CONCISE REPORT

Thrombin Proteolysis of Purified Factor VIII Procoagulant Protein: Correlation of Activation With Generation of a Specific Polypeptide

By Carol A. Fulcher, James R. Roberts, and Theodore S. Zimmerman

Factor VIII procoagulant protein (VIII:C) purified from commercial factor VIII concentrate contained multiple polypeptides ranging in mol wt from 79,000 to 188,000, all of which were removed from solution by a monoclonal anti-VIII:C antibody specific for a thrombin-sensitive epitope. In a time-course digest of the purified VIII:C using a trace amount of purified human α-thrombin, changes occurred in all VIII:C polypeptides during the activation and inactivation of VIII:C activity. The generation and destruction of a mol wt 92,000 polypeptide paralleled the increase and decrease in VIII:C activity, suggesting that this polypeptide represents an activated form. These results provide the basis for a working hypothesis for the mechanism of thrombin activation of VIII:C.

IN 1963 RAPAPORT ET AL. showed that treatment of plasma with trace amounts of thrombin enhanced coagulation by activating both the factor VIII procoagulant protein (VIII:C) and factor V. Later Hoyen and Trabold used partially purified VIII:C separated from von Willebrand factor (vWF) to show a rise and subsequent decrease in VIII:C activity and a decrease in apparent molecular weight after exposure to thrombin. Recently, when purified bovine, human, and porcine VIII:C were treated with thrombin and analyzed by NaDodSO4 PAGE, cleavage of high molecular weight polypeptides into lower molecular weight forms was demonstrated. We have now utilized a monoclonal anti-VIII:C antibody to confirm the identity of the purified human VIII:C polypeptides reported earlier and have correlated changes in them with changes in VIII:C activity during a thrombin activation time course analysis. This analysis has identified a mol wt 92,000 polypeptide, which appears to be an activated form. In addition, these studies have produced a working hypothesis for the mechanism of thrombin activation of VIII:C.

MATERIALS AND METHODS

Purification of VIII:C

VIII:C was purified from the VIII:C/vWF complex in commercial factor VIII concentrate using a monoclonal anti-vWF antibody bound to Sepharose, as previously described, with the following modifications: the bed volume of the immunoabsorbent column was increased from 1.5 liters to 3 liters and 17,000 U rather than 10,000 U of VIII:C activity were applied; the sodium chloride content of the buffer used to wash the immunoabsorbent after sample application was reduced from 0.5 M to 0.15 M; an Amicon model 8400 stirred cell (Amicon Corp., Danvers, Mass.) replaced the earlier model used, and the concentrated immunoadsorbent pool was adjusted to 0.25 M calcium chloride and adsorbed twice for 1 hr each time with 1/10 v/v of a mixture of monoclonal antifibrinogen, antifibrinectin, and anti-vWF antibodies that had been coupled to cyanogen-bromide-activated Sepharose (see below).

NaDodSO4 PAGE

Discontinuous NaDodSO4 PAGE of reduced VIII:C and staining with Coomassie blue R250 were as previously described, except that the polyacrylamide concentration was increased from 5% to 7.5% and the gel slab thickness was reduced from 1.0 mm to 0.8 mm. Gels were dried onto filter paper to increase contrast. Scanning and integration of a photographic print of the finished gel was done using a Zeineh soft laser scanning densitometer. Periodic acid-Schiff staining was by the method of Kapitany and Zebrowski.

Production of Monoclonal Antibody Against VIII:C

Monoclonal antibodies were produced as previously described using purified VIII:C as immunogen. The antibodies were selected with a solid-phase assay in Linbro-Titertek (Flow Laboratories, Inglewood, Calif.) plates and an enzyme-linked immunosorbent (ELISA) detection system using a peroxidase-antibody conjugate (Zymed Laboratories, Burlingame, Calif.). The plates were coated with 100 ng of purified VIII:C per well. Following repeated subcloning by limiting dilution, the ELISA-positive culture supernatant of the clone selected for use in this study also inhibited plasma VIII:C activity. The VIII:C inhibitory activity of the monoclonal anti-VIII:C antibody purified from ascites fluid (see below) was 15 Bethesda units per milligram of protein. Chain typing showed the antibody to be of the IgG1 subclass. The antifibrinectin monoclonal antibody was kindly provided by Dr. Deane Mosher and the antifibrinogen monoclonal antibody by Dr. Fred Jensen. The anti-vWF monoclonal antibody production has been previously described.

To prepare immunoabsorbents, the monoclonal antibodies were precipitated from ascites fluid using 50% ammonium sulfate, reprecipitated two more times, and then coupled to cyanogen-bromide-activated Sepharose 4B (Pharmacia, Piscataway, N.J.) according to the manufacturer's instructions at an antibody density of 2-4 mg/ml Sepharose. The immunoabsorbents were prewashed with 3 M sodium thiocyanate, washed with imidazole-saline buffer (0.02 M imid-
saline buffer containing 0.04 M A B C D E and treated twice with 1 mM p-APMSF prior to use.

Thrombin Activation Time Course Analysis of Purified VIII:C

Purified human \( \alpha \)-thrombin (specific activity 2534 U/mg, final concentration 0.5 U/ml), kindly provided by Dr. John W. Fenton II, was added to VIII:C (final concentration 167 \( \mu \)g/ml) in imidazole saline buffer containing 0.04 M CaCl\(_2\). Buffer alone was added to a control aliquot. The solutions were incubated at room temperature, and at various time intervals, samples of the VIII:C-thrombin mixture were added to tubes containing p-APMSF (Cal-Med, San Francisco, Calif.) to inactivate the thrombin rapidly and irreversibly.\(^4\) In order to minimize hydrolysis of p-APMSF, it was diluted 1:10 from a stock solution (100 mM in methanol) into imidazole saline buffer 60 sec before reaction with the VIII:C-thrombin samples. The final p-APMSF concentration was 1 mM. The control aliquot was treated similarly with p-APMSF at the start of the experiment. At the end of the 60-min time course, all VIII:C samples were assayed for VIII:C activity using an activated partial thromboplastin time assay as previously described\(^4\) and then prepared for NaDodSO\(_4\) PAGE.\(^4\)

RESULTS

Purification and Characterization of VIII:C

Modification of the monoclonal anti-vWF antibody immunoadsorbent technique by lowering the sodium chloride concentration in the washing buffer and use of a more efficient Amicon stirred cell (see Materials and Methods) improved the VIII:C recovery from 12% to 35% of starting activity, yielding 3 mg of protein from 17,000 U of VIII:C. The specific activity was 2000 U/mg. Inclusion of either 1 mM or 10 mM benzamidine in all buffers through application of VIII:C to the aminohexyl Sepharose column and treatment of VIII:C pools at all stages of the purification with 0.1 mM p-APMSF had no effect on the NaDodSO\(_4\) PAGE pattern of the purified VIII:C preparation, with the possible exception of a relative increase in the proportion of the mol wt 188,000 form. All forms of VIII:C contained carbohydrate, as evidenced by their positive reaction with the periodic acid-Schiff stain (not shown). VIII:C bands were identified as such by their adsorption to and elution from a monoclonal anti-VIII:C antibody immunoadsorbent column (Fig. 1, A, B, and C).

Thrombin Activation Time Course of Purified VIII:C

Thrombin activation of purified VIII:C activity was analyzed over a 60-min time course. Analysis of each time point by NaDodSO\(_4\) PAGE revealed sequential changes in VIII:C protein that correlated with changes in VIII:C activity (Fig. 2). Before thrombin exposure, the untreated VIII:C sample (Fig. 2, Un) showed the characteristic array of VIII:C forms ranging from a doublet at mol wt 79–80,000 to a band at 188,000. The band above 188,000 and the two bands below 79,000 did not bind to the monoclonal anti-VIII:C antibody immunoadsorbent and are presumably non-VIII:C contaminants.

During the first 5 min of the thrombin activation time course, all but one of the monoclonal anti-VIII:C antibody reactive bands with a mol wt greater than 92,000 gradually disappeared and were undetectable when VIII:C activity reached its peak at 5 min. The band at 122,000 appeared to be relatively thrombin-resistant in some experiments (Figs. 1 and 2) but not others.\(^4\) After extensive thrombin treatment, neither this band nor any other band was reactive with the immobilized monoclonal anti-VIII:C antibody (Fig. 1, D and E).

The mol wt 92,000 band increased in intensity as VIII:C activity increased and subsequently decreased...
Fig. 2. Changes in VIII:C activity and NaDodSO4 PAGE pattern of reduced VIII:C following treatment with thrombin. Gel lanes contained thrombin only (Thr), untreated VIII:C (Un), and VIII:C at various times after addition of thrombin (0.5 U/ml final concentration). Approximately 10 μg of protein were applied per lane. The anode is at the bottom. In the activity plot (upper panel), the point at which thrombin (Thr) was added is indicated with an arrow.

in intensity as VIII:C activity decreased (Fig. 2 top, bottom). The doublet at mol wt 79–80,000 appeared to be converted to a doublet at 71–72,000, with the latter form predominant from 5 to 60 min as VIII:C activity decreased. Two bands, at mol wt 54,000 and 44,000, became clearly visible from 5 to 60 min. The mol wt 44,000 band also appeared as a doublet in some experiments (Fig. 1, D and E). The 71–72,000 doublet, the 54,000 band, and the 44,000 band were not removed to a significant degree by the immobilized monoclonal anti-VIII:C antibody (Fig. 1, D and E).

Scanning and integration of the gel in Fig. 2 allowed correlation of changes in polypeptide concentration with changes in VIII:C activity (Fig. 3). As shown (Fig. 3, top), the mol wt 92,000 band increased and then decreased in concentration in parallel with VIII:C activity. This suggested that the 92,000 band was an active form of VIII:C, which was created by proteolysis of the higher molecular weight bands. The 54,000 and 44,000 bands increased steadily in concentration between 1 and 40 min (Fig. 3, middle). Considering that the sum of these two molecular weights equals 98,000, it is probable that the 54,000 and 44,000 bands were derived from ongoing thrombin proteolysis of the 92,000 band and are inactive.

Most of the 79–80,000 doublet (Fig. 3, bottom) was lost during the first 0.1–10 min as VIII:C activity peaked, while most of the 71–72,000 doublet appeared during this time and predominated as VIII:C activity decreased. These data suggest that the 71–72,000 doublet was derived from the 79–80,000 doublet and that the 71–72,000 doublet is by itself inactive. The data do not allow a conclusion to be drawn as to whether the 79–80,000 or the 71–72,000 doublets are necessary for VIII:C activity.

DISCUSSION

We have now used a heterologous monoclonal—rather than polyclonal—anti-VIII:C antibody to confirm the identification of the multiple VIII:C bands. The epitope recognized by the monoclonal antibody appears to be thrombin-sensitive, as evidenced by the lack of significant binding of thrombin-inactivated VIII:C to the immobilized antibody.
These results clearly demonstrate sequential changes in VIII:C polypeptides during thrombin activation and allow us to suggest a working hypothesis for the mechanism of VIII:C activation. We propose that native undegraded VIII:C has a molecular weight greater than or equal to 188,000. This form is cleaved by thrombin, possibly through intermediates, to generate the active 92,000 polypeptide. Accumulation of the 92,000 polypeptide is responsible, at least in part, for the rise in VIII:C activity seen upon activation. The mol wt 79–80,000 doublet, the function of which is uncertain at present, probably derives from an early scission of VIII:C not seen here (Fig. 2). It is then cleaved by thrombin to the 71–72,000 form. Because our purified VIII:C preparation already contains the 92,000 polypeptide and relatively large amounts of the 79–80,000 polypeptides, it has probably already been partially activated by thrombin or other proteases. This is further suggested by the fact that only a 3–4-fold activation of VIII:C activity was obtained with these preparations.

Cleavage of the mol wt 92,000 polypeptide, probably to form the 54,000 and 44,000 fragments, correlates with subsequent inactivation of VIII:C. The final thrombin-degraded VIII:C products include the 71–72,000 doublet, the 54,000, and the 44,000 polypeptides.

This hypothesis for thrombin activation of VIII:C has analogies with the activation sequences shown for bovine and human factor V. Both factors V and VIII:C function similarly as cofactors in the intrinsic coagulation pathway. Human factor V is present in plasma as a mol wt 330,000 precursor that is sequentially cleaved by thrombin to generate activated factor V, consisting of a mol wt 105,000 fragment noncovalently bound to a 71–74,000 doublet. The similarity of these polypeptides to the 92,000 fragment and the 79–80,000 doublet of VIII:C is striking. This hypothesis should provide a basis for further functional, biochemical, and immunologic studies of VIII:C.

ACKNOWLEDGMENT

We thank Roberta Novak-Buhrman for expert manuscript preparation; Drs. John H. Griffin, Bjorn Dahlback, and Mark J. Weinstein for helpful discussions; Dr. John W. Fenton II for purified human α-thrombin; Dr. Edward F. Plow for purified fibrinogen; and Armour Pharmaceutical, Tuckahoe, N.Y., for their generous gift of commercial factor VIII concentrate. This is publication no. 2809 of the Research Institute of Scripps Clinic.

REFERENCES

7. Engvall E, Perlmann P: Enzyme-linked immunosorbent assay
(ELISA), quantitative assay of immunoglobulin G. Immunochemistry 8:871, 1971


Thrombin proteolysis of purified factor viii procoagulant protein: correlation of activation with generation of a specific polypeptide

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