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CHARACTERIZATION OF SIALYLTRANSFERASE ACTIVITY FROM HUMAN PLACENTA

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Summary

Crude soluble sialyltransferase (CMP-*N*-acetylneuraminate:D-galactosylglycoprotein *N*-acetylneuraminyltransferase, EC 2.4.99.1) activity from human placenta is characterized with acid-treated fetuin and ceruloplasmin as acceptors. Two forms of sialyltransferase in human placental extract can be demonstrated by using either Sephadex G-200 or concanavalin-A-Sepharose column chromatography. The large species (apparent molecular weight larger than 200 000) does not bind to concanavalin-A Sepharose, while the small one (apparent molecular weight about 80 000) does bind to concanavalin-A-Sepharose, and can be eluted with 5% α-methylmannoside. The K_m values of the CMP-sialic acid substrate are different for these two forms of sialyltransferase. No divalent cation requirement can be demonstrated, and 10 mM EDTA stimulates the reaction 2—3-fold. Folic acid and its derivatives, including methotrexate, are inhibitory at concentrations of millimolar range. Kinetic studies indicate that folic acid acts as a competitive inhibitor with a K_i of 1.1 mM. While heparin inhibits the transferase reaction, protamine acts as a stimulator in this crude preparation. Sialyltransferase activity is inhibited by 0.1 M iodoacetate and 1 mM *p*-chloromercuribenzoyl sulfonate, suggesting that an intact sulfhydryl group of a methionine residue is involved in the active site of the enzyme.

Introduction

Sialyltransferase (CMP-*N*-acetylneuraminate:D-galactosyl-glycoprotein *N*-acetylneuraminyltransferase, EC 2.4.99.1) is involved in transfer of sialic acid resi-

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The abbreviations used are: sialyltransferase L, large form of sialyltransferase; sialyltransferase S, small form of sialyltransferase; CMP-sialic acid, cytidine 5′-monophosphate sialic acid.

dues from CMP-sialic acid to suitable glycoprotein acceptors [1]. This reaction is responsible for the biosynthesis of the terminal portion of the oligosaccharide prosthetic group of sialoglycoproteins [2]. Increasing interest in this enzyme is the result of the finding that neoplastic transformation of cells may be accompanied by alterations in the composition and metabolism of cellular sialoglycoproteins [3]. As compared with values in normal adults, elevated levels of sialyltransferase have been reported in human breast and colon tumor tissues [4], plasma of patients with neoplastic disease [5,6], kidney tissues of patients with chronic renal failure [7], and in the fetal liver [8]. The properties of normal human tissue sialyltransferase in regard to effectors and possible existence of isozymes have not been ascertained. In this paper, we have found in human placental extract two forms of sialyltransferase, and their properties are partially characterized.

Materials and Methods

Chemicals

Iodoacetic acid, *p*-chloromercuribenzoyl sulfonic acid, and α-methylmannoside were obtained from Sigma, concanavalin-A from Calbiochem, pteroylglutamic acid (folic acid), 5-formyl tetrahydrofolic acid (leucovorin) and methotrexate from Lederle, protamine sulfate and sodium heparin from Upjohn. 5-formyl folic acid was a kind gift from Dr. R.L. Blakely, The University of Iowa, Iowa City, Iowa.

Preparation of crude extract

Fresh human placenta was obtained directly from the nearby delivery room and cooled in crushed ice. After several washes with cold normal saline solution, the membrane was peeled off. The placenta was then sliced into small pieces, washed three times with 500 ml of 1 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA/0.05% KCL/0.8% NaCl, and once with 500 ml of 20 mM sodium phosphate buffer, pH 7.4, containing 1 mM $MgCl_2$/2 mM mercaptoethanol/0.1% Triton X-100. The slices were homogenized in a Waring Blendor for 5 min at high speed and the homogenate was centrifuged at 1500 × *g* for 30 min and 10 000 × *g* for 1 h. The supernatant thus obtained was designated placental extract. These procedures were carried out at 2—5° C.

Preparation of sialyltransferase L and sialyltransferase S by Sephadex G-200 column

A 2.5 × 90 cm colum was packed with Sephadex G-200 (Pharmacia Fine). After being equilibrated with 500 ml 20 mM sodium phosphate, pH 7.0 containing 1 mM $MgCl_2$/1 mM mercaptoethanol/0.1% Triton X-100 (Buffer T), the column was layered with 5 ml of placental extract (39 mg protein/ml). The column was eluted with Buffer T and every third fraction (5 ml per fraction) was assayed for sialyltransferase activity and for absorbance at 280 nm. Fractions 26—33 (see Fig. 1) were combined and concentrated to about 5 ml by an Amicon ultracentrifugation cell (Model 52, Amicon) using PM-10 Diaflo membranes. The concentrate is designated sialyltransferase L. Similarly, fractions 45—54 (see Fig. 1) were also combined and concentrated to about 5 ml, and designated sialyltransferase S.

Assay of sialyltransferase

The assay method of sialyltransferase activity was essentially as described by Grimes [9]. The method was modified by using Millipore filters to separate CMP-sialic acid from the sialylated protein product. A typical reaction mixture contained: 10 μl of 0.35 M sodium phosphate buffer, pH 6.9/7 mM $MgCl_2$/0.7% Triton X-100/10 μl of desialylated fetuin (50 mg/ml)/0.05—1.0 mg protein of placental extract/30 nmol of CMP-[^{14}C]sialic acid (New England Nuclear, about 5000 cpm/nmol). Final volumes were made to 75 μl with water. Incubations were carried out at 37°C for 1 h. The reaction was stopped by adding 1 ml 10% trichloroacetic acid, and the sialylated product was retained on Millipore filters (Millipore Corp., HAWP02500). The filters were then washed three times with 1 ml 10% trichloroacetic acid, put into vials with 10 ml of Aquasol (New England Nuclear) and counted in a liquid scintillation counter (Beckman, LS-250). Fetuin (Spiro method, GIBCO) and human ceruloplasmin (Sigma) were desialylated by acid hydrolysis as described by Bosmann [10].

We found that approximately 5% of the total radioactivity in the CMP-[^{14}C]-sialic acid preparation was in the form of sialic acid, using paper chromatography in a solvent consisting of 1.0 M ammonium acetate/ethanol (pH 7.5, 30 : 75, v/v) [11]. This value was not increased upon incubation with various fractions of enzyme. When purified CPM-[^{14}C]sialic acid was used in the assay mixture, the results were similar to the unpurified preparation.

Results

General characteristics of sialyltransferase

The assay system for sialyltransferase activity from human placenta was linear with respect to time for up to 2 h. The activity was also linear with respect to the amount of placental extract added in the assay mixture. On the basis of this data, all of the assays were conducted within the linear limits for time and amount of enzyme. The absolute requirements for enzyme activity with asialofetuin as acceptor are shown in Table I. There was some activity when non-

TABLE I

REQUIREMENTS FOR PLACENTAL SIALYLTRANSFERASE ACTIVITY

The complete assay mixture and assay conditions are described in Materials and Methods. The results are from a single experiment; duplicate assays were done with 1.5 μl of placental extract (39 mg protein/ml), at 37°C for 1 h.

Incubation mixture	Trichloroacetic acid precipitate (cpm)
Complete assay mixture	1013
Minus placenta extract	72
With heat-inactivated extract *	75
Minus asialo-fetuin	78
With 0.5 mg of fetuin	150
Minus Triton X-100	815
Minus $MgCl_2$	903
Plus 1 mM EDTA	1056
Plus 10 mM EDTA	2112

* The extract was boiled for 3 min.

treated fetuin was used, which may have been due to contamination of desialylated fetuin in the commercial preparation. Neither Triton X-100 nor $MgCl_2$ stimulated the activity. Dialysis of placental extract in the absence of 2 mM $MgCl_2$ caused 50% loss of enzyme activity. Inclusion of 2 mM $MgCl_2$ in the incubation mixture following dialysis did not recover the loss of activity. Preincubation of the placental extract with 2 mM EDTA at 37°C for 1 h resulted in a 50% decrease in activity. Other divalent cations (Ca^{2+}, Ba^{2+}) did not increase activity significantly either. Therefore, no divalent cation seems to be required but Mg^{2+} may help stabilize the enzyme. Interestingly, assay in the presence of 10 mM EDTA stimulated the activity 2—3-fold in the presence or absence of 10 mM $MgCl_2$. The mechanism of stimulation by EDTA is not clear.

The effects of CMP-[^{14}C]sialic acid and desialylated fetuin concentrations on the rate of sialoglycoprotein production using placental extract were studied. The results showed the general form expected for "saturation" of an enzyme by its substrates. Using the Lineweaver-Burk plot, the K_m values of CMP-sialic acid and asialo-fetuin were determined to be 0.27 mM and 0.3 mM respectively.

Two forms of sialyltransferase

When placental extract was fractionated on a calibrated G-200 Sephadex column, two peaks of sialyltransferase activity appeared (Fig. 1). One peak eluted immediately after the void volume, indicating an apparent molecular weight larger than 200 000 (sialyltransferase L), and the other peak was estimated to be 80 000 (sialyltransferase S). Only about 25% of the layered sialyltransferase activity was recovered from the G-200 column. To test the possibility that some unknown factors were separated from the sialyltransferase, assays with various combined fractions from the Sephadex G-200 column were performed and no increase in sialyltransferase activity was found. Regardless of the great loss of total sialyltransferase, equal amounts of sialyltransferase L and sialyltransferase S were routinely obtained. The specific activities of the sialyltransferase L and sialyltransferase S preparations from the G-200 were lower than that of placental extract.

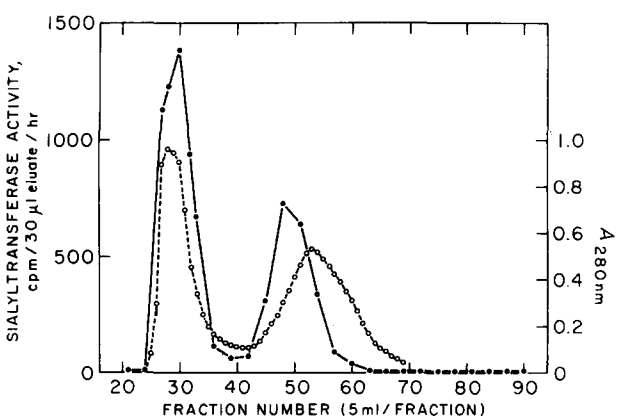
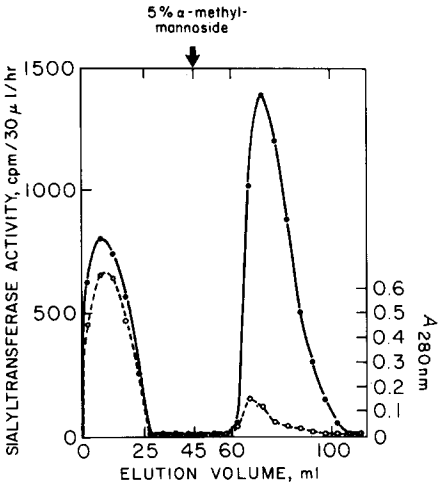


Fig. 1. The Sephadex G-200 elution pattern of placental sialyltransferase activity. Details are described in Methods and Materials. Every third (5-ml) fraction was measured for its sialyltransferase activity (●———●) and absorbance at 280 nm (○- - - - -○).



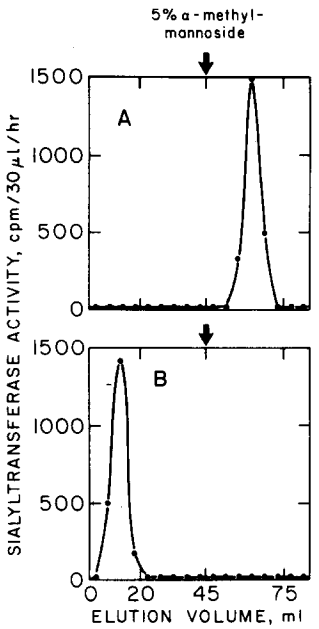


Fig. 2. The concanavalin-A-Sepharose elution pattern of placental sialyltransferase activity. A 25-ml column was packed with concanavalin-A-Sepharose (Pharmacia Fine), equilibrated with 60 ml of Buffer T and 10 ml of placental extract was added. The column was eluted with 45 ml of Buffer T and then eluted with 5% α-methylmannoside in Buffer T. 5-ml fractions were collected and assayed for its absorbance at 280 nm (●———●) and sialyltransferase activity (○- - - - -○).

Fig. 3. The concanavalin-A-Sepharose elution patterns of placental silayltransferase activity. The column was packed and eluted as described in Fig. 2. 5-ml fractions were collected and assayed for sialyltransferase activity. (A) Sialyltransferase S. (B) Sialyltransferase L. Preparations of sialyltransferase L and sialyltransferase S are described in Methods and Materials.

When placental extract was passed through a concanavalin-A-Sepharose column (Fig. 2), part of the sialyltransferase activity was bound to the column and was eluted by using 5% α-methylmannoside. However, regardless of the amount of extract added to the concanavalin-A-Sepharose column, there was always enzyme activity in the direct buffer eluate, and the ratio between bound and unbound forms of sialyltransferase was reproducibly found to be about 1 to 1. This suggested that there were at least two types of sialyltransferase in the placental extract, one binding to convanavalin-A-Sepharose, the other not. Concanavalin-A-Sepharose was therefore used to characterize the two forms of sialyltransferase from Sephadex G-200. Sialyltransferase S was found to be totally bound to the concanavalin-A-Sepharose column and could be eluted with 5% α-methylmannoside (Fig. 3A). In contrast, the sialyltransferase L from the G-200 Sephadex column did not bind to the concanavalin-A-Sepharose column under the conditions stated in Fig. 2. The specific activity of the sialyltransferase S preparation from concanavalin-A-Sepharose was 5 times higher than that of placental extract, while the sialyltransferase L preparation was lower. It is, therefore, possible that there are at least two forms of sialyltransferase in human placental extract with differences in molecular weight and affinity to concanavalin-A-Sepharose.

TABLE II

K_m VALUES CALCULATED WITH LINEWEAVER-BURK PLOT

Preparations of placental extract, sialyltransferase S and sialyltransferase L are described in Methods and Materials. All values are mM.

Substrate	Placental extract	Sialyltransferase L	Sialyltransferase S
CMP-Sialic acid	0.27 ± 0.08	0.53 ± 0.10	0.81 ± 0.04
Asialo-fetuin	0.30 ± 0.10	0.13 ± 0.05	0.13 ± 0.03
Asialo-ceruloplasmin		0.072 ± 0.008	0.065 ± 0.007

Kinetic parameters were measured in order to characterize these two forms of sialyltransferase (Table II). The two forms of sialyltransferase exhibited different K_m values for CMP-sialic acid as substrate (sialyltransferase S, 0.81 mM; sialyltransferase L, 0.53 mM). In contrast, sialyltransferase L and sialyltransferase S showed no significant differences in protein substrate specificity for asialo-fetuin and asialo-ceruloplasmin.

Effect of inhibitors and activators on sialyltransferase activity

Folic acid has been shown to inhibit rat liver and kidney sialyltransferase activity [12]. Folic acid and its derivatives, including Methotrexate, at 1.5 mM also inhibited human placental sialyltransferase activity (Table III). Sialyltransferase S was more sensitive to folic acid inhibition than sialyltransferase L. Kinetic studies using Dixon's method [13] indicated that folic acid acted as a competitive inhibitor acting at the CMP-sialic acid site, with a K_i of 1.1 mM (Fig. 4).

p-Chloromercuribenzoyl sulfonate, a sulfhydryl inhibitor, at 1 mM decreased the enzyme activity to about 20% of the original activity (Table III). The data

TABLE III

THE EFFECT OF VARIOUS REAGENTS ON SIALYLTRANSFERASE ACTIVITIES OF PLACENTAL EXTRACT, SIALYLTRANSFERASE L AND SIALYLTRANSFERASE S

The preparations of placental extract, sialyltransferase L and sialyltransferase S are described in Materials and Methods. The results are from a single experiment; duplicate assays were done at 37°C for 1 h.

Reagent added	% activity		
	Extract	Sialyltransferase L	Sialyltransferase S
None	100	100	100
Concanavalin A, 1 mg/ml	97	—	98
Folic acid, 1.5 mM	54	59	40
N5-Formyltetrahydrofolate, 1.5 mM	60	—	—
Formyl folate, 1.5 mM	61	—	—
Methotrexate, 1.5 mM	69	—	—
Protamine, 60 μg/ml	150	165	130
Heparin, 60 μg/ml	28	29	27
Iodoacetate, 10 mM	75	—	—
Iodoacetate, 0.1 M	9	—	—
p-Chloromercuribenzoyl sulfonic acid, 1 mM	23	—	—

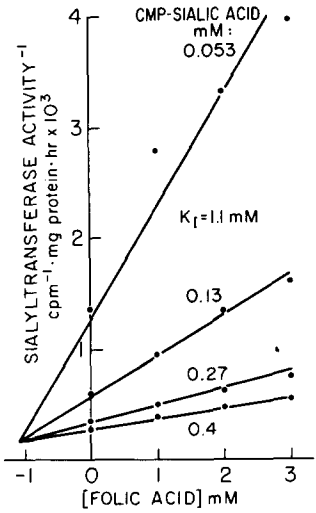


Fig. 4. Dixon plot of the folic acid inhibition of placental silayltransferase activity with different concentrations of CMP-sialic acid.

suggested that an intact sulfhydryl group was important for the enzymatic activity. Iodoacetate, which specifically carboxymethylates methionine residues [14] also inhibited most of the enzymatic activity at a concentration of 0.1 M. It is possible, therefore, that the active site of sialyltransferase contains methionine residues and that the active form of the enzyme requires an intact sulfhydryl group of methionine.

Heparin at a concentration of 60 μg/ml inhibited the sialyltransferase activity, while protamine at 60 μg/ml activated the enzymatic activity (Table III). Sialyltransferase S was less stimulated by protamine. When both heparin and protamine at equal concentrations were included in the incubation mixture, the activity was unaffected, as shown in Table IV. Both heparin and protamine

TABLE IV

THE EFFECTS OF HEPARIN AND PROTAMINE ON PLACENTAL SIALYLTRANSFERASE ACTIVITY

The assay mixture and assay conditions are described in Materials and Methods, but placenta extract and asialo-fetuin were added only when indicated. The results are from a typical experiment; duplicate assays were done at 37°C for 1 h.

Placental extract (5 μl)	Asialo-fetuin (0.5 mg)	Protamine (60 μg6ml)	Heparin (60 μg/ml)	Trichloroacetic acid precipitate (cpm)
+	+			3444
	+			68
+				150
+	+	+		5474
+		+		151
	+	+		63
+	+		+	1304
+			+	88
	+		+	62
+	+	+	+	3369

acted minimally as acceptors, and neither contained sialyltransferase activity. Although sialyltransferase S binds to the concanavalin-A-Sepharose, concanavalin-A does not inhibit sialyltransferase S activity (Table III).

Discussion

Two forms of sialyltransferase with differences in molecular size and affinity to concanavalin-A-Sepharose are demonstrated in human placenta. In contrast, human colostrum [15], human serum and culture medium from a human hepatocellular carcinoma cell line [6], which are extracellular fluids, contain only the small form of sialyltransferase. We speculate that the large form is the cellular component and responsible for synthesis of sialoglycoprotein, while the small form is a secretory protein whose function is not understood. It is unlikely that the sialyltransferase L is membrane-bound since centrifugation of the placental extract at 100 000 $\times g$ for 1 h does not sediment sialyltransferase L significantly and addition of 0.1% Triton X-100 [16] to the assay mixture caused only slightly increased activity.

Sialyltransferase S may have less affinity to CMP-sialic acid than sialyltransferase L, since it has a higher K_m value for CMP-sialic acid (Table II). This is further supported by the fact that sialyltransferase S may be more sensitive than sialyltransferase L to inhibition by folic acid (Table III) which competes with CMP-sialic acid at the CMP-sialic acid site. However, we should note that the differences between sialyltransferase L and sialyltransferase S in K_m for CMP-sialic acid (0.53 mM and 0.81 mM) and in folate inhibition (41 and 60%) were small. Since CMP-sialic acid is the only known sialic acid donor in the sialylation reaction, a decrease in affinity to the CMP-sialic acid would decrease the sialylation of glycoproteins in general.

Sialyltransferases may also differ in their specificity to various asialoglycoproteins. The existence of these sialyltransferases would result in more sialylation of certain glycoproteins. For example, virus-transformed chick embryo fibroblasts contain a sialyltransferase which is relatively specific toward endogenous acceptors but non-specific toward desialylated fetuin or bovine submaxillary mucin [17]. Although we do not find significant differences with respect to ability to sialylate exogenous acceptors between these two forms of enzyme, further studies using suitable endogenous acceptors should be made.

The K_m values of the CMP-sialic acid substrate for sialyltransferase L and sialyltransferase S are two to three times higher than when unfractionated placental extract is used as the enzyme source. Sialyltransferase K_m values in crude lymphocyte preparation [18] and serum [19] are similar to the K_m for unfractionated placental extract. It is possible that some unknown activator is lost in the fractionation process, which may explain why many purification methods have given low yields in terms of activity. However, by recombination assay of various fractions from Sephadex G-200, we failed to find any stimulatory factor of sialyltransferase activity.

The inhibitory effect of heparin on placental sialyltransferase activity may be due to its polyanionic character, causing it to combine with many basic proteins. Viral and bacterial neuraminidase have been shown to be inhibited by heparin through its polyanionic character [20]. Polycationic protamine may

cause activation of placental sialyltransferase by preventing heparin-like mucoproteins in the placental extract from taking effect. A similar result is found in human serum. Serum mucoprotein has been shown to have heparin-like properties [21]. Therefore, the levels of sialyltransferase activity in the serum could be significantly effected by the presence of varied amounts of serum mucoproteins associated with different diseases. Thus, reduction or elevation of serum mucoprotein levels in patients with uncomplicated hepatitis, portal cirrhoris, multiple myeloma or with advanced cancer [22] could affect sialyltransferase activity.

As this manuscript was being revised for publication, two reports appeared in the literature. We reported [23] that the sialyltransferase secreted from a human hepatoma cell line also exists in at least two forms with molecular weights of about 80 000 and 60 000. Since these two forms differ in their affinity to concanavalin-A-Sepharose, we suggest that the carbohydrate contents of these sialyltransferase are different. Paulson, Beranek and Hill [24] have extensively purified the sialyltransferase from bovine colostrum (44 000-fold purification) and found two species with molecular weights of 56 000 (sialyltransferase I) and 43 000 (sialyltransferase II) as determined by sodium dodecyl sulfate gel electrophoresis and sedimentation equilibrium. By gel filtration, the sialyltransferase I had a molecular weight of 80 000. There is no difference between these two species with regard to substrate specificity and kinetics. However, the purified sialyltransferase (both sialyltransferase I and sialyltransferase II) forms only sialyl $\alpha 2 \rightarrow 6$ lactose while partially purified preparations from bovine or goat colostrum have been found to synthesize both sialyl $\alpha 2 \rightarrow 6$- and sialyl $\alpha 2 \rightarrow 3$-lactose [25]. This suggests that a species of sialyltransferase activity may have been lost during purification.

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