Blood Clotting Factor IX

LOSS OF ACTIVITY AFTER CLEAVAGE OF SIALIC ACID RESIDUES

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Enzymatic cleavage of sialic acid from human blood clotting factor IX results in a loss of factor IX clotting activity. The loss of clotting activity and the rate of release of sialic acid follow the same time courses. Control experiments have ruled out several explanations for the loss of factor IX activity: proteolytic degradation, inhibitory effects of free sialic acid, and non-specific inhibition of the clotting assays. Furthermore, no inhibition was seen when similar enzymatic cleavage was carried out on factor X and factor VIII. Therefore, we suggest that the loss of factor IX activity is the direct result of cleavage of sialic acid from the protein.

Most of the inhibition appeared to be an effect on the activity of factor IX, itself, and thus far, little or no effect has been shown on the activation of factor IX to IXa. The structural basis for this unusual effect of sialic acid on protein function currently is being investigated.

The function(s) of sialic acid and other carbohydrate constituents of plasma glycoproteins are not well understood. In the case of many glycoproteins, the cleavage of 1-2 residues of sialic acid/molecule of protein results in rapid clearance of the protein from circulation (McFarlane, 1983). The rapid clearance is a consequence of uptake by a hepatic membrane receptor with specificity for galactose residues (Ashwell and Morrell, 1974). Removal of sialic acid residues generally has little or no effect on the structure or in vitro function of the asialoglycoproteins, although several reports have suggested that there may be some proteins in which removal of sialic acid has some effect (usually inhibitory) on enzymatic activity (Warren et al., 1978).

Factor IX, a plasma glycoprotein constituent of the clotting cascade, is a vitamin K-dependent serine protease which contains γ-carboxyglutamic acid residues. In this paper, we have investigated the effects of removal of terminal sialic acids on the clotting activity of factor IX.

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MATeRIALS AND METHODS

All chemicals were either reagent grade or the highest available purity.

Sodium chloride, sodium azide, benzamidine hydrochloride, soybean trypsin inhibitor (Type I-S), imidazole, Russell's viper venom (Vipera russelli), neuraminidase from Clostridium perfringens (Type X, 125-150 units/mg containing less than 0.001 Sigma unit of protease activity/mg of protein), calcium chloride, diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, triethylamine, monooctanolamine, and N-acetylneuraminic acid (Type IV) were purchased from Sigma Chemical Company, St. Louis, MO. Sodium citrate, sodium acetate, and acetone were purchased from Fisher Scientific Co., Rochester, NY. Plasma deficient in factor IX or factor X was purchased from George King Biomedical, Overland Park, KS. Activated partial thromboplastin reagent was purchased from General Diagnostics, Morris Plains, NJ. Centeroxel P phospholipid was a gift from Central Soya, Gibson City, IL, and was stored in a desiccator at 4 °C. Cyanogen bromide was purchased from Eastman Kodak Company, Rochester, NY. Factor IX concentrate was a kind gift from Cutter Laboratories, Berkeley, CA.

All procedures were carried out at room temperature unless otherwise noted.

Sulfated Sephadex and heparin-Sepharose were synthesized according to the methods of Miletich et al. (1980) and Pepper and Prowse (1977), respectively.

Covalent coupling of Russell's viper venom to Sepharose was done according to Kohn and Wilchek (1982), with the following modifications. Ten grams of wet Sepharose 4B-Cg were washed and resuspended in 10.8 ml of 60% acetone, and then cooled to 0 °C. 0.25 g of cyanogen bromide in 2.3 ml of acetone was added with vigorous stirring. Then 0.2 g of triethylamine in 2.3 ml of 60% acetone was added slowly with vigorous stirring over the course of 2 min. The slurry was then added to 100 ml of acetone, 0.1 M hydrochloric acid (1:1, v/v) at 0 °C and washed in a sintered glass funnel with cold 60% acetone, 30% acetone, and water. Finally, the washed Sepharose was resuspended in 20 ml of 0.1 M sodium acetate containing 10 mg of crude venom and agitated overnight. The slurry was filtered to separate bound from unbound venom proteins and then mixed with 20 ml of 1 M monooctanolamine and stirred for 2 days.

Purification of Human Factor IX

This was accomplished by modifications of the methods of Miletich et al. (1980) and Pepper and Prowse (1977).

Barium Citrate Precipitation—Ten g of the factor IX concentrate, containing about 10,000 units of factor IX, was dissolved in 10 liters of 0.02 M sodium citrate, pH 6.7, containing 0.25 M sodium chloride, 1 mM benzamidine hydrochloride, 20 mg/liter of soybean trypsin inhibitor, 0.02% sodium azide and was precipitated with saturated barium chloride. The slurry was then separated in a continuous flow centrifuge and diisopropyl fluorophosphosphate and phenylmethylsulfonyl fluoride were added to final concentrations of 10-4 M each. The suspension was mixed 1:1 with the protein fraction which precipitated between 20 and 70% (w/v) saturation. The precipitate was collected. The precipitate was dissolved in a small volume of 0.2 M sodium citrate, pH 6.7, containing 0.25 M sodium chloride, 1 mM benzamidine hydrochloride, 20 mg/liter of soybean trypsin inhibitor, 0.2% sodium azide, and was dialyzed overnight against the same buffer at 4 °C.

Ion Exchange Chromatography on Sulfate-Sepharose—Sulfate-Sepharose was equilibrated with 0.02 M sodium citrate, pH 6.7, containing 0.02 M sodium chloride, 1 mM benzamidine, 20 mg/liter of soybean trypsin inhibitor, 0.02% sodium azide, and was packed in a glass column (10 × 20 cm). The column was eluted with a linear gradient composed of 4 liters each of 0.25 and 1.0 M sodium chloride, in 0.02 M sodium citrate, pH 6.7, 1 mM benzamidine hydrochloride, 20 mg/liter of soybean trypsin inhibitor, 0.02% sodium azide. The fractions which contained factor IX activity but no factor X activity were pooled, concentrated by hollow fiber ultrafiltration, and dialyzed against 0.03 M imidazole HCl, pH 6.0, 1 mM benzamidine, 2.5 mM calcium chloride, 0.02% sodium azide.

Chromatography on Heparin-Sepharose—A column (5 × 50 cm)
was packed with heparin-Sepharose equilibrated in 0.05 M imidazole HCl, pH 6.0, 1 mM benzamidine, 2.5 mM calcium chloride, 0.02% sodium azide. The chromatograph was developed with a linear gradient composed of 1 liter each of 0 and 0.7 M sodium chloride in 0.05 M imidazole HCl, pH 6.0, containing 1 mM benzamidine hydrochloride, 2.5 mM calcium chloride, 0.02% sodium azide. The peak fractions containing the highest concentrations of factor IX activity were pooled and concentrated by membrane ultrafiltration to 0.5 mg/ml and dialyzed against 0.02 M sodium citrate, pH 6.7, 1 mM benzamidine hydrochloride, 0.02% sodium azide. After dialysis, the protein was stored at -80 °C.

The purified factor IX consisted of a single major polypeptide when analyzed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 20 μg/well. Occasionally, small amounts of factor X (by clotting activity) or other unidentified polypeptides were seen. The mean specific activity was 408 ± 82 units/mg (n = 6). An absorbance at 280 nm of 13.3 was assumed to be equal to 1.0 mg/ml of factor IX (DiScipio et al., 1978).

Activation of Factor IX with Russell’s Viper Venom-Sepharose
To 0.1 ml of factor IX at 0.5 mg/ml was added 50 μl of Russell’s viper-venom-Sepharose and 10 μl of 0.1 M calcium chloride. This was incubated for 18 h in a vertical rotator to achieve complete conversion of factor IX to IXα. The Sepharose beads were separated from the factor IXα by centrifugation on a bench top centrifuge.

Cleavage of Sialic Acids from Factor IX Using Neuraminidase
To 0.1 ml of factor IX at 0.49 mg/ml was added 5 μl of neuraminidase at 10.0 units/ml (final solution contained 10 units of neuraminidase/mg of factor IX). This was then incubated at either 25 or 37 °C for various lengths of time.

Electrophoretic Analysis
Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970).

Sialic Acid Assay
Free sialic acid was measured by the procedure of Warren (1959) after protein-bound sialic acid was released by enzymatic cleavage.

Clotting Assays for Factor IX, IXα, and X
A 0.1-ml aliquot of sample, appropriately diluted in 0.02 M imidazole HCl buffer, pH 7.0, was mixed with 0.1 ml of factor IX-deficient plasma and 0.1 ml of activated partial thromboplastin reagent. The mixture was incubated for 5 min at 37 °C. Then 0.1 ml of 25 mM CaCl2 was added and mixed and the clotting time was recorded. Factor IX was measured using the same technique, except that 0.1% (w/v) Centrolex P phospholipid was used in place of the activated partial thromboplastin time reagent. For each set of assays, a standard curve was prepared from the clotting times of dilutions of normal pooled plasma, plotted on semilog paper. Factor IX activity was calculated by interpolation. One unit of factor IX is the amount of factor IX protein present in 1.0 ml of plasma. The assay for factor X was done as for factor IX, except for use of plasma deficient in factor X instead of factor IX.

RESULTS
Approximately half of the total sialic acid was released enzymatically from factor IX during the first 60 min, and thereafter the rate decreased slowly (Table I). After incubation of protein with enzyme for 18 h at 37 °C, 11 residues of sialic acid/mol of protein were released, in good agreement with the total of 10–12 residues reported by DiScipio et al. (1978). There was a progressive loss of clotting activity; after incubation for 6 h, approximately 13% of the original activity remained (Table I). No further decrease in clotting activity was seen, even after incubation for a total of 18 h. The loss of factor IX clotting activity and the enzymatic release of sialic acid followed the same time course. When the decrease in clotting activity is plotted against the loss of sialic acid residues, the experimental points fall on a line with a slope of 0.97, consistent with a direct relationship between the two phenomena (Fig. 1).

In order to see whether proteolytic degradation (rather than desialylation) might be responsible for the loss of factor IX activity, the NH2-terminal amino acid sequence of factor IX was determined before and after incubation with neuraminidase.
The effect on factor IX <sub>III</sub> clotting activity of cleavage of sialic acid

Factor IX was converted completely to factor IX <sub>III</sub> by incubation for 18 h with Russell’s viper venom-Sepharose. The activating enzyme was removed by centrifugation, and the factor IX <sub>III</sub> was incubated with neuraminidase as follows. At 1-h intervals, one aliquot was removed by centrifugation, and the factor IX, was incubated with neuraminidase; was maximal within the first 60 min of incubation and no further changes were seen, even after incubation for 48 h. A similar change in migration was seen with two other glycoproteins, antithrombin III and clotting factor X (result not shown). When ovalbumin (a nonsialylated glycoprotein (Kornfeld and Kornfeld, 1980) was similarly treated with neuraminidase, there was no detectable change in electrophoretic migration (Fig. 2). Thus, the change in electrophoretic migration was seen only with sialylated proteins. These results indicate that proteolytic degradation was unlikely to be responsible for the loss of factor IX activity.

Another possible explanation for the loss of factor IX clotting activity is a decreased solubility of the asialoglycoprotein. This possibility has been ruled out by high pressure liquid chromatography gel permeation chromatography of factor IX. The elution time of the untreated factor IX was 7.36 min and that of the asialylated protein was 7.48 min. The amount of protein recovered was essentially the same in the two preparations. These results provide no evidence for aggregation and/or precipitation of factor IX as a consequence of removal of the negatively charged sialic acid residues.

In order to show that the changes in activity were due specifically to removal of sialic acids from factor IX, several control experiments were performed. First, similar treatment of purified factor X for 6 h resulted in release of 54% of the total sialic acid residues, but no detectable change in the factor X clotting activity. Second, incubation of highly purified factor VIII (Fay et al., 1982) with neuraminidase with or without several other exo- and endoglycosidases did not cause any change in the factor VIII clotting activity. When factor IX was incubated for 6 h at 25°C without neuraminidase, there was no detectable loss of clotting activity. Thus, the loss of clotting activity was seen only with factor IX, and this loss of activity required treatment with neuraminidase.

When the assay reagents were preincubated with neuraminidase for only 10 min, and then used to assay untreated factor IX, no change in measurable factor IX activity was seen. The slopes of the assay times for normal versus desialylated factor IX were parallel. Finally, the addition of free sialic acid to the assay mixture at a concentration of 6 μM (equivalent to the concentration of sialic acid released after 2 h of enzymatic digestion) had no effect on the assay. Taken together, these controls showed that the loss of factor IX activity was not due to effects on the clotting assay.

**DISCUSSION**

The data we have presented show that cleavage of sialic acid results in a decrease in the activity of factor IX (when activation occurs during the assay) and of factor IX <sub>III</sub> (when the active enzyme itself is desialylated). There is no evidence that this effect is caused by proteolysis or by nonspecific inhibitory effects on the clotting assay. Therefore, we propose that the loss of activity is caused in some way by the removal of sialic acids from the factor IX.

Factor IX consists of a single polypeptide chain with a total molecular mass of about 57,000 daltons, comprising a 47,500-dalton polypeptide and a 9,500-dalton carbohydrate (17% w/w), including 10–12 mol of sialic acid/mol of factor IX (DiScipio et al., 1978; Katayama et al., 1979).

The carbohydrate, including the sialic acid residues, of factor IX appears to be in at least 3 different regions of the protein. Twenty-seven per cent is located on the COOH-terminal polypeptide (M<sub>c</sub> = 28,000), 13% is on the NH<sub>2</sub>-terminal polypeptide (M<sub>c</sub> = 18,000), and the remaining 60% is attached to the activation peptide (M<sub>c</sub> = 11,000) (DiScipio et al., 1978). Two potential carbohydrate binding sites on the activation peptide have been identified at Asn 157 and Asn

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1 S. I. Chavin, and S. M. Weidner, unpublished experiments.
2 P. J. Fay, S. I. Chavin, and V. J. Marder, manuscript in preparation.
167, based on the sequence of cDNA for human factor IX (Kurachi and Davie, 1982), but the locations of the other carbohydrate groups are not yet known. In the case of IXα, the activation peptide remains covalently attached to the NH2-terminal end of the protein (Lindquist et al.), so that all sialic acid remains associated with the activated protein and may influence its structure and function. In the case of IXa, it is not known whether the activation peptide remains associated. The activation peptide may or may not remain with the protein, depending upon how tightly it is associated with heavy and light chains; therefore, the influence of the large amount of carbohydrate associated with this peptide is not known. Nevertheless, at least 40% of the original carbohydrate remains covalently attached to the light and heavy chains and may play some role in the structure of the protein.

There are 2 ways in which the sialic acid may influence the structure and function of factor IX. First, because factor IX must be activated in order for it to express its enzymatic activity, any structural changes which affect its interaction with the activating enzymes would result in a decrease in measurable factor IX activity. We have shown that the rates of activation of normal and of desialylated factor IX by Russell’s viper venom are essentially the same. Although we have not yet studied directly the rate of activation of factor IX by factor X, we think that this rate will not be affected significantly by the loss of sialic acid. Second, the enzymatic activity of factor IX itself may be affected by a loss of sialic acid residues. We have shown here that the clotting activity of the factor IXa is reduced markedly by removal of the sialic acid residues, indicating that this second possibility appears to be the more important one.

Many studies have failed to show consistent effects following removal of sialic acids and other carbohydrate constituents on the structure and function of glycoproteins (Warren et al., 1978). In one report, the rate of “autoactivation” of prothrombin to thrombin was considerably faster with desialylated prothrombin than with untreated prothrombin, although the final specific activities were the same (Schwick and Schultz, 1959). Another study showed that following 6-h incubation with neuraminidase, there was cleavage of about 64% of the protein-bound sialic acid, and a reduction of 41% in the specific activity of prothrombin (Tishkoff et al., 1980). A third study also reported a 30% reduction in clotting activity of asialo-prothrombin, although the reduction was attributed to “nonspecific inactivation” (Nelsestuen and Suttie, 1971). A fourth study of asialo-prothrombin failed to show any change of activity following removal of sialic acids (Henriksen et al., 1976).

There have been several recent studies on the sialic acid residues of von Willebrand protein (factor VIII-related protein). Although one group reported that enzymatic removal of more than 90% of the sialic acid from von Willebrand protein resulted in a 50% loss of its platelet aggregating activity (Sodetz et al., 1977), other laboratories have been unable to confirm these results (Gralnick, 1978; DeMarco and Shapiro, 1981; Gralnick et al., 1983).

An acquired abnormality of fibrinogen recently has been reported in which 1.4–3.4 extra residues of sialic acid/molecule of protein were present and appeared to cause a moderate (12–22 s) prolongation in thrombin clotting time and a reduced rate of fibrin monomer aggregation (Martines et al., 1983). Enzymatic removal of the excess sialyl residues apparently restored normal function to the fibrinogens, and complete desialylation appeared to have no further effects on the thrombin time.

Although it is clear, for many proteins, removal of sialic acid does not significantly affect in vitro biological activity (as opposed to survival in circulation), alterations have been noted for some plasma glycoproteins. Factor IX, with an unusually high content of carbohydrate and sialic acid, appears to possess activity which is unusually sensitive to the loss of sialic acids. This uncommon effect deserves further investigation in order to ascertain its structural basis and whether it has functional significance.

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