PROPERTIES OF ASIALO AND AGLYCOPROTHROMBIN

G. L. Nelsestuen* and J. W. Suttie**
Department of Biochemistry, College of Agricultural and Life Sciences
University of Wisconsin, Madison, Wisconsin 53706

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SUMMARY

Native bovine prothrombin was shown to have a circulatory half-life of 90 minutes when injected into the circulatory system of rats. Removal of sialic acid resulted in a circulatory half-life of only 9 minutes and further enzymatic degradation of the carbohydrate restored the circulatory half-life to more nearly that of intact prothrombin. These observations broaden the generality of this phenomenon displayed by some other glycoproteins and therefore the possibility that sialic acid may function in determining the circulatory life of glycoproteins.

The generalized function of the carbohydrate moiety of glycoproteins is not well understood, although a possible function for the sugars (sialic acid - galactose) in the terminal position of several circulatory glycoproteins has been reported (1-3). It has been observed that when the terminal sialic acid residues are removed enzymatically or chemically, the asialoglycoprotein which remains is rapidly removed from the circulation by the liver. If, however, the galactose residue is also removed or altered, this rapid uptake is completely reversed and the glycoprotein displays a normal circulatory half-life. This suggests that the function of the sialic acid residue is to determine the circulatory life of glycoproteins and that galactose is necessary for the recognition of the desialylated glycoprotein by the liver. This property appears to be a common but not universal characteristic of glycoproteins (3).

The physiologically important plasma glycoprotein, prothrombin, contains about 10% carbohydrate and has a terminal carbohydrate sequence of sialic acid -

* National Institutes of Health Postdoctoral Fellow 110 49,281-01
** To whom inquiries and requests for reprints should be addressed.

galactose (5). The rate of removal of administered prothrombin, or prothrombin derivatives, from the circulation can easily be determined as the prothrombin concentration in the recipient animal can be depressed by warfarin administration. Physiological levels of prothrombin can then be injected, and its rate of disappearance followed without a large correction for the animal's own prothrombin. A simple and highly specific assay for this glycoprotein also eliminates the necessity of forming a radioactivity derivative by labeling exposed galactose residues (4) or by alteration of the sialic acid (6).

MATERIALS AND METHODS

Enzymes: Prothrombin was purified from bovine plasma to electrophoretic homogeneity (2600 units/mg) by the method of Ingwall and Sheraga (7), and assayed by a modification (8) of the Ware and Seegers two stage method. Glycosidase preparations containing α-mannosidase (13.1 units/A_{280}), β-N-acetylglucosaminidase (1.2 units/A_{280}), α-galactosidase (.04 units/A_{280}) and β-galactosidase (.08 units/A_{280}) activities were prepared from jack bean meal by the method of Li (9) and assayed by his procedures. Neuraminidase (3 units/mg) and protease from S. griseus (4 units/mg) were obtained from Sigma.

Prothrombin derivatives: Asialoprothrombin was prepared by incubating 0.4 mg of neuraminidase with 30 mg of prothrombin in 2 ml of 01 M phosphate buffer (pH 6.0) for 24 hours. At this time 85% of the sialic acid had been removed as assayed by the thiobarbituric acid assay (10). The prothrombin was selectively adsorbed onto barium citrate (11) washed and dissolved in EDTA and dialyzed exhaustively against 0.15 M NaCl - 1 mM potassium phosphate (pH 7.0).

Aglycoprothrombin was prepared by incubating 7.5 mg of asialoprothrombin with 0.3 units of α-galactosidase, 0.65 units of β-galactosidase, 12.6 units of α-mannosidase, and 1.9 units of β-N-acetylglucosaminidase in 0.9 ml of 0.3 M potassium phosphate buffer (pH 5.4) containing 20 mM diisopropylfluorophosphate (Sigma). After 47 hours at room temperature, about 40% of the hexosamine had been liberated as indicated by the increase in free hexosamine assayed by

the Elson-Mogan reaction (12). This modified prothrombin was then adsorbed onto barium citrate and purified as before.

Asialoglycopeptides were prepared from prothrombin by mild acid hydrolysis (80° for 1 hr in 0.1 N H_2SO_4) of glycopeptides prepared by exhaustive protease digestion of prothrombin. The glycopeptides were purified from amino acid and peptide material by gel filtration and ion exchange chromatography.

Injection procedures: Male albino rats weighing 200 gm were injected intraperitoneally with 5 mg of warfarin per kg body weight about 27 hours before the experiment was begun. The plasma levels of prothrombin had dropped to less than 25 Iowa units per ml. Approximately physiological concentrations of the prothrombin preparations (1 mg) were injected into the exposed jugular vein of the anesthetized rat. At various times, 0.5 ml samples of oxylated blood were withdrawn from the jugular wein or by heart puncture, and prothrombin activity was determined in the plasma.

RESULTS AND DISCUSSION

The asialoprothrombin prepared by the action of neuraminidase retained 100% of its clotting activity per mg of protein and the appearance and disappearance of thrombin activity in the assay system were exactly the same as for normal prothrombin. The aglycoprothrombin retained 70% of its specific activity and, again, the appearance and disappearance of thrombin activity closely paralleled normal prothrombin. The loss of 30% of the specific activity in the aglycoprothrombin may have been due to nonspecific inactivation during the long room temperature incubation. Very long incubations at room temperature resulted in complete loss of activity. Asialoprothrombin has previously been reported to retain nearly full clotting activity (13,14).

The results in Fig. 1 demonstrate that while intact prothrombin had a circulatory half-life of about 90 minutes, the asialoprothrombin had a half-life of only about 9 minutes. The presence of asialoglycopeptides isolated from prothrombin was observed to slightly inhibit the initial disappearance of asialoprothrombin but it did not affect the half-life of asialoprothrombin

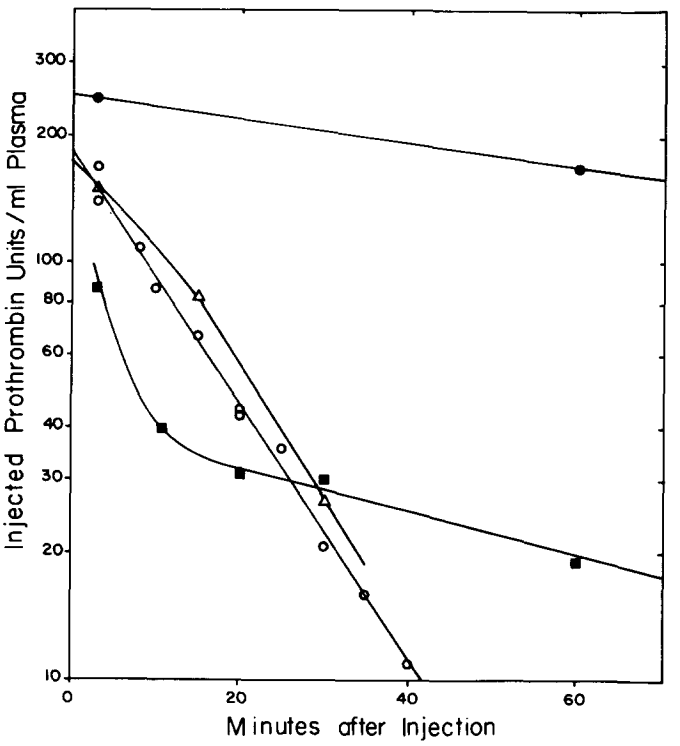

Fig. 1. Disappearance of prothrombin and its derivatives from the circulatory system. The rats were anesthetized with ether, and the jugular vein was exposed. After removing a 0.5 ml sample of blood for determination of the rat's own prothrombin which was subtracted as a blank, about 1 mg (0.25 ml volume) of prothrombin, asialoprothrombin or aglycoprothrombin were injected at zero time. Samples (0.5 ml) were removed at the times indicated and prothrombin activity was determined.

●——●, 2 rats receiving intact prothrombin (samples taken at longer times agreed very well with the slope drawn; o——o, results from 3 different rats receiving asialoprothrombin; Δ——Δ, a single rat receiving 1 mg of asialoprothrombin plus asialoglycopeptide derived from 10 mg of prothrombin (total volume of injection 0.75 ml); ■——■, a single rat receiving aglycoprothrombin.

at longer time periods. These results agree with those reported by Morell et al. (1), who observed that asialoglycopeptides inhibited the uptake of asialoglycoproteins by the liver until the glycopeptides had been substantially removed from the bloodstream.

In the case of the aglycoprothrombin, there was an initial rapid disappearance of prothrombin followed by a more gradual decline. This biphasic disappearance of prothrombin was probably due to lack of complete removal of

carbohydrate by the glycosidase enzymes. Those prothrombin molecules retaining galactose would be rapidly taken up by the liver while those from which galactose and other sugars had been removed would disappear from the circulation at a rate more similar to intact prothrombin. It is clear from this observation that removal of additional carbohydrate residues from asialoprothrombin resulted in a protein with a longer circulation half-life and that in this respect prothrombin behaved similar to ceruloplasmin (3).

The rapid removal of asialoprothrombin from rat circulatory system supports the postulation that the sugar residues function in determining the circulatory life of glycoproteins. The presence of a terminal galactose residue appeared however to be essential for removal by the liver.

It is interesting to note that removal of substantial quantities of the carbohydrate (and essentially all of the sialic acid) had no determinable effect on the catalytic activity of prothrombin nor on the very selective adsorption of this protein by certain barium salts. Evidently then, neither the enzymes responsible for activation of prothrombin nor those responsible for inactivation of thrombin depend on an interaction with the carbohydrate moiety. It has previously been reported that partial removal of the carbohydrate of thrombin has no effect on its clotting activity (15).

It has been shown that the action of vitamin K in regulating prothrombin biosynthesis involves the conversion of a preformed polypeptide chain to prothrombin (16), and the glycosylation system has been suggested as the specific site involved (17,18). This demonstration that substantial carbohydrate removal does not affect prothrombin activity would suggest that such a precursor should have biological activity. However, prothrombin produced by warfarin treated animals has been reported to have a substantially lowered specific activity (19,20).

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