PROTEOLYSIS OF HUMAN FACTOR II BY FACTOR Xa IN THE PRESENCE OF HIRUDIN

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SUMMARY

Human Factor II was reacted with Factor Xa in the presence of hirudin, a highly specific thrombin inhibitor. Under these conditions, two new proteins with apparent molecular weights of 39000 and 37000 daltons are generated. Purification of the 39000 dalton species and subsequent incubation with either Factor Xa or thrombin revealed that only thrombin could cleave this protein producing two polypeptides with apparent molecular weights of 27000 and 14000 daltons. These findings suggest that the intermediates observed when Factor II is reacted with Factor Xa in the absence of a thrombin inhibitor arise from a combination of Factor Xa and thrombin proteolysis at different sites on the Factor II molecule.

INTRODUCTION

Numerous studies have focused on the mechanism of the in vitro conversion of human(1-4) and bovine(5-9) Factor II(prothrombin) to thrombin by Factor Xa. When Factor II is mixed with small amounts of Factor Xa and the reaction monitored by sodium dodecyl sulfate(SDS) gel electrophoresis, one observes an apparent sequential conversion of Factor II to two thrombogenic intermediates (Intermediates 1 and 2*) and two nonthrombogenic fragments(Fragments 1 and 2*) prior to the formation of thrombin. Stenn and Blout(6) originally proposed that bovine Factor II can be activated via two pathways: a two-step, Factor Xa catalyzed pathway, and a three-step, thrombin initiated pathway. Recently, Esmon et al. (10) unambiguously confirmed this proposal by incubating bovine Factor II with Factor Xa in the presence and absence of diisopropylfluorophosphate(DFP), isolating the intermediates and testing the purified intermediates for possible proteolysis by Factor Xa and thrombin.

Inasmuch as thrombin cleaves human Factor II at a specific peptide bond yielding products(Intermediate 1 and Fragment 1) indistinguishable from two

*See references 9 and 10 for proposed nomenclature of activation products

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species observed when Factor II is incubated with Factor Xa(4), we investigated the proteolysis of human Factor II by Factor Xa in the presence of purified hirudin, a highly specific thrombin inhibitor. The purpose of this communication is to report the findings of this study which essentially corroborate those of Stenn and Blout(6) and Esmon et al.(10) using bovine Factor II.

MATERIALS AND METHODS

Human Factors II and Xa were prepared as previously described(4,11). Human thrombin was purified according to Lundblad(12) from mixtures containing 20-30 mg Factor II, 200 μg Factor Xa, 30 Ortho Units of partially purified bovine Factor V and 1 mg PS/PC* dissolved in 15 ml 0.1 M Tris-HCl(5 mM $CaCl_2$), pH 7.5 previously incubated at 25° for 2 hr. Thrombin activity was determined from a standard log-log plot of clotting time vs. thrombin concentration constructed with an NIH standard thrombin preparation(Lot B-3; 21.7 NIH U/mg) at 28° according to Shapiro and Waugh(13). Purified thrombin possessed a specific activity of 1800 NIH U/mg and consisted of approximately 95% α-thrombin and 5% β-thrombin when examined by SDS gel electrophoresis(14).

Hirudin was purified to homogeneity(as judged by disc and SDS gel electrophoresis) from commercial preparations(Sigma-Grade II) by DEAE cellulose (Whatman DE52) chromatography as described by Fritz et al.(15). As noted by Fritz et al.(15), commercial preparations of hirudin are rather heterogeneous and contain large quantities of low molecular weight inhibitors for trypsin, chymotrypsin and plasmin which apparently form active site acyl intermediates with these enzymes. Our purified preparations of hirudin were functionally free of trypsin inhibitors as judged by their inability to affect the activity of trypsin(Worthington) toward the chromogenic substrate, benzoyl arginine p-nitroanilide(16). One μg of the purified hirudin inhibited 7 NIH Units (4 μg) of human thrombin.

SDS gel electrophoresis was performed according to Swank and Munkres (17) in 10% acrylamide gels (1:30 bis:acrylamide ratio) containing 0.1% SDS and

*Prepared as previously described(4).

8 M urea for 16-20 hr at a constant current of 2 ma/gel. Molecular weight estimates of test proteins were obtained by interpolation from a linear, semi-logarithmic plot of molecular weight vs. migration distance for reduced samples of bovine serum albumin (68000), catalase (60000), ovalbumin (45000), aldolase (40000), pepsin (35000), carbonic anhydrase (30000) and myoglobin (17200).

The proteolysis of Factor II by Factor Xa in the presence of purified hirudin was conducted in polycarbonate containers at room temperature. On an analytical scale, 2-3 mg Factor II was mixed with 150 μg hirudin and 40-50 μg Factor Xa (500 Ortho U/mg) in a total volume of 1.5 ml 0.1 M Tris-HCl, pH 7.5. The progress of this reaction was monitored by SDS gel electrophoresis of incubation mixture aliquots withdrawn at selected times between 0 and 300 min after the addition of Factor Xa. Aliquots of the activation mixture (100 μl) were removed, diluted with 300 μl 0.05 M Tris-HCl, pH 7.5 containing 4 mg SDS and 192 mg urea (Mann-Ultrapure), and subsequently analyzed by SDS gel electrophoresis. Ten μl aliquots of the incubation mixture were also removed at the selected times and mixed with 0.5 ml fibrinogen solution (5 mg/ml Fraction I-2(11) dissolved in 0.1 M Tris-HCl, pH 7.5) to check for the presence of free, unneutralized thrombin.

The products formed in the reaction of Factor II with Factor Xa in the presence of hirudin were isolated from preparative scale incubation mixtures by preparative disc gel electrophoresis using a Buchler Poly-Prep 200 apparatus as previously described (11). These incubation mixtures consisted of 20-30 mg Factor II, 1-2 mg hirudin and 500 μg Factor Xa dissolved in 15 ml 0.1 M Tris-HCl, pH 7.5 and were incubated 4 hr at room temperature. Prior to eletrophoresis the incubation mixture was made 20 mM in DFP, incubated at 25° for 2 hr and excess DFP removed by dialysis against 0.05 M Tris-HCl, pH 7.5.

RESULTS AND DISCUSSION

When human Factor II is incubated with small quantities of Factor Xa in the presence of purified hirudin, two new proteins are generated coincident with a decrease in Factor II concentration. Figure 1 represents a time course

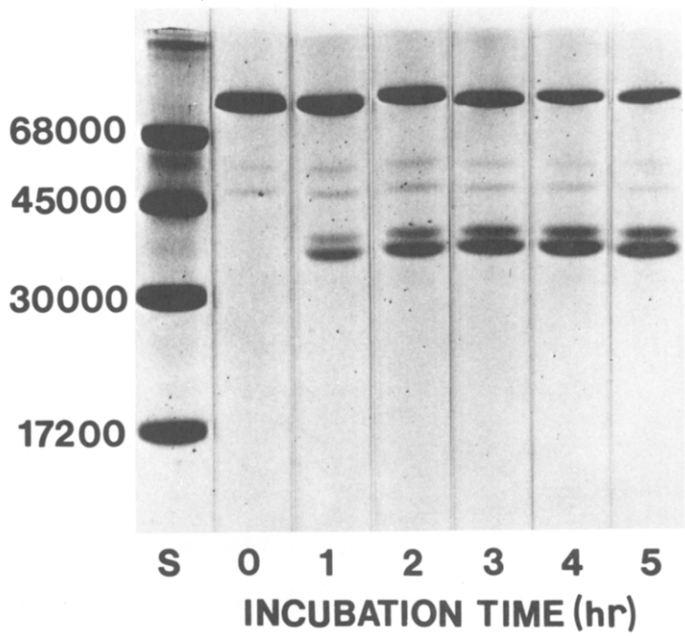

Figure 1. Time course of the proteolysis of Factor II by Factor Xa in the presence of hirudin as monitored by SDS gel electrophoresis. Gel S is a mixture of bovine serum albumin, ovalbumin, carbonic anhydrase and myoglobin.

study of this reaction as monitored by SDS gel electrophoresis. The apparent molecular weights of these new proteins are 39000 and 37000 daltons as determined by SDS gel electrophoresis. The two minor protein bands with molecular weights of 55000 and 48000 daltons were identified as Intermediate 1 (a contaminant of this Factor II preparation) and added Factor Xa, respectively. From densitometry tracings, the concentration of these minor components remained essentially unchanged throughout the incubation period.

At no time in the course of analytical or preparative scale incubations were aliquots of the incubation mixture observed to clot fibrinogen, indicating the absence of free thrombin. Presumably, the observed fragmentation of Factor II resulted solely from Factor Xa proteolysis.

When preparative scale incubation mixtures of Factor II, Factor Xa and hirudin were subjected to preparative disc electrophoresis, three protein

peaks emerged. The most anionic peak was not thrombogenic and was shown by SDS gel electrophoresis to be the 39000 dalton species. Approximately 2-3 mg of the 39000 dalton protein could be isolated from 20 mg starting Factor II by this procedure. The next peak in the electropherogram was unreacted Factor II which was followed by a broad peak presumed to be an Intermediate 2-hirudin complex. This last peak did not contain thrombin activity nor could thrombin activity be generated by the addition of Factor Xa.

The isolated 39000 dalton protein was incubated either with purified human Factor Xa or thrombin for 1 hr at 25° and the products of these reactions analyzed by SDS gel electrophoresis. Approximately 300 μg quantities of the 39000 dalton species were incubated with thrombin or Factor Xa in final concentrations of 5-130 NIH U/ml or 14-140 Ortho U/ml, respectively. At all Factor Xa concentrations, no apparent proteolysis of the 39000 dalton protein was observed, while thrombin at all concentrations studied cleaved this protein producing polypeptides with apparent molecular weights of 27000 and 14000 daltons. As shown in Figure 2, these latter polypeptides (Fig. 2,B) were electrophoretically indistinguishable from Fragment 1 (Fig. 2,C) and Fragment 2 (Fig. 2,D) which had been purified from mixtures of Factor II and Factor Xa in the absence of any thrombin inhibitor (4).

While the exact relationship between the Factor II fragmentation products observed in this study and those observed in the absence of a specific thrombin inhibitor (4) is not known at this time, a reasonable tentative statement is that Factor Xa, in the presence of a substance that specifically and rapidly inhibits newly-formed thrombin, cleaves Factor II to produce Intermediate 2 and a non-thrombogenic 39000 dalton fragment most probably equivalent to the F_x or Fragment 1·2 species observed by Stenn and Blout (6) and Esmon *et al.* (10), respectively. Interestingly, while a molecular weight of 39000 daltons was observed for this species by the procedure of Swank and Munkres (17), an apparent molecular weight of 34000 daltons was observed when estimated according to Weber and Osborn (18) and is consistent with molecular

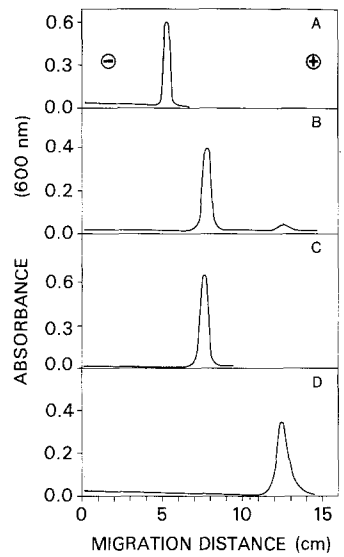

Figure 2. Absorbance scans of SDS gels of the 39000 dalton protein before (A) and after (B) thrombin treatment. Gels A and B each contain 30 μg protein. Gels C and D contain human Fragment 1 (20 μg) and Fragment 2 (40 μg), respectively.

weight values reported with SDS electrophoretic systems in the presence (6) and absence of urea (10). Assuming little molecular differences between bovine and human Factor II, this anomaly may stem from the relatively high carbohydrate content known to be in the Fragment 1 portion of the bovine Factor II molecule (9, 19).

The 37000 dalton species generated concomitantly with the 39000 dalton protein is thought to be Intermediate 2 inasmuch as its apparent molecular weight remained unchanged after 2-mercaptoethanol treatment and the fact that it migrated to a position identical to that of purified, unreduced α-thrombin (data not shown). This would suggest that hirudin forms a complex with Intermediate 2 and either prevents or hinders its conversion to thrombin by Factor Xa.

The data presented here are consistent with an activation scheme originally proposed by Stenn and Blout (6) for bovine Factor II. In their proposal, Factor Xa converts Factor II to thrombin and a nonthrombogenic fragment,

Fragment 1·2. Newly-formed thrombin rapidly converts Factor II to Intermediate 1 and Fragment 1, and in addition, cleaves Fragment 1·2 to produce Fragment 1 and Fragment 2. Intermediate 1 is converted to Intermediate 2 (and subsequently α-thrombin) and Fragment 2 by Factor Xa. This proposed activation scheme provides one possible explanation for the low yields of thrombin activity observed in our earlier experiments (4). In the intial two-stage assay of Factor II, a relatively low Factor II/Xa ratio exists which ensures that little Factor II will be remaining for newly-formed thrombin to act on. Conversely, when one goes to systems with relatively high Factor II/Xa ratios (4), considerable Intermediate 1 and Fragment 1 is produced by low concentrations of thrombin. In view of the 30 min half-life observed for thrombin (20) and the fact that Intermediate 1 appears to be a poor substrate for Factor Xa (4, 21), the consequential protracted incubation time could result in significant thrombin denaturation.

It should be emphasized that while these data reflect a mechanism essentially identical to that described for bovine Factor II preparations (6, 10), further work is required to establish the physico-chemical properties of the human Factor II fragments and intermediates. Whether the proposed activation scheme is operative *in vivo* is an interesting question in view of the slow activation rate of Intermediate 1 (4, 21) and the inhibitory properties of Fragment 1 (7, 19). Thrombin conceivably could limit the rate of its own formation simply by converting Factor II to Intermediate 1 and Fragment 1.

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