown to be a specific impairment of one of the two factor Xa-catalyzed cleavages, whereas in the case of prothrombin Quick [8] the abnormality lies in the thrombin part of the molecule.

Here we present the purification and partial characterization of prothrombin Metz. The purified material is indistinguishable from normal prothrombin by all the studied physicochemical criteria. However, activation by factor Xa in purified system generates only amidolytic activity but no clotting activity. An abnormal cleavage pattern is observed which is different from those described for prothrombin Barcelona and prothrombin Madrid.

A preliminary report of this study has been presented at the 7th Int. Cong. Thrombosis and Haemostasis, July 1979, London [20]

2. Material and methods

For comparison purposes, normal prothrombin and prothrombin Metz were purified in parallel, starting from small quantities of plasma (30–60 ml), using barium citrate adsorption/elution, ammonium sulfate precipitation [9] and DEAE–Sephadex chromatography [10]. Prothrombin was assayed by double immunodiffusion [11] and quantitated by the Laurell method [12] using specific anti-human prothrombin antibodies.

Urea–SDS gel electrophoresis was performed according to [13] in 12.5% acrylamide with a 40/1 acrylamide bisacrylamide ratio. Disc gel electrophoresis was carried out at pH 8.9 according to [14,15] and at pH 7.2 and 5.5 according to [16].

The N-terminal residue was determined by dansylation [17].

Factor Xa was obtained from Sigma. Crude human brain cephalin was used as source of phospholipids. Activation of prothrombin was performed in Tris 0.025 M, NaCl 0.15 M (pH 7.4). Thrombin activity was measured by clotting assay [18] and spectrophotometrically using the synthetic substrates S_{2160} and S_{2238}(KABI) [19].

3. Results and discussion

3.1. *Purification and physicochemical characterization*

For the purification of prothrombin Metz, advantage has been taken of the existence in the family of 3 siblings who, being double heterozygotes for pro-

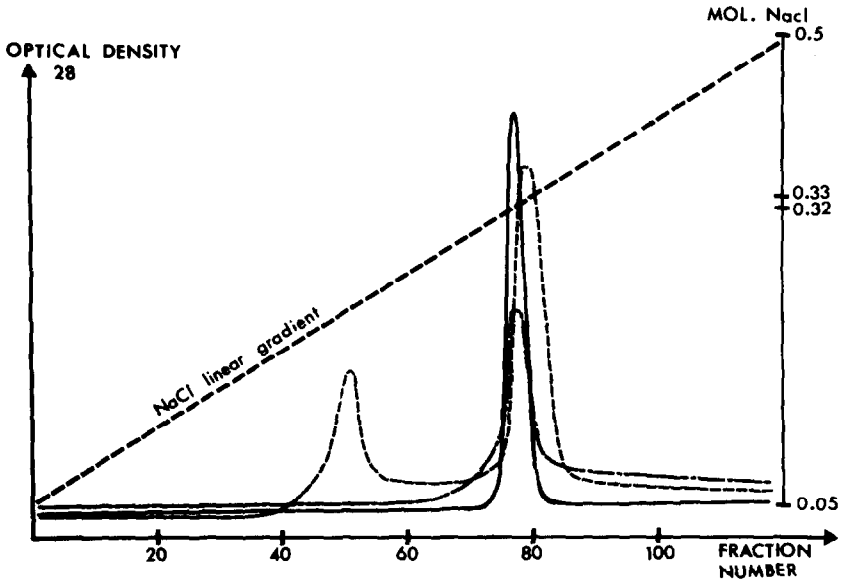

Fig.1. Elution pattern of normal and abnormal prothrombins from DEAE–Sephadex. The column (1.5 × 30 cm) was eluted at a 10 ml/h flowrate with a linear gradient of NaCl (0.05–0.5 M) in Tris–citrate buffer 0.05 M (pH 6.5) (200/200 ml). Normal prothrombin (——); prothrombin Barcelona (– – –); prothrombin Metz (– · – ·).

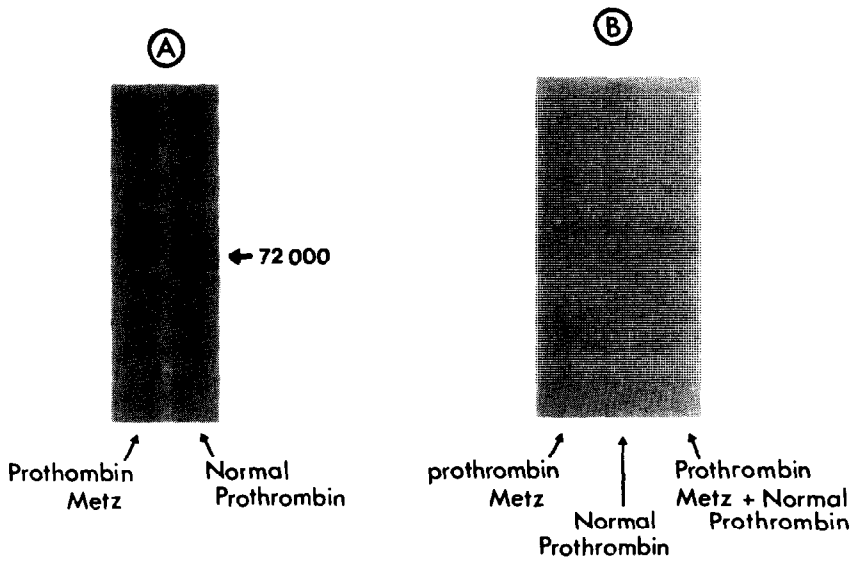

Fig.2. Polyacrylamide gel electrophoresis of normal and abnormal prothrombins. (A) Urea–SDS gel electrophoresis. (B) Disc-gel electrophoresis at pH 8.9 (12% acrylamide, height 10 cm) performed during prolonged time (2 h at 2 mA/gel after the marker reached the end of the gel).

thrombin Metz and hypoprothrombinemia, have no normal prothrombin. Prothrombin Metz is totally adsorbed onto barium citrate, as shown by immunological assay. The usual technique for prothrombin purification has therefore been used to isolate the abnormal molecule. One single symmetrical peak containing prothrombin is obtained from the DEAE–Sephadex column eluted at the same ionic strength as normal prothrombin (fig.1). This result is in contrast with what is observed with Barcelona plasma where two peaks are obtained, a slightly delayed prothrombin Barcelona peak and a second one containing prothrombin crossreacting material.

Pure material is obtained as shown by SDS–gel electrophoresis (fig.2A) which reveals a single band with the same mobility as normal prothrombin corresponding to mol. wt 72 000. As for normal prothrombin, alanine is the only N-terminal residue revealed by dansylation. No charge difference is found by gel electrophoresis in a native system neither at pH 8.9 (fig. 2B) nor at pH 7.2 and 5.5.

Prothrombin Metz therefore is indistinguishable from normal prothrombin by the studied physicochemical criteria. In comparison prothrombin Barcelona exhibits a more anionic mobility at alkaline pH.

3.2. *Biological activity*

Upon activation by factor Xa, in the presence of Ca^{2+} and phospholipids, prothrombin Metz generates in 60 min < 4% of the clotting activity observed with normal prothrombin (fig.3A).

However amidolytic activity on S_{2238} and S_{2160} (not shown) is generated (fig.3B) with an initial rate identical to that of normal prothrombin but reaching a plateau at 70% of the normal maximum activity. This at least shows that the factor Xa-catalyzed cleavage, responsible for the unmasking of the active site, has occurred, leading to some form(s) of thrombin showing no activity toward fibrinogen and with modified catalytic properties toward small synthetic substrates.

3.3. *Cleavage pattern*

The cleavage pattern of prothrombin Metz by factor Xa has been monitored by SDS–gel electrophoresis (fig.4). Two main abnormal features are observed.

(1) Prethrombin 1 (P_1) appears as for normal prothrombin for short incubation times, but remains the major species at 120 min.

(2) An abnormal intermediate with app. mol. wt 25 000 is formed.

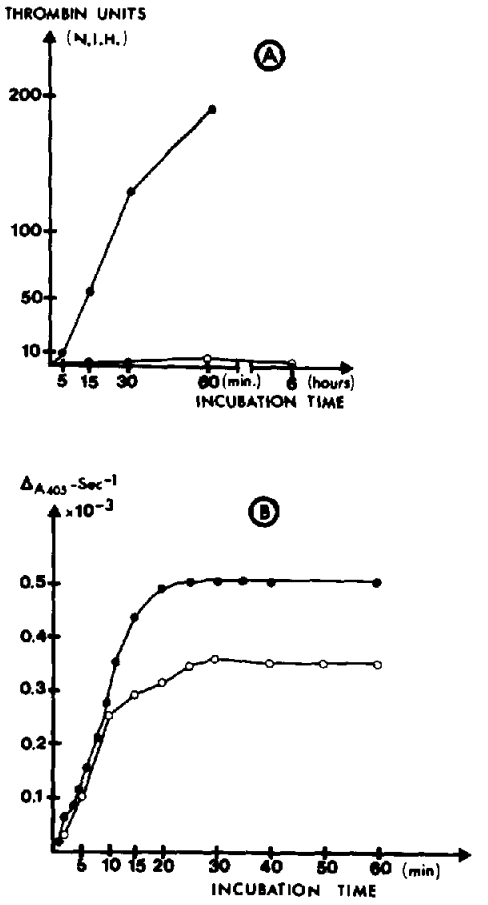

Fig.3. Generation of thrombin activity upon activation of prothrombin Metz (o—o) and normal prothrombin (•—•) by factor Xa (5 U/mg prothrombin) in the presence of Ca^{2+} (2 mM) and phospholipids (40 μg/mg prothrombin). (A) Clotting activity. (B) Amidolytic activity on S_{2238} (B_Z–Phe–Pip–Arg–*p*-nitroanilide).

The scanning of the gels clearly shows that in the case of normal prothrombin after 60 min incubation, P_1 decreases concomitantly with an increase of prethrombin 2 whereas the amount of P_1 remains constant for prothrombin Metz. These results show that the cleavage between F_1 and P_1 occurs normally. Furthermore, this cleavage being catalyzed by throm-

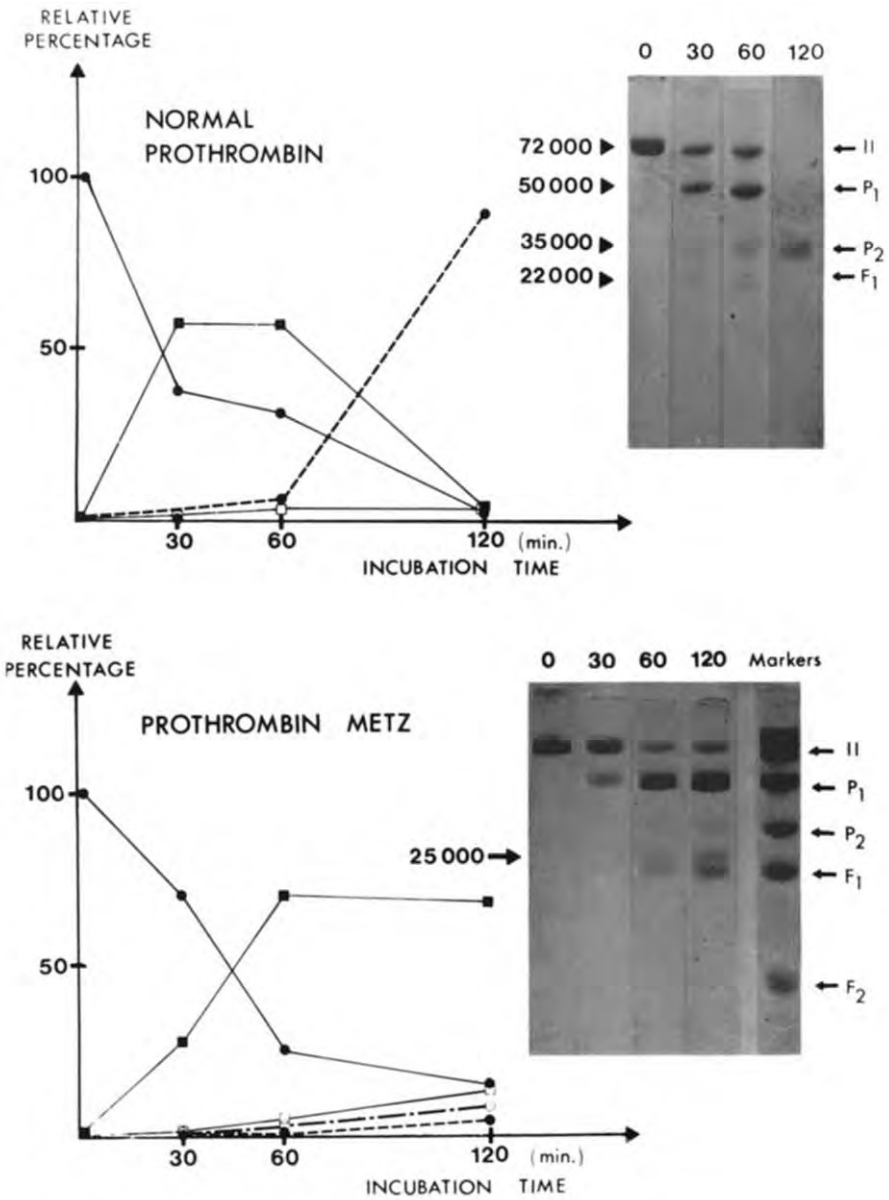

Fig.4. Cleavage pattern of normal prothrombin and prothrombin Metz by factor Xa. The products of proteolysis have been analysed by urea–SDS gel electrophoresis and their relative percentage measured by scanning of the gels. Prothrombin (II, •—•); prethrombin 1 (P1 ▪—▪); prethrombin 2 (P2 •– –•); fragment 1 (F1 □—□); abnormal additional fragment 25 000 (○–.–.–○). All samples are unreduced.

bin, one may conclude that the abnormal form(s) of thrombin Metz still recognizes prothrombin as a substrate. The nature of the 25 000 mol. wt product is not clear at the present time. One possibility to be tested is to check if this abnormal intermediate might correspond to a degraded form of thrombin analogous to β thrombin with no clotting activity and a reduced activity on synthetic substrates.

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