SIALIC ACID RESIDUES INHIBIT PROTEOLYTIC DEGRADATION OF DOPAMINE β-HYDROXYLASE

D. AQUINO, R. WONG, R. U. MARGOLIS and R. K. MARGOLIS

Department of Pharmacology, New York University Medical Center, New York, NY 10016 and *Department of Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, NY 11203, USA*

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1. Introduction

Dopamine β-hydroxylase (EC 1.14.2.1) is a tetrameric glycoprotein with a subunit mol. wt 75 000, and a carbohydrate content of ~4% [1,2]. It is present in catecholamine storage vesicles of both adrenal medulla and sympathetic nerve terminals, where it catalyzes the biosynthesis of norepinephrine from dopamine. Approximately equal amounts of the enzyme occur in soluble and membrane-bound forms in adrenal chromaffin granules [3], whereas most of the dopamine β-hydroxylase in large, dense-cored sympathetic nerve vesicles is soluble [4]. Dopamine β-hydroxylase is also present in the plasma [5] due to its release by exocytosis from storage organelles.

The soluble and membrane-bound forms of the enzyme isolated from adrenal chromaffin granules appear to be identical in many respects [3] including their carbohydrate composition [2], and it has been reported that enzymatic removal of 4 of the 7 mannose residues present in the dopamine β-hydroxylase monomer does not result in the loss of any catalytic activity [6]. Therefore, in spite of the extensive interest from a number of standpoints in this key enzyme in catecholamine biosynthesis, the function of its carbohydrate moiety is presently unknown.

There is, however, recent evidence that glycosylation decreases the sensitivity of certain proteins to proteolytic degradation [7,8]. It has also been reported that mild trypsin treatment of the soluble proteins from adrenal chromaffin granules resulted in the rapid degradation of the chromogranins, whereas dopamine β-hydroxylase survived this mild proteolysis without change in affinity for tyramine, loss of immunoreactivity or change in electrophoretic mobility [9]. Since the carbohydrate moiety may serve to protect it from proteolytic degradation, we have examined the effect of enzymatic deglycosylation on the susceptibility of dopamine β-hydroxylase to tryptic proteolysis.

Address correspondence to: Dr Richard U. Margolis, Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, NY 10016, USA

2. Experimental

Chromaffin granules were isolated from bovine adrenal medullae by differential centrifugation followed by a simplified sucrose density gradient [10] modified as in [2]. The granules obtained from 20 adrenals were lysed by homogenizing in 90 ml 5 mM Tris–sodium succinate buffer (pH 5.9) followed by freezing and thawing. The suspension was then centrifuged for 1 h at 190 000 × g, the pellet rehomogenized in 90 ml buffer, and the freezing, thawing and centrifugation steps repeated as above. The combined supernatants were either dialyzed against deionized water and lyophilized for the large-scale isolation of dopamine β-hydroxylase and other soluble granule proteins, or the supernatant resulting from the initial lysis step was used directly for ion-exchange chromatography and measurement of enzyme activity [11].

The soluble chromaffin granule proteins were dissolved (at ~3 mg/ml) in 50 mM Tris–HCl buffer (pH 8.2 at 4°C) containing 90 mM NaCl. This was then applied to a column of DEAE-cellulose (Whatman DE-52) equilibrated with the same buffer. The first of

the 3 peaks eluted at this [NaCl] contained all of the dopamine β-hydroxylase activity, and migrated as a single Coomassie Blue-stained band (with a subunit mol. wt 73 000) in 7% SDS–polyacrylamide gels [12], or in 6% polyacrylamide gels using a Tris–glycine buffer system [13]. The chromogranins and two species of chondroitin sulfate proteoglycans [14] were later eluted from the DEAE-cellulose column using higher [NaCl].

Dopamine β-hydroxylase (DBH), at 10 mg/ml, was incubated for 8 h at 37°C with *Vibrio cholerae* neuraminidase (13.5 units/mg DBH) in 50 mM sodium acetate buffer (pH 6.0) containing 0.15 M NaCl and 5 mM $CaCl_2$. The incubation mixture was then dialyzed for 2 days against 2 changes (100 vol. each) of distilled water at 4°C, and lyophilized.

In some experiments the DBH was first treated (in the pH 6 buffer) with both neuraminidase and rat epididymis L-α-fucosidase (0.012 units/mg), and after dialysis the lyophilized material was redissolved at the original concentration in 50 mM sodium citrate buffer (pH 4.5) for a further 8 h incubation at 37°C with Jack Bean β-galactosidase (0.026 units/mg), β-*N*-acetylglucosaminidase (0.05 units/mg) and α-mannosidase (0.18 units/mg).

Sialic acid was determined by the periodate–resorcinol method [15] and glucosamine was determined using the amino acid analyzer (after deacetylation in 2 N HCl for 3 h at 100°C, in the case of *N*-acetylglucosamine released by glycosidase treatment). Mannose, galactose and fucose were assayed by automated ion-exchange chromatography of their borate complexes [16] using a gradient containing 100 ml each of 0.27 M sodium borate buffer (pH 7.7) and 0.4 M borate buffer (pH 10) with a 0.3 × 70 cm column of Hamilton HA × 6 resin. The neutral sugar concentration of the original DBH sample was determined after hydrolysis for 3 h in 2 N trifluoroacetic acid at 100°C, and amino sugars were determined after hydrolysis for 22 h in 6 N HCl at 110°C (with correction for destruction during hydrolysis).

The glycosidase-treated DBH was incubated with trypsin for varying times at 37°C in 20 mM Tris–HCl buffer (pH 7.5) containing 3.6 mM $CaCl_2$. The tryptic digestion was terminated by the addition of soybean trypsin inhibitor in a ratio of 2.5 mg inhibitor/mg trypsin.

Neuraminidase was obtained from Behringwerke/Calbiochem. All other enzymes and soybean trypsin inhibitor were purchased from Sigma, St Louis, MO.

3. Results and discussion

Treatment of dopamine β-hydroxylase with neuraminidase, L-α-fucosidase, β-galactosidase, β-*N*-acetylglucosaminidase, and α-mannosidase under optimum conditions released >90% of the sialic acid but <20% of the fucose or other (mostly internal) sugar residues. The resistance of intact glycoproteins, as compared to glycopeptides, to enzymatic deglycosylation is a well-known phenomenon, and was therefore not unexpected in this case. However, 24–48 h incubation with an α-mannosidase of unspecified origin removed over half of the mannose residues from DBH without prior or concomitant treatment with any other glycosidases [6], suggesting that many of the mannose residues are present in terminal positions, or that their enzyme preparation was contaminated with other glycosidase activities.

Dopamine β-hydroxylase treated with neuraminidase alone or neuraminidase followed by other glycosidases migrated on polyacrylamide gels as a single band with the same mobility as untreated DBH (fig.1). However, whereas the original DBH was largely resistant to trypsin proteolysis, as in [9], desialylated DBH was almost completely degraded. Shorter periods of incubation or lower ratios of trypsin to desialylated DBH resulted in less digestion of the DBH but did not produce degradation products having a greater electrophoretic mobility. The slight effect of trypsin on 'native' DBH before treatment with neuraminidase can probably be attributed to an action on those molecules lacking their full complement of sialic acid residues, in agreement with the known microheterogeneity of glycoproteins.

The oligosaccharide structure of glycoproteins may be of considerable importance in determining their biological activities. In other cases the carbohydrate moiety appears to function as a signal for the secretion of certain proteins, or for their recognition and/or uptake by cells and tissues. The extensive studies of Ashwell, Morell and coworkers (reviewed in reference [17]) indicate a generalized role for the terminal sialic acid residues of circulating glycoproteins. These investigations have demonstrated that terminal galactose residues, exposed by enzymatic removal of sialic acid, constitute a structural configuration recognizable by receptors present on hepatocyte plasma membranes, and capable of initiating prompt clearance and catabolism of such asialoglycoproteins. However, in the case of many proteins the functional significance

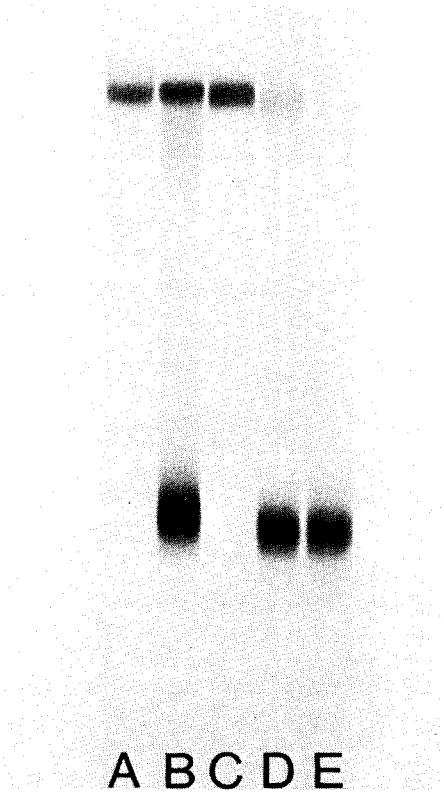

Fig.1. Polyacrylamide gel electrophoresis in Tris–glycine buffer of: (A) 30 μg dopamine β-hydroxylase; (B) 30 μg DBH incubated for 6 h with 3 μg trypsin; (C) 30 μg desialylated DBH incubated for 6 h; (D) 30 μg desialylated DBH incubated for 6 h with 3 μg trypsin; (E) 3 μg trypsin plus soybean trypsin inhibitor. Incubation and electrophoresis conditions were as in section 2.

of glycosylation is unknown.

The studies reported here demonstrate that the presence of sialic acid residues greatly increases the resistance of dopamine β-hydroxylase to tryptic proteolysis. This may be a general function of glycosylation in many glycoproteins. The addition of carbohydrates at the β-turns of proteins was proposed to result in the masking of the turn conformations [18], and could therefore be an important factor in protecting the molecule from proteolysis. It was later shown that inhibition of glycosylation does not affect the biosynthesis or secretion of the common precursor to ACTH and β-lipoprotein, but did result in an enhanced proteolysis of the unglycosylated precursor, and its processing to a set of atypical peptides which were secreted by the pituitary [7]. It has also been reported [8] that inhibition of glycosylation of fibronectin, a major cell surface glycoprotein, did not affect its synthesis, secretion or biological activity, but greatly increased its sensitivity to proteolysis. These reports, together with these findings concerning dopamine β-hydroxylase, support the concept that in many glycoproteins the carbohydrate moiety may serve to inhibit proteolytic degradation. In the case of dopamine β-hydroxylase it is apparently only the terminal sialic acid residues which are required for this effect, a situation analogous to the specific role of sialic acid in regulating the circulation time of serum glycoproteins [17]. Our data also provide the first indication concerning the functional role of sugar residues in this central enzyme of catecholamine metabolism.

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