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PURIFICATION AND CHARACTERIZATION OF A VARIANT OF HUMAN PROTHROMBIN: PROTHROMBIN SEGOVIA

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Abstract: A dysprothrombin designated prothrombin Segovia was isolated from the plasma of an individual with normal prothrombin antigen and prothrombin activity lesser than 25% of the control prothrombin activity. Activation by prothrombinase complex showed a lower amidolytic than clotting activity, which suggests a lesser generation of active intermediates than normal prothrombin. When prothrombin Segovia was activated by prothrombinase complex in the absence of factor Va, no thrombin formation was found by functional activities. SDS-PAGE analysis of the molecules derived by activation with prothrombinase complex, Taipan snake venom and *Echis carinatus* venom showed an accumulation of molecules not cleaved at bond Arg320-Ile321. This was more evident with *Echis carinatus* venom, which only acts on this bond. Our data suggest that the alteration of prothrombin Segovia impairs the scission of bond Arg320-Ile321.

Dysprothrombinemias represent one of the most uncommon coagulation defects; so far, 21 variants, all of them characterized by a decrease in the functional level of prothrombin compared to the immunologically detectable prothrombin (1), have been reported (2-7).

The conversion of human prothrombin to thrombin requires two cleavages (8): one at bond A (Arg271-Thr272), that splits prothrombin into fragment 1+2 (F1+2) and prethrombin 2 (P2), and another at bond B (Arg320-Ile321), which exposes the active site (9) and yields the disulfide-linked two-chain (A and B chains) enzyme thrombin. These cleavages can be produced in the opposite order, yielding first the disulfide-linked two-chain active intermediate meizothrombin (MT), and then, thrombin. There are also two more peptide bonds susceptible to autocatalysis; bond C (Arg155-Ser156), and bond D, (Arg286-Thr287). Cleavage of bond C from prothrombin produces prethrombin

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1 (P1), and from MT produces meizothrombin-desF1 (MT-desF1), another disulfide linked two chain active intermediate (10, 11).

Eleven of the prothrombin variants have been purified and characterized (2, 12). Prothrombins Barcelona and Madrid, have a functional defect affecting bond A; prothrombin Clamart has a functional defect affecting bond B; prothrombin San Juan is thought to have a calcium binding defect, and finally, an abnormality of the thrombin active site has been demostrated for prothrombins Molise, Quick, Metz, Salakta, Tokushima, Habana and Himi (2, 7, 13, 14).

The specific molecular defect has been identified as a single amino-acid substitution in the dysprothrombinemias Barcelona and Madrid (Arg271→Cys) (15, 16), Tokushima (Arg418→Trp) (17, 18) and Salakta (Glu466→Ala) (19) and as a double amino-acid substitution in protrombins Quick (Arg382→Cys and Gly588→Val) (20, 21) and Himi (Met337→Thr and Arg388→His) (22), which are the two unique cases known to be compounds heterozygous for two dysfunctional prothrombin molecules.

Prothrombin Segovia (1), characterized by abnormally low levels of plasma prothrombin activity using both physiologic activators as well as Russell, *Notechis scutatus* or *Echis carinatus* venoms, showed a more cathodic mobility in plasma immunoelectrophoresis than normal prothrombin, and had in the bidimensional electrophoresis of the propositu's serum three peaks instead of the normal two. These data suggested that prothrombin Segovia was a new dysfunctional prothrombin molecule. Therefore, the aim of this work was its purification and functional characterization.

MATERIAL AND METHODS

Blood was collected from the proband using plastic syringes that contained 1/10 (v:v) of 3.8% trisodium citrate. Platelet-poor plasma was obtained after centrifugation at 2500 g for 30 minutes at 4°C and stored a -80°C.

Prothrombin obtained from the already refered (1) heterozygous patient and named, for the remaining of this manuscript, as prothrombin Segovia, was isolated by barium citrate adsortion/elution, ammonium sulphate precipitation (23) and DEAE Sephadex (Pharmacia) chromatography (24). It was then studied by immunoelectrophoresis performed according to Scheidgger et al (25), bidimensional electrophoresis carried out according to a modification (26) of the Clarke and Freeman method (27), and SDS-PAGE according to Weber and Osborn (28).

Prothrombin activation:
Experiments with prothrombin Segovia were done in parallel with control prothrombin, using purified bovine factors Xa and V (Diagen), rabbit brain cephaline (Sigma), *Echis carinatus* and *Oxyuranus scutellatus* (Taipan snake) venoms (Sigma).

Thrombin clotting activity was determined by its ability to clot human purified fibrinogen (Kabi-Vitrum), using human thrombin (Roche) for the calibration curve. Amidolytic activity of thrombin and thrombin-like enzymes was determined by following spectrophotometrically the hydrolysis of the chromogenic substrate S-2238 (H-D-Phe-Pip-Arg-pNA) (Kabi-Vitrum) at 405 (29).

Prothrombin activation products were identified by SDS-PAGE with and without dithiothreithol (DTT), in order to identify the following molecules: prothrombin and MT (72000), P1 and MT-desF1

(57000) and P2 and thrombin (38000). When *Echis carinatus* venom was used as activator, prothrombin activation products were analyzed by Western blot (30, 31). Determination of F1+2 was performed according to the method of Pelzer (32) using a commercially available immunoassay (Behring).

The incubation mixtures were the following:
a. Prothrombinase complex: prothrombin (4.2-8.4 μg/ml) was incubated with factor Xa (0.02 U/ml), factor V (0.012 U/ml) and rabbit brain cephalin (1:5 v/v) in 0.1 M Tris, 0.1 M NaCl, 0.01 M $CaCl_2$, pH=7.4. In absence of factor V, the concentration of factor Xa was 2 U/ml. In SDS-PAGE and F1+2 determination, prothrombin (25 μg/ml) was incubated with factor Xa (0.35 U/ml), factor V (0.024 U/ml) and rabbit brain cephalin (1:3.5 v/v) in 0.1 M Tris, 0.1 M NaCl, 0.04 M $CaCl_2$, pH=7.4.

b. Taipan snake venom: prothrombin (2.1-4.2 μg/ml) was incubated with the venom (0.2 μg/ml) and rabbit brain cephalin (1:10 v/v) in 0.05 M Tris, 0.1 M NaCl, 0.01 M $CaCl_2$, pH=7.4. In SDS-PAGE and F1+2 determination, prothrombin (50 μg/ml) was incubated with the venom (16 μg/ml) and rabbit brain cephalin (1:9 v/v) in 0.05 M Tris, 0.1 M NaCl, 0.04 M $CaCl_2$, pH=7.4.

c. *Echis carinatus* venom: prothrombin (4.2-8.4 μg/ml) was incubated with the venom (2.5 μg/ml) in 0.02 M Tris, 0.1 M NaCl, pH=7.4. In SDS-PAGE and F1+2 determination, prothrombin (60 μg/ml) was incubated with the venom (35 μg/ml) in the above buffer.

RESULTS

After the adsorption of propositu's plasma onto barium citrate and the elimination of most plasma proteins, two peaks were obtained from the DEAE-Sephadex column. The second of them containing prothrombin Segovia, similary to normal prothrombin, eluted at the same ionic strength, migrated as a single band, had the same mobility in SDS-PAGE and its molecular weight was estimated as 72000 (fig. 1). Nevertheless, the immunoelectrophoretic mobility of this prothrombin was more cathodic than that of normal prothrombin, already confirmed by bidimensional electrophoresis.

Activation of the propositu's purified prothrombin, (prothrombin Segovia), by the prothrombinase complex showed a delay of thrombin production, determined by clotting as well as amidolytic activity (fig. 2). The thrombin activity generated by prothrombin Segovia was 85% of the normal activity upon fibrinogen and 35% upon synthetic substrate S-2238. However, similary to normal prothrombin, prothrombin Segovia reached maximum amidolytic activity faster than maximum clotting activity. When the activation was carried out in absence of factor Va, no clotting activity appeared from prothrombin Segovia and amidolytic activity reached a plateau at less than 5% of the normal maximum activity (fig. 2). The rate of F1+2 production by prothrombin Segovia was similar to that of normal prothrombin (fig. 3a). Although the cleavage pattern of both prothrombins on SDS-PAGE was similar, the appearance of 57,000 (P1 or MT-desF-1) and 38,000 Da (P2 or thrombin) fragments occurred later in prothrombin Segovia. DTT reduction had a more intensive effect on the activation of normal prothrombin fragments producing B chain, while on prothrombin Segovia, only a decrease of intensity of the fragments was seen (data non shown).

After activation with Taipan snake venom, Segovia' and normal prothrombins reached clotting and amidolytic activity plateaus at the same time (45 and 20 minutes respectively) (fig. 4) but the former generated 36% and 42.8% of clotting and amidolytic activity in relation to the latter. F1+2 production was similar to that of normal prothrombin, and greater than with the rest of activators (fig. 3b).

Cleavage pattern by SDS-PAGE showed a delay in prothrombin Segovia activation and an accumulation of prothrombin, P1 and P2 non susceptible to reduction.

Activation of prothrombin Segovia with *Echis carinatus* venom was very impaired. As shown in fig. 4, the production of thrombin from prothrombin Segovia, measured by clotting as well as amidolytic activity, was less than 5% of the produced by normal prothrombin, even after one hour incubation. F1+2 concentration was one-fifth that generated in normal prothrombin (fig. 3c). Very slow activation of prothrombin Segovia was observed in SDS-PAGE, because while the formation of thrombin was clearly appreciated in normal prothrombin, it was not observed in prothrombin Segovia. After reduction, the intermediate fragments of normal prothrombin activation produced B chain, while in prothrombin Segovia there was a decrease in the intensity of transitory products, without B chain formation (fig. 5). Nevertheless, the cleavage pattern of prothrombin Segovia activation studied by Western blot, showed minute thrombin formation in the last periods of incubation (fig. 6).

DISCUSSION

The fact that there is less amidolytic than clotting activity in prothrombin Segovia after activation with prothrombinase complex, suggests that the molecule generates less active intermediates than normal prothrombin. This can be explained because while the action on substrate S-2238 is produced by active intermediates (MT and MT-desF1) (33) as well as thrombin, the clotting activity is only generated by thrombin (13). Moreover, the great resistance of prothrombin Segovia to reduction of intermediates, points to an accumulation of intermediates lacking a cleaved bond B (Arg320-Ile321), and therefore, not expressing the active site. However, the clotting activity and the formation of the 57000 Da intermediate, which is a product of autolysis (34), shows thrombin formation, although in a lesser degree than in normal prothrombin.

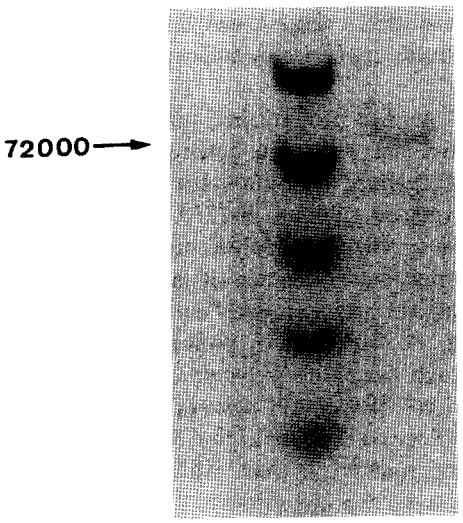

FIG. 1

Polyacrilamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) of purified and prothrombin Segovia.

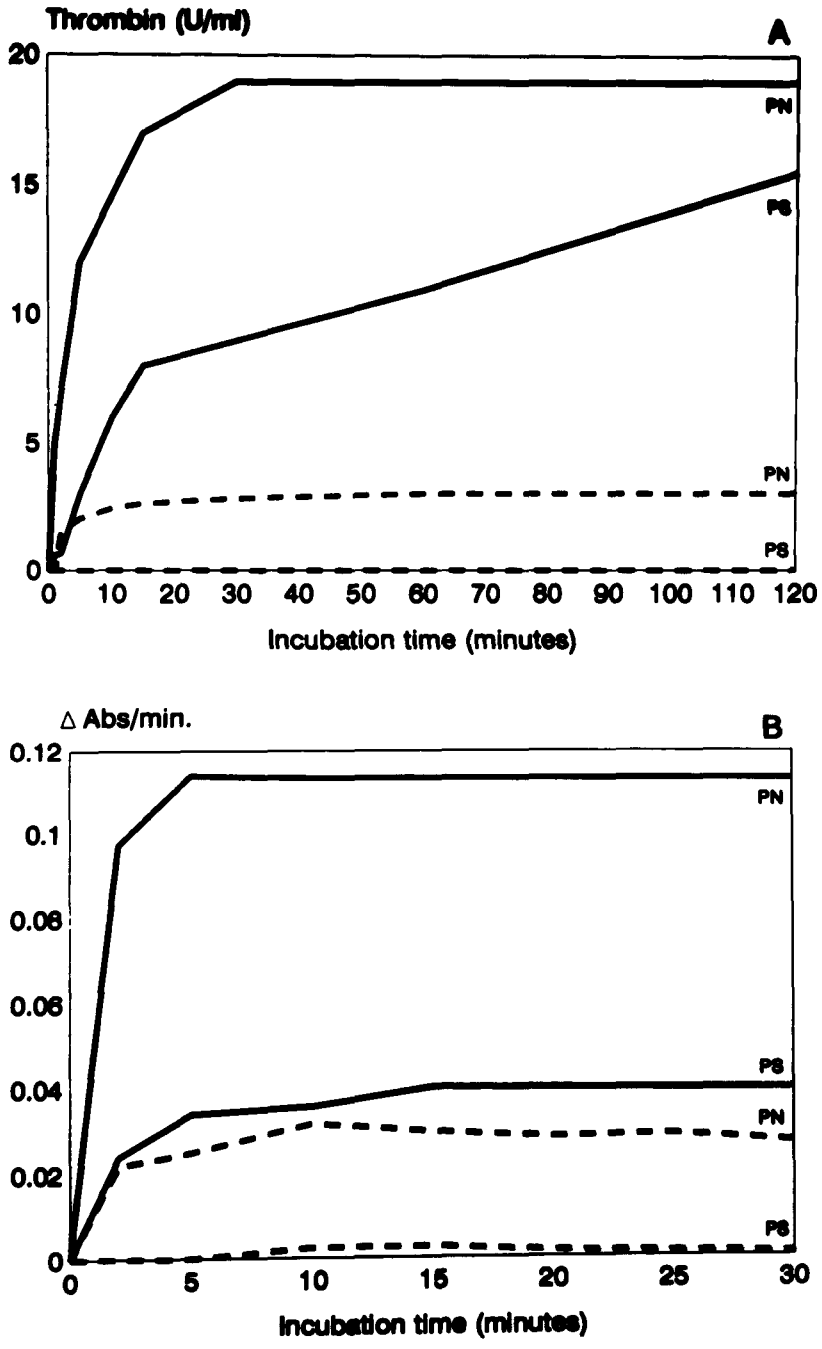

FIG. 2

Activities generated during activation of prothrombin Segovia (PS) and normal prothrombin (PN) by prothrombinase complex in the presence (black line) and absence (striped line) of factor V. A) Clotting activity; B) amidolytic activity.

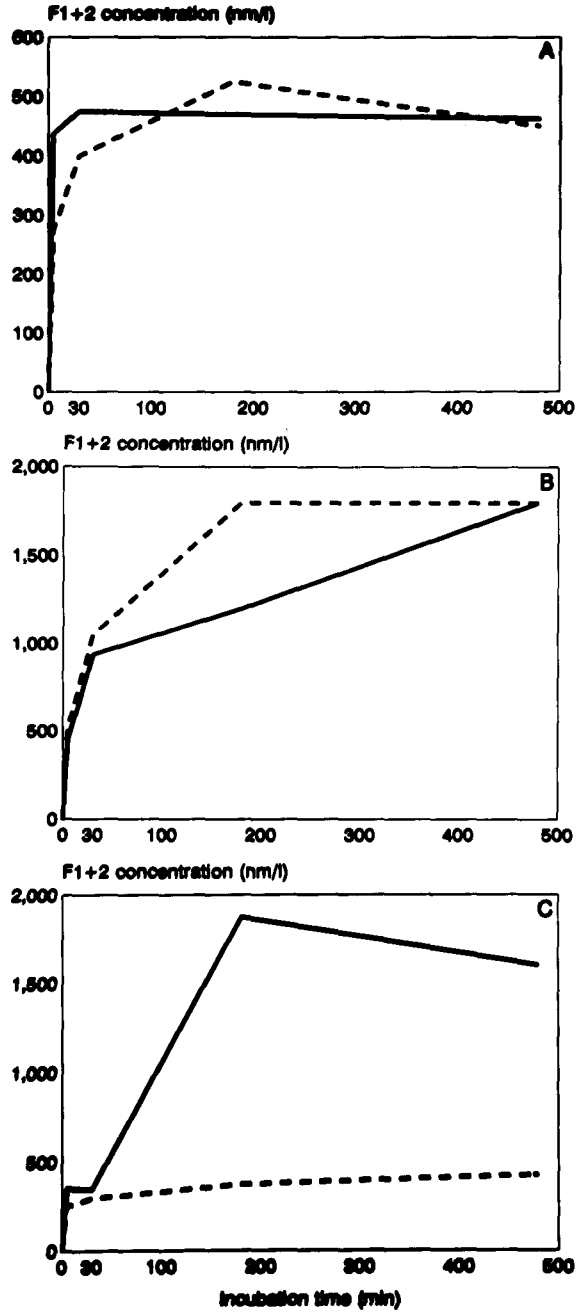

FIG. 3

Fragment F1+2 concentration generated during activation of prothrombin Segovia (striped line) and normal prothrombin (black line) by: A) prothrombinase complex; B) Taipan snake venom and C) *Echis carinatus venom.*

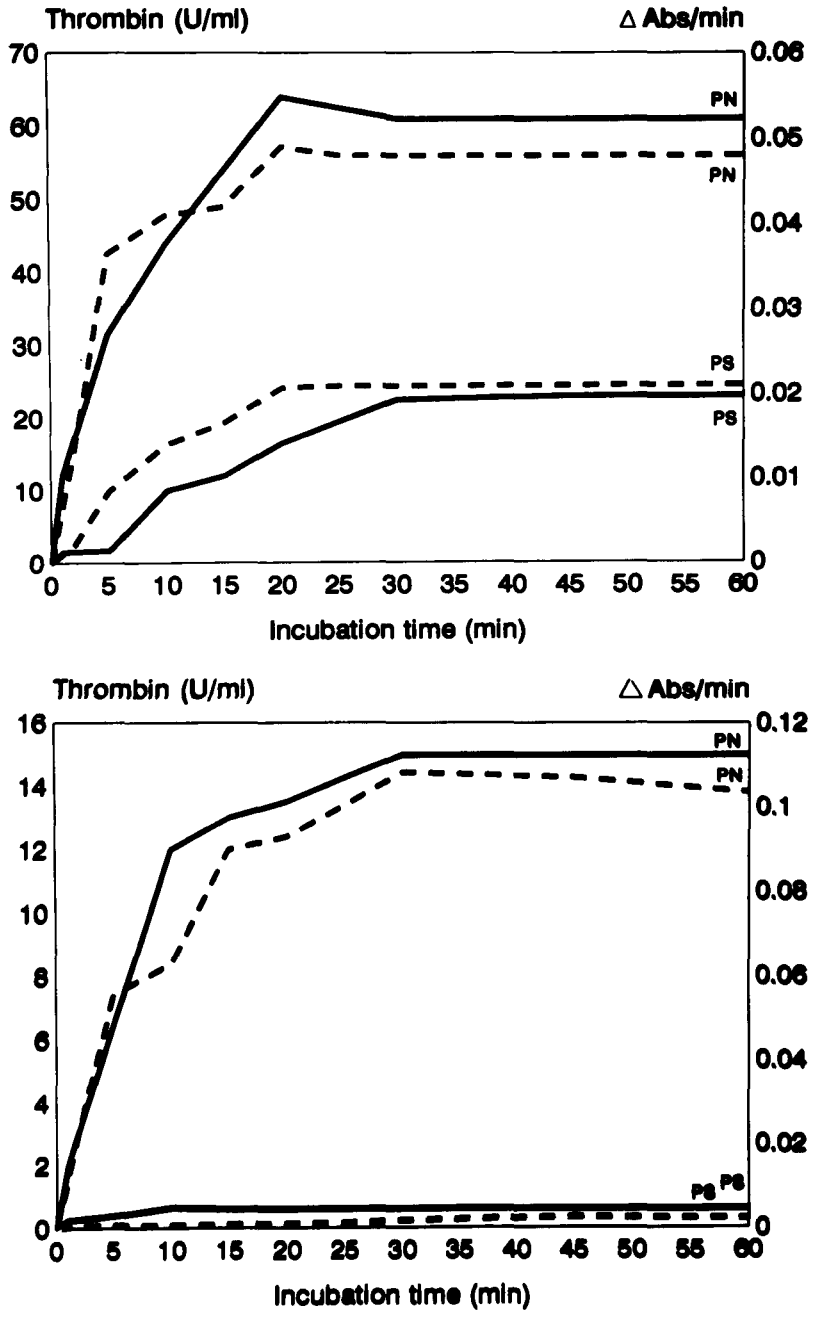

FIG. 4

Activities generated during activation of prothrombin Segovia (PS) and normal prothrombin (PN) by Taipan snake venom (top) and *Echis carinatus* venom (bottom). Clotting activity in black line and amidolytic activity in striped line.

Upon activation by Taipan snake venom, prothrombin Segovia reached a plateau of clotting and amidolytic activities at the same time, thus demonstrating that thrombin is the only active enzyme (35) and that the activation pathway is through P2 (36). Similar to prothrombinase complex, resistence to reduction is reflected by the accumulation of P2. Nevertheless, the clotting activity and the production of the 57000 Da intermediate, result of autolysis, points out to a small thrombin production.

The dramatically decrease thrombin formation from prothrombin Segovia with *Echis carinatus* venom suggests an impairement of proteolysis at bond B, because this venom cleaves only the single Arg320-Ile321 bond forming meizothrombin, with the rest of reactions being autocatalytic (37, 38). However

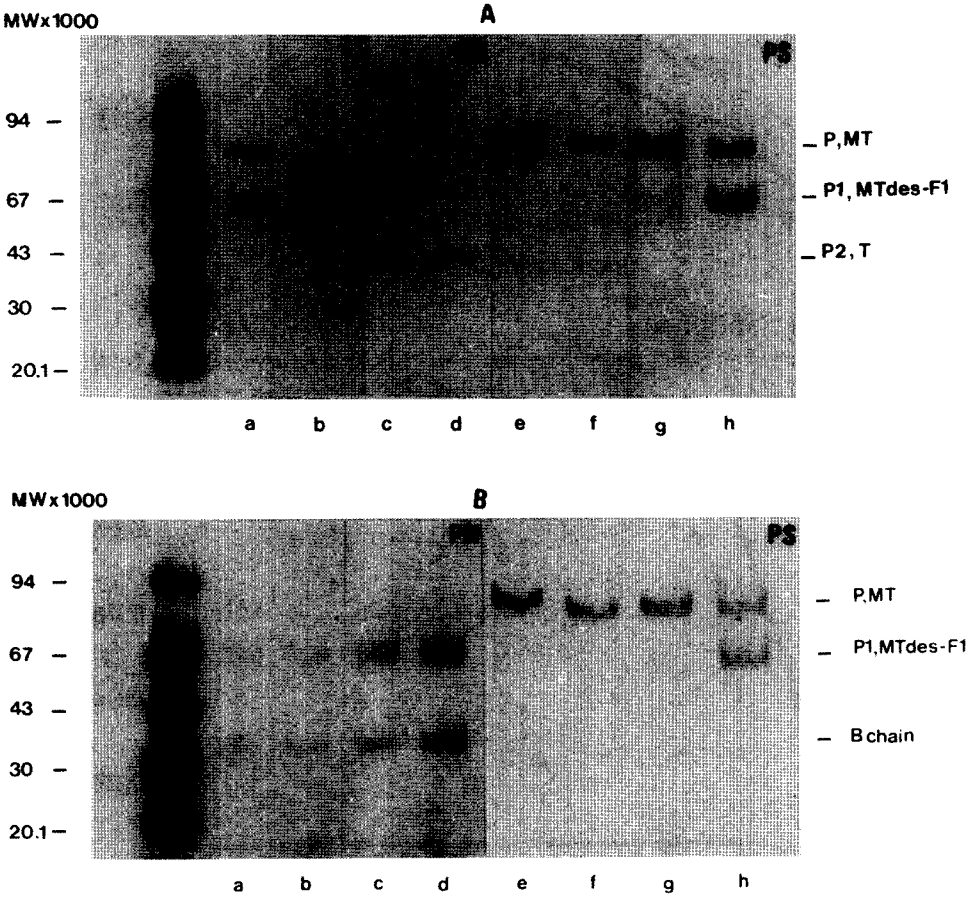

FIG. 5

Cleavage patterns of normal prothrombin (PN) and prothrombin Segovia (PS) by *Echis carinatus* venom under A) non reduced and B) reduced conditions. Lanes a to d in PN and lanes e to h in PS: time 5, 30, 480 minutes and 24 hours. P, prothrombin; MT, meizothrombin; P1, prethrombin-1; MT-desF1, meizothrombin-desF1; P2, prethrombin 2; T, thrombin.

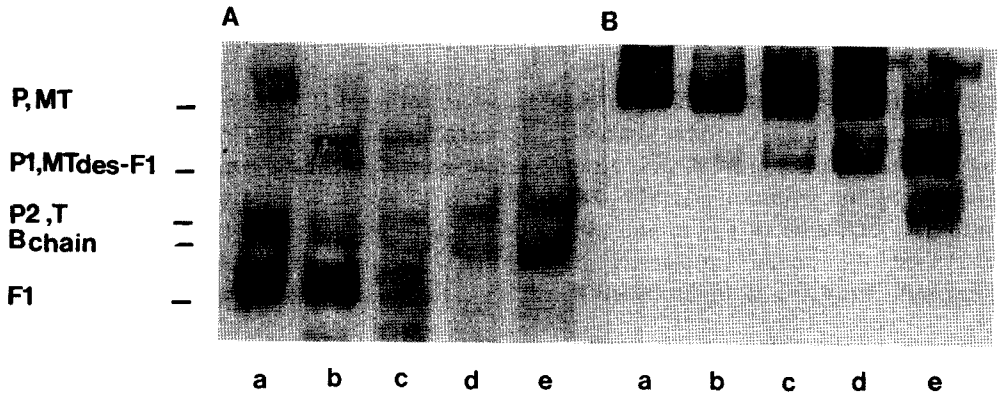

FIG 6

Western blot under reduced conditions of cleavage patterns of A) normal and B) prothrombin Segovia by *Echis carinatus* venom. Lanes a to e: time 5, 30, 180, 480 minutes and 24 hours. P, prothrombin; MT, meizothrombin; P1, prethrombin 1; MT-desF1, meizothrombin-desF1; P2, prethrombin 2; T, thrombin; F1, fragment 1.

SDS-PAGE analysis insinuated some degree of activation, shown by the parallelism between the increase in the 57000 Da intermediate intensity and the decrease in the 72000 Da intermediate intensity, which suggests that the former is formed from the latter. This activation was also confirmed by the appearance of thrombin in the Western blot, and by the generation of F1+2.

Up until now, the known dysprothrombinemias have been characterized by defects in the following sites: a) scission of bond A; b) fragment 1; c) thrombin part of the molecule (dysthrombinemia); and d) scission of bond B. The formation of P2, thrombin and F1+2 with prothrombinase complex and Taipan snake venom, suggests that scission of bond A, Arg271-Thr272 in prothrombin Segovia is normal. Moreover, in prothrombins Barcelona (39) and Madrid (40), which are characterized by this defect, the activation with *Echis carinatus* venom was normal, in contrast to prothrombin Segovia. We can also discard an abnormality in fragment F1 like that of prothrombin San Juan (41), because prothrombin Segovia is quantitatively adsorved in barium salt and its immunoelectrophoretic pattern in the presence of calcium is normal.

There are some similarities between prothrombin Segovia and some well recognized dysthrombinemias, i.e., a decrease of clotting and amidolytic activities after activation with prothrombinase complex, similar to prothrombins Molise (42), Quick (43), Metz (44), Tokushima (45), Salakta (46), Habana (14) and Himi (7). However, in these dysthrombinemias, the decrease with prothrombinase complex and Taipan snake venom was more drastic that in prothrombin Segovia. On the other hand, the behaviour of intermediates of prothrombin Segovia after reduction was different than that found in dysthrombinemias where B chain was produced. In prothrombin Himi (7), after activation with *Echis carinatus* venom, the cleavage pattern was normal, which is very different to prothrombin Segovia. We can not discard a defect in the thrombin part of prothrombin Segovia; such a defect, would explain the delay in its activation with prothrombinase complex and *Echis carinatus* venom.

An alteration in the scission of bond B, was first postulated by Rocha et al (1986) who found a distinctive pattern of peaks in bidimensional eletrophoresis (1), generally related with a defect in the activation process (2, 12).

The only dysprothrombinemia where an altered cleavage of bond B, probably induced by an affinity impairement of factor Xa to prothrombin (47), has been found, is prothrombin Clamart which has the following characteristics: the thrombin generation rate by prothrombinase complex is twice as slow as that of normal prothrombin and SDS-PAGE analysis of the final activation products reveals an abnormal accumulation of P2, resistant to reduction, with a lesser degree of thrombin formation. Similar results were found in prothrombin Segovia with the prothrombinase complex and the Taipan snake venom. The defect in prothrombin Clamart lies in the scission of bond B, Arg320-Ile321.

The lesser degree of amidolytic than clotting activity observed in prothrombin Segovia and the accumulation of Arg320-Ile321 intermediates not cleaved with the prothrombinase complex, suggest fewer intermediates with an uncovered active site. The more evident result was produced with *Echis carinatus* venom, which acts solely on bond B (48), because when it was used as activator, thrombin formation was practically absent and after the reduction of activation intermediates, the B chain did not appear in the SDS-PAGE analysis. Nevertheless, the production of clotting and amidolytic activities as well as autolysis reactions (49), suggests a small cleavage of this bond.

In order to study factor Va influence, prothrombin Segovia was activated with prothrombinase complex in the absence of factor V. Very poor or no thrombin generation was found by amidolytic activity or by clotting activity, respectively. Several studies have shown that factor Va not only binds to the substrate, prothrombin, but also modulates the conformation of its protease domain, determining the proteolysis efficiency of factor Xa (50, 51, 52, 53). Since factor Va produced a moderately active thrombin molecule from prothrombin Segovia, it is possible that factor Va made the scission of bond B easier, probably by inducing a more appropiate conformation of prothrombin.

Our results suggest that the defect of prothrombin Segovia impairs the correct scission of bond B, Arg320-Ile321, although it is unlikely that it is located in one of these amino-acids.

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