CONCISE REPORT

Proteolytic Inactivation of Human Factor VIII Procoagulant Protein by Activated Protein C and its Analogy With Factor V*

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Purified human factor VIII procoagulant protein (VIII:C) was treated with purified human activated protein C (APC) and the loss of VIII:C activity correlated with proteolysis of the VIII:C polypeptides. APC proteolyzed all VIII:C polypeptides with mol wt = 92,000 or greater, but not the doublet at mol wt = 79-80,000. These results and our previous thrombin activation studies of purified VIII:C, are analogous with similar studies of factor V and form the basis for the following hypothesis: activated VIII:C consists of heavy and light chain polypeptides [mol wt = 92,000 and mol wt = 79-80,000 (or 71-72,000), respectively] which are similar in M, to the heavy and light chains of activated factor V. Thrombin activates VIII:C and V by generating these polypeptide chains from larger precursors and APC inactivates both molecules by cleavage at a site located in the heavy chain region of activated VIII:C and V.

Although factor VIII procoagulant protein (VIII:C) has been highly purified by a number of investigators, its structural characterization is still very preliminary. Most reports of purified VIII:C from bovine, porcine, and human sources have described preparations containing multiple molecular weight forms. However, two laboratories have reported the isolation of homogeneous VIII:C at mol wt = 365,000 or mol wt = 100,000. In the case of porcine VIII:C, Fass et al. have shown that the molecular weight heterogeneity is due to proteolysis and that there is sequence homology with bovine factor V chains.

The human VIII:C we have purified from commercial factor VIII concentrate showed extensive molecular weight heterogeneity, with polypeptides ranging from mol wt = 79,000-188,000. Studies of its proteolytic activation by thrombin demonstrated notable polypeptide similarities with thrombin-activated factor V (V,). The thrombin activation study we reported has been reproduced by others who purified human VIII:C by our method. Fass et al. have performed similar thrombin proteolysis studies with porcine VIII:C.

We have now examined proteolysis of VIII:C with the anticoagulant enzyme (APC). The results of this study, as well as those of thrombin proteolysis, are analogous with similar work on factor V and, when taken in conjunction, suggest a working hypothesis for the structure of activated VIII:C (VIII:C ).

MATERIALS AND METHODS

Purification of Human VIII:C and Human Protein C were as previously described except that a column of Cibacron-blue Sepharose was substituted for dextran-sulfate agarose and a mono S column of a Pharmacia FPLC system was used to separate APC from thrombin.

Discontinuous NaDodSO4 7.5% Polyacrylamide Gel Electrophoresis (PAGE) of reduced VIII:C, staining with Coomassie blue R250 and scanning and integration of the gel were as previously described.

Sample Preparation

A 339 μg sample of VIII:C in 0.3 ml of VIII:C buffer containing 0.3 M calcium chloride was dialyzed overnight against buffer (50 mM Tris-chloride, 0.15 M sodium chloride, 5 mM calcium chloride, 0.02% sodium azide, pH 7.4). To the dialyzed VIII:C sample was added 1.095 ml of buffer, 90 μl of rabbit brain cephalin (Sigma Chemical Co., St. Louis, MO, reconstituted, stored and thawed according to manufacturer’s instructions) and 15 μl of 1 mM dansylarginine N-(3-ethyl-1,5-pentanediyl) amide (DAPA), to give a final DAPA concentration of 10 μM. The DAPA was included to inhibit any trace amounts of thrombin present in the APC since 10 μM DAPA does not significantly inhibit APC. The final volume of the sample was 1.5 ml and the final VIII:C concentration was 226 μg/ml. Four hundred microliters of the 1.5 ml VIII:C sample were withdrawn and set aside as a control (designated VIII:C). To the remaining 1.1 ml was added 20 μl (10 μg) of APC giving a final APC concentration of 9 μg/ml (designated VIII:C + APC). A second control sample was prepared containing all components at similar concentrations except that VIII:C was omitted (designated APC).

Timepoints

VIII:C alone, the mixture of VIII:C and APC, and APC alone were placed in a 37°C waterbath and at given timepoints aliquots were withdrawn for NaDodSO4 PAGE and/or VIII:C activity assay. It was also determined in a control experiment that APC remained active in the hydrolysis of the synthetic substrate S-223822 after prolonged incubation at 37°C.


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Assays

Samples were assayed for VIII:C activity as previously described using an activated partial thromboplastin time assay with hemophilia A plasma substrate.

Preparation of Samples for Electrophoresis

Since calcium ions are required for APC activity, APC was stopped at various times by addition of 1/10 volume of 100 mM EDTA containing 10 uM DAPA to the VIII:C + APC aliquots as well as to the control VIII:C and APC aliquots. A 1/10 volume of 10% NaDodSO₄ was then added to these aliquots. They were then heated in a boiling waterbath for 5 min and subsequently dialyzed for NaDodSO₄ PAGE as previously described.

RESULTS

Digestion of purified VIII:C (Fig. 1, left) with either APC (Fig. 1, middle) or thrombin (Fig. 1, right) was associated with loss of approximately 85% of the control VIII:C activity in both cases, but each enzyme produced different VIII:C polypeptide cleavage products. APC inactivation of VIII:C activity (Fig. 1, middle) resulted in the diminution of all VIII:C polypeptides of mol wt between 92,000 and 188,000, and generation of a polypeptide of mol wt = 45,000, while leaving the doublet of mol wt = 79,000–80,000 intact.

Unlike APC, thrombin treatment of VIII:C activity (Fig. 1, right) resulted in the disappearance of the doublet of mol wt = 79,000–80,000 and generation of a doublet of mol wt = 71,000–72,000. However, like APC, thrombin caused a diminution of the polypeptides of mol wt = 92,000–188,000 (with the exception of one thrombin-resistant polypeptide) and the generation of two lower molecular weight products at mol wt = 54,000 and 44,000. A small amount of what may be the polypeptide of mol wt = 45,000 also appeared in the thrombin digest.

A time course inactivation of purified VIII:C by APC showed the progressive disappearance of specific VIII:C polypeptides (Fig. 2, bottom) as VIII:C activity decreased (Fig. 2, top) over a 360-min time period.
course. Scanning and integration of the gel showed that the polypeptide of mol wt = 188,000 and the polypeptide of mol wt = 92,000 decreased in parallel with VIII:C activity (Fig. 3). One other polypeptide of intermediate mol wt was cleaved by APC but it was not easily quantitated by gel scanning. In this experiment, unlike that in Fig. 1, some VIII:C polypeptides of mol wt = 92,000–188,000 were resistant to the APC digestion. However, like Fig. 1, the doublet polypeptide at mol wt = 79,000–80,000 was not proteolyzed by APC.

A polypeptide of mol wt = 45,000 appeared to increase in concentration as the polypeptides of mol wt = 188,000 and 92,000 decreased suggesting that it is a proteolytic fragment derived from them. No other digestion products were visualized with Coomassie blue.

We have previously shown that during thrombin activation of VIII:C the mol wt = 92,000 polypeptide increased and decreased in parallel with VIII:C activity. In order to determine whether a linear relationship existed between proteolysis of the polypeptide of mol wt = 92,000 and the loss of VIII:C activity, the data of Figs. 2 and 3 were replotted to examine percent VIII:C activity versus percent of polypeptide of mol wt = 92,000 (Fig. 4). The amount of VIII:C activity appeared to be proportional to the amount of the polypeptide of mol wt = 92,000.

DISCUSSION

The VIII:C purified from commercial factor VIII concentrate used in these studies is heterogeneous, consisting of a doublet polypeptide at mol wt = 79,000–80,000 and at least six larger polypeptides up to mol wt = 188,000. This heterogeneity is most likely due to proteolysis of a high molecular weight form of VIII:C which occurred before or during the purification process. Despite the heterogeneity of purified VIII:C, our thrombin and APC proteolysis studies bear a close resemblance to similar studies of factor V and suggest a probable structure for the activated form of VIII:C (VIII:Ca).

We have previously shown that the generation of the polypeptide of mol wt = 92,000 during thrombin activation of VIII:C was associated with the increase in VIII:C activity, a result which has recently been confirmed. At the time of maximal VIII:C activity, most of the higher molecular weight polypeptides had apparently been converted to the mol wt = 92,000 form. At this point, VIII:C polypeptides of mol wt = 92,000, 79,000–80,000 and 71,000–72,000 predominated. We also noted the structural similarity between the polypeptides of mol wt = 92,000 and the mol wt = 79,000–80,000 (or 71,000–72,000) of VIII:C and the heavy and light chains of factor Va. APC selectively cleaves the heavy chain of human and bovine factor Va, leaving the light chain intact. Similarly the proposed VIII:C heavy chain of mol wt = 92,000 is...
cleaved by APC leaving the proposed VIII:C light chain of mol wt = 79-80,000 (or 71,000–72,000 doublet(s) intact. Thus, the cleavage pattern of the proposed VIII:C polypeptides by APC appears analogous with that of factor V.a

The degree of cleavage of high molecular weight VIII:C polypeptides (mol wt greater than 92,000) by thrombin and APC is variable, suggesting that some may be precursors of the mol wt = 92,000 form and are thus susceptible to APC cleavage whereas others are precursors of the doublet of mol wt = 79,000–80,000 and are therefore resistant.

The destruction of the polypeptide of mol wt = 92,000 of purified human VIII:C by APC, leaving the doublet of mol wt = 79–80,000 intact, is analogous not only with human or bovine factor V, but also with purified bovine VIII:C. Vehar and Davie purified bovine VIII:C to homogeneity and showed it to consist of three polypeptides of mol wt = 93,000, 88,000, and 85,000. They demonstrated that thrombin cleaved all three polypeptides, while activated protein C destroyed only the polypeptide of mol wt = 93,000, leaving the mol wt = 88,000 and 85,000 forms intact. This appears very similar to our results and suggests that the purified bovine VIII:C described by these investigators may represent activated VIII:C.

Thus, based on our studies on the proteolysis of purified human VIII:C with thrombin1 and APC and their analogy with similar studies of factor V,15,18,21 we propose a working hypothesis for the structure of VIII:C; human VIII:C consists of a heavy chain and a light chain which appear structurally similar and might function similarly to the heavy and light chains of factor V.a

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