ABNORMAL ACTIVATION OF A HUMAN PROTHROMBIN VARIANT: PROTHROMBIN BARCELONA

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1. Introduction

Prothrombin Barcelona is a variant of human prothrombin described in four siblings of a Spanish family [1]. In homozygous patients' plasma, this variant is characterized by a very low functional activity but normal prothrombin level by immunochemical and staphylocoagulase assays.

Prothrombin Barcelona which is able to bind calcium and to be adsorbed onto barium citrate has been purified. It was demonstrated to have the same molecular weight but a slightly more anodic electrophoretic mobility than normal prothrombin [2]. The purified variant is converted by prothrombinase complex at a much lower rate than normal prothrombin.

The assumption was made that this slow activation rate could be due to the impairment of one of the proteolytic cleavages known to occur in the normal activation process.

Experiments using various activation systems were performed in order to test this hypothesis, and their results suggest a specific defect in the cleavage by factor Xa between fragment 2 (F_2) and prethrombin 2 (P_2).

2. Materials and methods

Normal prothrombin was purified from human plasma using barium citrate adsorption/elution and ammonium sulfate precipitation as in [3] and DEAE Sephadex A50 chromatography as in [4]. Prothrombin Barcelona was purified as in [2]. Factor Xa was prepared from bovine factor X [3] and human thrombin from purified prothrombin [5]. Crude human brain cephalin preparation was used as phospholipid source. Hirudin (Lab. STAGO) was used at the final concentration of 200 AT U/mg prothrombin.

Generation of thrombin clotting activity was measured by a two stage assay as in [6]. Treatments of prothrombin by thrombin and by Xa were separately performed as in [7]. The appearance of the products of proteolysis was monitored by acrylamide–SDS gel electrophoresis as in [8].

The esterase activity was tested spectrophotometrically on TAMe (*p*-toluenesulfonyl L arginine methyl ester) as in [9].

3. Results and discussion

In the present study, prothrombin activation was tested using various partial activation systems (legend fig.1). In the presence of Xa, Ca^{2+} and phospholipids, no thrombin clotting activity was generated within 4 h, a minute amount of such an activity being found later.

The cleavage of prothrombin by thrombin (fig.2) gave rise to the appearance of prethrombin 1 (P_1) and fragment 1 (F_1). It was found to be roughly the same for both normal and abnormal proteins with a slight delay in the latter case. The disappearance of prothrombin was complete within 1 h in the case of prothrombin Barcelona. Thus the sensitivity of the variant molecule to the action of thrombin can be considered to be fairly normal.

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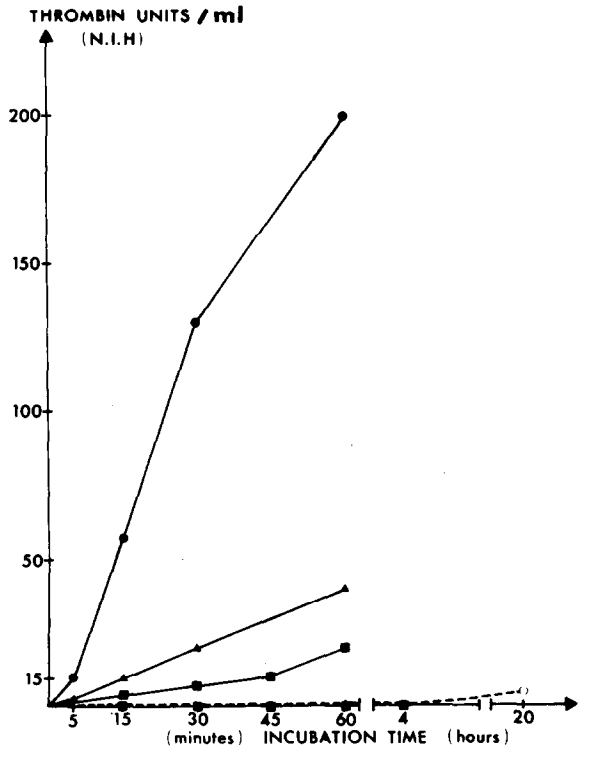

Fig.1. Activation of prothrombin by factor Xa (5 U/mg prothrombin): generation of thrombin-clotting activity. Normal prothrombin is represented in continuous line and prothrombin Barcelona in dashed line. Activation system: (□——□) Xa alone; (△——△) Xa + 2 mM calcium; (○——○) Xa, calcium + phospholipids 40 μg/mg prothrombin.

Conversely the proteolytic cleavage by factor Xa (fig.3) was found to be abnormal in prothrombin Barcelona. When compared to the control, the main difference observed was the absence of P_2 after 1h, and simultaneously a persistence of a prothrombin band and an accumulation of P_1. After 2 h prothrombin disappeared, P_1 was still accumulating and a faint band could be seen migrating like P_2. Its significance is not clear; it could be due either to a very late cleavage by Xa or to the second thrombin cleavage which occurs 13 residues further as shown [7] to be specific of human prothrombin. The F_2–P_2 cleavage seems therefore to be specifically impaired in prothrombin Barcelona. P_1 is generated from normal prothrombin by a thrombin cleavage between F_1 and P_1. Therefore the accumulation of P_1 during the activation of prothrombin Barcelona is in discrepancy with the absence of any thrombin activity detectable by clotting assay (fig.1). Then prothrombin activation by Xa was performed in the presence of hirudin, a specific thrombin inhibitor. No formation of P_1 was observed and in the case of prothrombin Barcelona, the prothrombin band remained unchanged. Finally the generation of an esterase activity was checked on

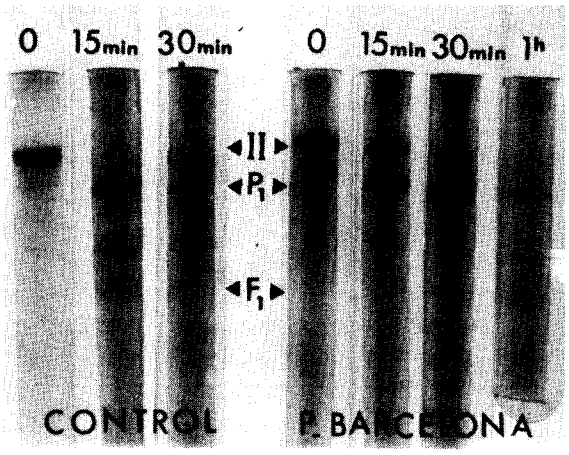

Fig.2. Cleavage of prothrombin by thrombin (10 NIH units/mg prothrombin). Identification of products formed by SDS–polyacrylamide gel (10%) electrophoresis. II, prothrombin; P_1, prethrombin 1; F_1, fragment 1.

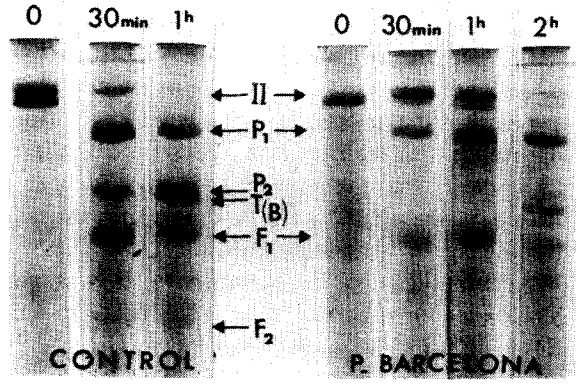

Fig.3. Activation of prothrombin by factor Xa (5 U/mg prothrombin). SDS–polyacrylamide gel (10%) electrophoretic identification of the intermediates of activation with disulfide bonds reduced. P_2, prethrombin 2; T_B, thrombin chain B.

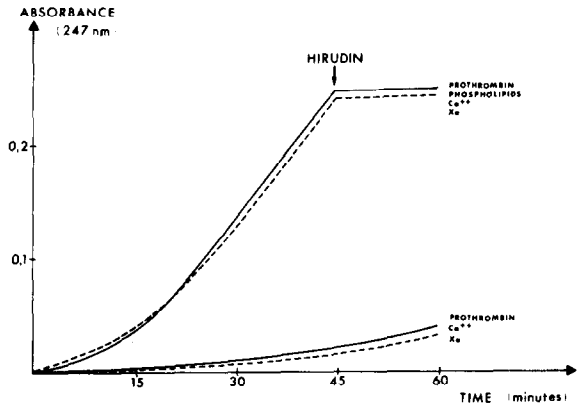

Fig.4. Esterase activity on TAMe during the activation of prothrombin by factor Xa. Normal prothrombin is represented in continuous line and prothrombin Barcelona in dashed line. Prothrombin 0.1 mg/ml; Xa 5 U/mg prothrombin; Ca^{2+} 10 mM; phospholipids 40 μg/mg prothrombin. TAMe 10^{-3} M in buffer 0.04 M Tris, 0.01 M $CaCl_2$, pH 8.1. Hirudin 200 AT units/mg prothrombin was added after 45 min incubation.

TAMe as a result of the treatment of prothrombin by factor Xa (fig.4). In these conditions, the generated activity was identical with both normal and abnormal prothrombin and was similarly inhibited by hirudin.

Two main abnormal features therefore characterize the activation of prothrombin Barcelona by factor Xa:

(i) The absence of the F_2P_2 Xa-catalyzed cleavage.

(ii) The generation of a thrombin-like activity responsible for the F_1P_1 cleavage and for the esterase activity, inhibited by hirudin, but without any activity toward fibrinogen.

No definite mechanism can be ascertained up to now, but a possible hypothesis can be advanced comparing our results with the known activation process of prothrombin by snake venoms. In these cases the generation of high molecular weight intermediates with thrombin activity has been shown [10,11]. These observations indicate that the generation of thrombin activity requires cleavage between the two chains of thrombin but not the removal of the 'pro' part of the molecule (F_1 and F_2). Similarly the thrombin-like activity observed during the activation of prothrombin Barcelona by Xa could be related to a single Xa catalyzed cleavage between the two chains of thrombin, without the F_2P_2 cleavage, giving rise to an active high molecular weight intermediate.

Acknowledgements

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