Glycoconjugate Journal (1993) **10**:109–115

Regulation of biosynthesis of *N*-glycolylneuraminic acid-containing glycoconjugates: characterization of factors required for NADH-dependent cytidine 5′monophosphate-*N*-acetylneuraminic acid hydroxylation

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Received 17 November 1992

The hydroxylation of CMP-NeuAc has been demonstrated to be carried out by several factors including the soluble form of cytochrome b_5. In the present study, mouse liver cytosol was subjected to ammonium sulfate fractionation and cellulose phosphate column chromatography for the separation of two other essential fractions participating in the hydroxylation. One of the fractions, which bound to a cellulose phosphate column, was able to reduce the soluble cytochrome b_5, using NADH as an electron donor. The other fraction, which flowed through the column, was assumed to contain the terminal enzyme which accepts electrons from cytochrome b_5, activates oxygen, and catalyses the hydroxylation of CMP-NeuAc. Assay conditions for the quantitative determination of the terminal enzyme were established, and the activity of the enzyme in several tissues of mouse and rat was measured. The level of the terminal enzyme activity is associated with the expression of *N*-glycolylneuraminic acid in these tissues, indicating that the expression of the terminal enzyme possibly regulates the overall velocity of CMP-NeuAc hydroxylation.

Keywords: CMP-NeuAc hydroxylase, *N*-glycolylneuraminic acid, H-D antigen, cytochrome b_5

Abbreviations: CMP, cytidine 5′-monophosphate; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; DTT, dithiothreitol.

Introduction

Sialic acids of glycoconjugates play significant roles in biological phenomena involving recognition [1], such as cell adhesion [2], lymphocyte homing [3], tumour metastasis [4], viral infection [5], and the clearance of serum glycoproteins [6]. Sialic acid is a generic name given to various kinds of derivative of neuraminic acid and constitutes a large family [1, 7]. Among these derivatives, *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) are two major members in terms of *N*-acyl modification.

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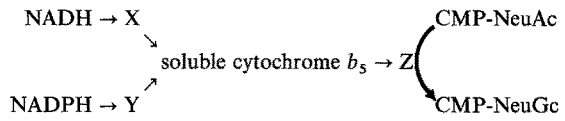
The amount of NeuGc relative to NeuAc in glycoconjugates varies among animal species, tissues, and sometimes strains of one species [8–10]. Among mammals, man is remarkable, because NeuGc is not detected at all in normal conditions, and the expression of NeuGc is oncofetal [11]. A change of NeuGc expression in normal development was also reported in rat [12]. These observations suggest that there must be several different types of regulation mechanism that control the expression of NeuGc in glycoconjugates.

NeuGc is a hydroxylated derivative of NeuAc, and the hydroxylation was proposed to take place at free NeuAc, CMP-NeuAc and NeuAc-containing glycoconjugates. It has been demonstrated that the hydroxylation of CMP-NeuAc

is the most active and is carried out by soluble proteins in the cytosolic fraction of porcine submandibular gland [13]. In addition, a correlation between the level of CMP-NeuAc hydroxylation activity in the cytosol and the expression of NeuGc-gangliosides has been shown in the small intestine of rat [14]. Similarly, the magnitude of the hydroxylation activity of rat and mouse liver was reported to be correlated with the amount of NeuGc expressed [15]. Thus, the activity of CMP-NeuAc hydroxylation is assumed to play a key role in the regulation of the biosynthesis of NeuGc-containing glycoconjugates.

CMP-NeuAc hydroxylation was reported to require NADH or NADPH [16]. Our previous studies with the mouse liver cytosolic fraction demonstrated that the hydroxylation of CMP-NeuAc was carried out by more than one enzyme, and was inhibited by anti-cytochrome b_5 antibody [17]. Further studies indicated that the required factors were separated into two fractions on DEAE-Sepharose chromatography. Both fractions were necessary to reconstitute the CMP-NeuAc hydroxylation reaction, and we succeeded in reconstituting the hydroxylation reaction with the soluble type of cytochrome b_5 purified from horse erythrocyte lysate in place of one of the two fractions [18]. On the basis of these results together with others, we proposed that CMP-NeuAc hydroxylation is a monooxygenase type of reaction and is carried out by an electron transport system involving soluble cytochrome b_5, as indicated below.



where X, Y and Z are unidentified components.

The hydroxylation is carried out by such a complex system that we need to establish assay conditions giving quantitative results. This is also required for the purification of unidentified components. In this paper, we report the assay conditions for the hydroxylation and the characterization of factors involved. We also report that Z, the terminal enzyme, is the rate-limiting factor determining the expression of NeuGc in several tissues of mouse, rat and man.

Materials and methods

Materials

CMP-NeuAc was purchased from MECT (Tokyo, Japan); NADH and NADPH from Oriental Yeast Co., Ltd. (Tokyo, Japan); cellulose phosphate P11 from Whatman Scientific Ltd. (Kent, UK); DEAE-Sepharose CL-6B and a Superdex 75 column from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden); a TSK-gel ODS-80TM column (4.6 mm ID × 250 mm) from Tosoh (Tokyo, Japan); Spectra/por 2 dialysis tubing from Spectrum Medical Industries, Inc. (Los Angeles, CA, USA); a BCA protein assay kit from Pierce Chemical (Rockford, IL, USA); and bovine serum albumin, ovalbumin, α-chymotrypsinogen and myoglobin, as molecular mass markers, from Sigma (St. Louis, MO, USA). Soluble cytochrome b_5 was purified from horse erythrocyte lysate as described in a previous paper [18]. All the other reagents were purchased from Wako Chemicals (Osaka, Japan).

Fractionation of cytosolic proteins of mouse liver

Frozen livers (330 g batches) of DBA/2 mice (5–7 weeks) were thawed in 990 ml of 250 mM sucrose, and then minced with scissors and divided into eight batches. Each batch was homogenized with a Polytron tissue grinder at low speed by two 20 s bursts with a 20 s interval. The resulting homogenates were centrifuged at 3300 × g for 10 min in a Sorval GSA rotor and the supernatants were saved. Each pellet was suspended in 330 ml of 250 mM sucrose, homogenized with a Dounce homogenizer, and then centrifuged as above. To the combined supernatants (1150 ml) 1 M $MgCl_2$ was added to a final concentration of 10 mM and then the suspension was centrifuged at 27 000 × g for 1 h in a GSA rotor. After discarding the pellet (microsomes) the cytosolic fraction was collected.

The cytosolic fraction (1 l) was diluted with 1 l of 0.2 M Tris-HCl buffer, pH 7.5, containing 2 μg ml^{-1} pepstatin A and 0.2 mM dithiothreitol (DTT). To the solution, 486 g of ammonium sulfate was added to obtain 40% saturation, followed by stirring for 30 min. After centrifugation at 12 500 × g for 15 min in a GSA rotor, the supernatant (2 l) was made to 60% saturation with ammonium sulfate (260 g). The resulting precipitate was collected by centrifugation as above, dissolved in a minimal volume of 10 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM DTT, and then dialysed against the same buffer. The dialysate (160 ml) was divided into aliquots and stored at −80 °C.

A 100 ml aliquot of this fraction was diluted with 650 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 12.5% glycerol and 0.1 mM DTT, and then applied to a cellulose phosphate P11 column (5.0 cm ID × 13 cm) equilibrated with the same buffer. After the column had been washed with 2 l of the same buffer, the bound material was eluted with 10 mM sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl, 12.5% glycerol and 0.1 mM DTT. Fractions of 100 ml were collected in flasks, and a 2 μl aliquot of each fraction was used for assay of hydroxylation (assay 1) or assay of the terminal enzyme (assay 2). The conditions for assays 1 and 2 are given later. The elution of protein was monitored as the absorbance at 280 nm, and the fractions containing factors required for the hydroxylation and exhibiting absorbance of over 0.1 were pooled. Two pooled fractions, named the P11 bound and unbound fractions, were concentrated by ammonium sulfate precipitation, and then dialysed against 10 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM DTT and 12.5% glycerol. The protein concentrations of the dialysed fractions were 24 and

22 mg ml^{-1} (P11 unbound and bound fractions, respectively). All procedures described were performed at 4 °C.

Preparation of cytosolic fractions from various tissues

The livers and brains of DBA/2 mice and DA rats were frozen in liquid nitrogen and stored at −80 °C before use. The thymuses from three WHT mice were minced and then filtered through a stainless steel mesh to obtain a single cell suspension. The erythrocytes in the suspension were lysed by the ammonium chloride method. Small intestines were obtained from DA rats of 3 to 5 weeks old as described by Bouhours and Bouhours [14]. All the procedures described below were performed at 4 °C. Livers, brains, small intestines (0.2 g wet weight each) and packed thymocytes (0.1 ml) were placed in 0.8 ml of buffer containing 0.25 M sucrose, 0.1 mM DTT and 10 mM Tris-HCl, pH 7.5, and then homogenized with a Polytron-type microhomogenizer (Niti-on, Tokyo, Japan) by two 30 s bursts with a 1 min interval. The homogenates were centrifuged at 10000 × g for 5 min and the supernatants were further centrifuged at 105000 × g for 15 min. Each resulting supernatant was dialysed against 10 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM DTT. Aliquots of the dialysate were subjected to the assay for the terminal enzyme (assay 2).

The assay of hydroxylation

Assay 1. The method described previously [17] for the determination of CMP-NeuAc hydroxylation activity was modified. The modification comprises the addition of an excess amount of soluble cytochrome b_5 purified from horse erythrocyte lysate. Thus, the assay mixture contained 40 μM CMP-NeuAc, 5 μM soluble cytochrome b_5, 0.7 mM NADH, 1 mM DTT, 10 mM Tris-HCl buffer, pH 7.5, and the enzyme source, in a total volume of 50 μl. After incubation at 37 °C for 1 h, the reaction was stopped by the addition of 6 volumes of cold ethanol and then the products recovered in the ethanol supernatant were separated on an ODS-80TM column as reported previously [17].

To verify the effect of the P11 bound fraction upon hydroxylation, a fixed amount of the pooled P11 unbound fraction (4.8 μg protein) and various amounts of the pooled P11 bound fraction (0–6.5 μg protein) were included in the assay mixtures.

Assay 2. To determine the activity of the terminal enzyme, the assay was performed as Assay 1 except that the reaction mixtures included an excess amount of the P11 bound fraction (5.5 μg protein).

Measurement of cytochrome b_5 reducing activity

The assay mixtures contained 10 mM Tris-HCl buffer, pH 7.5, 2.9 μM soluble cytochrome b_5, 44 μM NAD(P)H or 220 μM NAD(P)H, and the P11 bound fraction (2.6 μg protein) or P11 unbound fraction (2.9 μg protein), in a total volume of 50 μl. The reactions were performed at 25 °C and the increase in absorbance at 424 nm was monitored using a DU-64 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA). The initial rate of the increase was calculated with a Kinetics Soft-Pac module distributed by Beckman Instruments, Inc.

Estimation of the molecular masses of the factors

The molecular masses of the factors were estimated by gel filtration chromatography after partial purification on a DEAE-Sepharose column. A 40 ml aliquot of the fraction obtained on 40–60% ammonium sulfate precipitation from mouse liver cytosol was applied on a DEAE-Sepharose column (26 mm ID × 380 mm) and the bound material was eluted with a linear sodium chloride gradient, as reported previously [18]. The fractions eluted between 0.1 M and 0.2 M sodium chloride (designated as DE1) were pooled. DE1 contained both components X and Z, and was concentrated to 1.8 ml and applied to a Superdex 75 column (16 mm ID × 600 mm) equilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 0.15 M sodium chloride and 0.1 mM DTT. The flow rate was 1 ml per min and fractions of 1 ml were collected. The protein concentration was determined by the BCA method. The elution positions of the following molecular mass markers were determined; bovine serum albumin (68 kDa), ovalbumin (44 kDa), α-chymotrypsinogen (24.5 kDa) and myoglobin (17 kDa). Aliquots of the fractions (2 μl each) were used for assaying of the terminal enzyme (assay 2).

NADH dependent reducing activity was measured as the reduction of potassium ferricyanide. The reaction mixtures contained 220 μM potassium ferricyanide, 100 μM NADH, 0.1 M potassium phosphate buffer, pH 7.5, and a 20 μl aliquot of each fraction, in a total volume of 600 μl. The mixtures were incubated for 10 min at 25 °C and the decrease in absorbance at 420 nm was measured. In order to confirm that the factor carrying the reducing activity of potassium ferricyanide is able to participate in the hydroxylation of CMP-NeuAc, a 2 μl aliquot of each fraction was used for the assay, and the assay conditions other than the addition of the P11 unbound fraction (6 μg protein) were the same as assay 1.

Protein determination

Protein concentrations were determined with a BCA protein assay kit with bovine serum albumin as a standard.

Results

Amount of soluble cytochrome b_5 in the hydroxylation assay

As we reported previously [18], the CMP-NeuAc hydroxylation reaction was reconstituted *in vitro*, using mouse liver cytosol and purified soluble cytochrome b_5 from horse erythrocyte lysate. Enhancement of the hydroxylase activity was observed when the amount of soluble

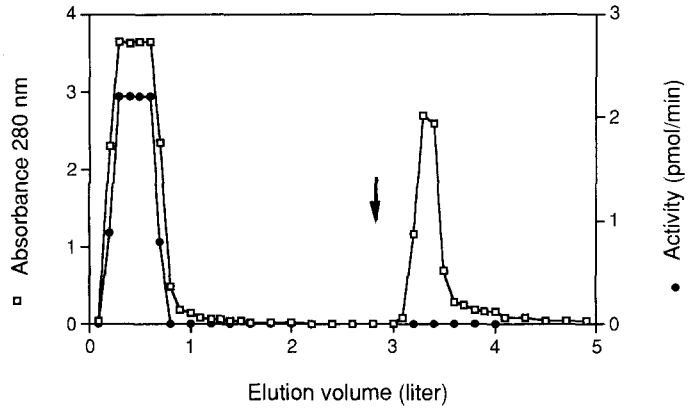


Figure 1. Cellulose phosphate P11 column chromatography of the cytosolic proteins of mouse liver: □, protein (absorbance at 280 nm); ●, activity of the terminal enzyme, as measured by assay 2 (see the Materials and methods section). Results obtained with assay 1 are omitted. The arrow indicates the position where the buffer was changed to that containing 0.5 M sodium chloride.

cytochrome b_5 in the reaction mixture was increased [18, 19]. In a kinetic study using crude cytosolic fraction of mouse liver, the K_m value for the soluble cytochrome b_5 was estimated to be 0.19 μM (H. Takematsu *et al.*, unpublished data). In the presence of an excess amount of exogenously added soluble cytochrome b_5, the velocity of the hydroxylation depends on the amounts of the other factors participating in the reaction. Thus, in order to identify the other factors, assays were performed in the presence of 5 μM exogenous soluble cytochrome b_5 (assay 1).

Fractionation of essential factors

Using the assay conditions given above, the hydroxylation activity in the cytosol was recovered quantitatively in the precipitated fraction on 40–60% saturation with ammonium sulfate. This fraction was subjected to chromatography on a cellulose phosphate P11 column and, as shown in Fig. 1, most proteins were recovered in the flow-through fraction (the P11 unbound fraction), and bound proteins were eluted with the buffer containing 0.5 M sodium chloride (the P11 bound fraction). When an aliquot of each fraction was subjected to assay 1, almost no activity was detected. The hydroxylase activity was restored when the bound and unbound fractions were mixed together. Therefore, the essential factors were assumed to be separated into two fractions.

NADH-dependent cytochrome b_5 reducing activity

Our previous proposal was that one of the essential factors involved in the hydroxylation is a cytochrome b_5 reducing factor. Therefore, in the presence of NADH or NADPH, aliquots of the P11 bound and unbound fractions were mixed separately with the solution of purified soluble cytochrome b_5, and then reduction of the soluble cytochrome b_5 was monitored as the increase in absorbance at

Table 1. Reduction of cytochrome b_5 with the P11 bound and unbound fractions.

Fraction[a]	*Electron donor*	(μM)	ΔA_{424} (min^{-1})
P11 bound	NADH	(44)	0.91
	NADPH	(44)	0.02
	NADPH	(220)	0.08
P11 unbound	NADH	(220)	<0.01
	NADPH	(220)	<0.01

[a] The 50 μl incubation mixtures contained 2.6 μg protein of the P11 bound fraction and 2.9 μg protein of the P11 unbound fraction.

424 nm. Only the P11 bound fraction contained cytochrome b_5 reducing activity, with NADH as an electron donor (Table 1). NADPH was less effective than NADH. These results indicate that the P11 bound fraction contains NADH-dependent cytochrome b_5 reducing activity and the P11 unbound fraction might contain the terminal enzyme, which accepts electrons from the reduced form of soluble cytochrome b_5, hydroxylates CMP-NeuAc and converts it to CMP-NeuGc.

The terminal enzyme activity

The terminal enzyme recognizes the substrate and, therefore, one of the regulation mechanisms for the production of NeuGc is possibly a change in the amount of the terminal enzyme. In order to assess this possibility, the activity of the terminal enzyme must be measured accurately and excess amounts of the other factors should, therefore, be added to the reaction mixture. Figure 2 shows the effect of the P11 bound fraction on the hydroxylation. Amounts of protein higher than 3 μg in 50 μl incubation mixtures gave a

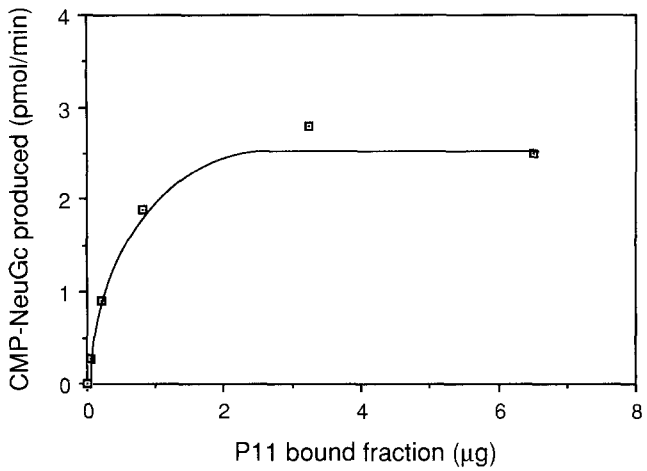


Figure 2. Effect of the P11 bound fraction on the CMP-NeuAc hydroxylation. With the indicated amounts of the P11 bound fraction, the hydroxylation reaction was performed in the presence of 4.8 μg of P11 unbound fraction as an enzyme source, in a total volume of 50 μl. The production rate of CMP-NeuGc is plotted.

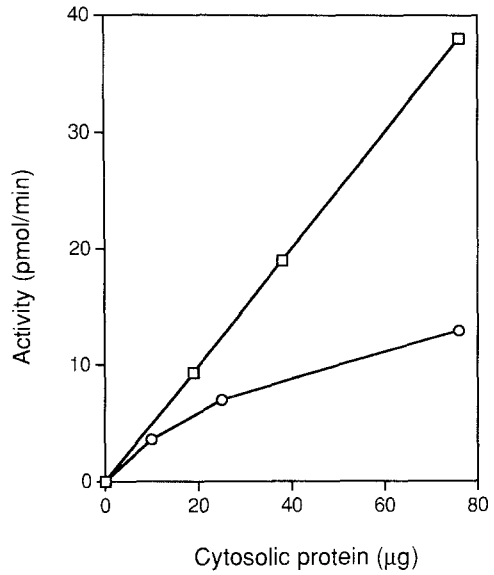


Figure 3. Linear dependence of the CMP-NeuAc hydroxylation reaction on the amount of cytosolic proteins. The hydroxylation reaction was performed in the presence of □, 5 μM (Assay 2) or ○, 0.5 μM soluble cytochrome b_5.

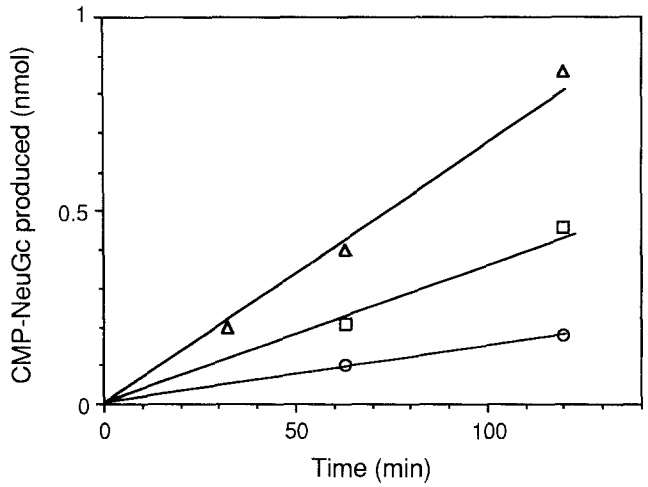


Figure 4. Time dependence of the CMP-NeuAc hydroxylation. Cytosolic proteins of mouse liver (○, 3, □, 6 and △, 12 μg) were used as the enzyme source. The hydroxylation reaction was performed by assay 2.

saturated level of the reaction. Then, the assay conditions were modified as follows (assay 2); 5.5 μg protein of the P11 bound fraction and 5 μM of the purified soluble cytochrome b_5. When we applied these conditions to measure the activity of the terminal enzyme in the cytosolic fraction of mouse liver, up to 80 μg protein, a linear reaction was observed (Fig. 3). The time dependence is also shown in Fig. 4, with the same conditions.

Hydroxylation activity in various tissues

Using the assay conditions given above (assay 2), the activity of the terminal enzyme in the cytosolic fractions of various tissues of mouse and rat was measured (Table 2). A high level of terminal enzyme activity was detected in mouse liver, in which most of ganglioside sialic acid is NeuGc [8]. Mouse thymocytes contained one-fifth the activity in the liver. On the other hand, no activity was detected in the cytosol of mouse and rat brains, which expressed a quite small amount of NeuGc-containing gangliosides [8, 20]. It was reported that the amount of NeuGc-containing GM3 in the small intestine of rat increased during development after birth [14]. Thus, the terminal enzyme activity was measured to determine whether or not it is related to the NeuGc-containing GM3 expression. The activity increased with development after birth. These results suggest that the regulation of the terminal enzyme activity determines the expression of NeuGc.

Table 2. The activity of the terminal enzyme for CMP-NeuAc hydroxylation in various tissues.

Tissue	*Specific activity* (mU mg^{-1})[a]
Mouse liver	0.50
0.11 thymocytes	
ND[b] brain	
Rat liver	0.002
ND brain	
small intestine	
0.14 age 3 weeks	
0.23 4 weeks	
0.26 5 weeks	

[a] U is defined as the amount of enzyme catalysing the formation of 1 μmol product per min at 37 °C.
[b] ND = not detected.

Estimation of the molecular masses of the terminal enzyme and the factor exhibiting NADH-dependent cytochrome b_5 reducing activity

The partially purified fraction from mouse liver was analysed by gel filtration chromatography on a Superdex 75 column (Fig. 5). The molecular mass of the terminal enzyme was estimated to be 58 kDa from its elution position. The factor exhibiting NADH-dependent cytochrome b_5 reducing activity was monitored as to the reduction of potassium ferricyanide. At the same time, it was monitored by means of the hydroxylation assay in the presence of soluble cytochrome b_5 and the P11 unbound fraction. Both methods gave almost the same elution profile, and its molecular mass was estimated to be 30 kDa.

Discussion

We proposed that components X and Z in addition to soluble cytochrome b_5 were required for the reconstitution

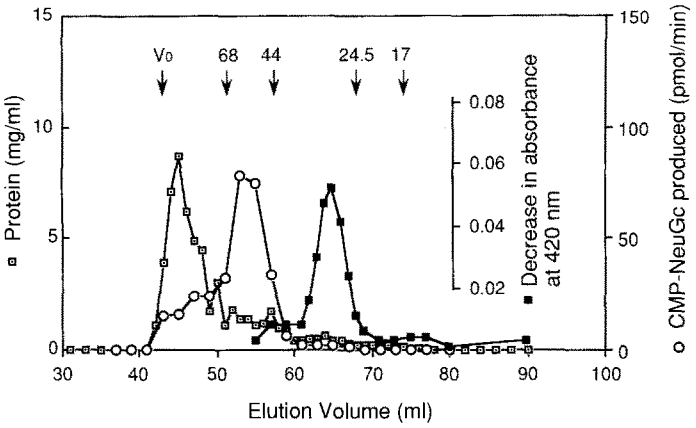


Figure 5. Gel permeation chromatography of the cytosolic proteins of mouse liver on a Superdex 75 column: ⊡, protein concentration determined by the BCA method; ○, activity of the terminal enzyme determined by assay 2; ■, activity of cytochrome b_5 reducing factor determined as the reduction of potassium ferricyanide. Arrows indicate the elution positions of molecular mass markers: bovine serum albumin (68 kDa), ovalbumin (44 kDa), α-chymotrypsinogen (24.5 kDa) and myoglobin (17 kDa).

of the CMP-NeuAc hydroxylation reaction when NADH was used for an electron donor [17]. In this study, we obtained two fractions containing components X and Z, respectively, from the cytosolic fraction of mouse liver. One of the two fractions, the one that bound to a cellulose phosphate column, contained component X, which was assumed to be a soluble type of NADH-dependent cytochrome b_5 reducing factor for the following reasons. 1, The fraction reduces the soluble cytochrome b_5 and ferricyanide, using NADH as an electron donor. NADPH poorly supports the reaction. 2, The soluble cytochrome b_5 reducing activity of the fraction was suppressed by salt [18], as was the case for an NADH-dependent cytochrome b_5 reductase, which was found in human erythrocytes, bovine brain, etc. [21, 22]. 3, The molecular mass of the factor is 30 kDa, which is quite similar to that of the NADH dependent cytochrome b_5 reductase solubilized by acid proteolysis from microsomes [22]. 4, The stability of the enzyme is greatly enhanced by the addition of flavin (data not shown). Flavin is the prosthetic group of the enzyme reported in human erythrocytes [23]. However, definite identification of the factor requires its purification and structural determination.

The terminal enzyme, component Z, recovered in the flow-through fraction from a cellulose phosphate column accepts electrons from the reduced form of soluble cytochrome b_5, recognizes CMP-NeuAc, and catalyses the hydroxylation of CMP-NeuAc. This factor should play a critical role in the expression of NeuGc-containing glycoconjugates. Therefore, in this study, the conditions for a quantitative assay for the terminal enzyme activity were established. Using this assay, the activity of the terminal enzyme in various tissues was measured. Several lines of evidence suggest that this enzyme is the key which regulates the overall velocity of the CMP-NeuAc hydroxylation, as follows. 1, The specific activities of this enzyme in various tissues of mouse and rat are correlated with the expression of NeuGc-containing gangliosides in the tissues. 2, The specific activity of the enzyme in rat intestine increased during development, as did the amount of NeuGc-containing GM3 ganglioside [14]. 3, No detectable activity was found in the cytosolic fractions of human lymphocytes and chicken liver, both of which do not express NeuGc (data not shown). On the contrary, the cytochrome b_5 reducing activity was present at a sufficient enough level to support the hydroxylation reaction of the terminal enzyme, if any, in every tissue examined, including the human and chicken tissues.

Recently, Muchmore reported the amount of NeuGc expressed and cytosolic CMP-NeuAc hydroxylation activity in various tissues of rats at various developmental stages [24]. The conclusion of her study was that CMP-NeuAc hydroxylation activity was not correlated with the amount of NeuGc expressed, suggesting that another regulation mechanism is involved in the determination of the amount of NeuGc expressed. However, the hydroxylation is a complex of reactions, as shown in this study, and accurate determination of the activities is quite difficult when one has not characterized the complex of reactions precisely. Therefore, we need to reexamine and reconsider her conclusion. In this study, we determined the activity of the terminal enzyme in the presence of excess amounts of soluble cytochrome b_5 and soluble cytochrome b_5 reducing factor, and we are quite sure that the determination gave quantitative results. Therefore, we can conclude at present that the terminal enzyme activity regulates NeuGc expression, at least, in the all-or-nothing-type of regulation seen in the liver and brain of rats and mice.

There is a possibility that the amount of soluble cytochrome b_5 regulates the production rate of CMP-NeuGc. However, the actual involvement of the soluble form of cytochrome b_5 *in situ*, in cells other than erythroid cells, is questioned, because soluble form cytochrome b_5 has so far only been found to occur in erythrocytes and probably their progenitor cells. Studies to answer this question are in progress in our laboratories.

The oncofetal expression of NeuGc-containing glycoconjugates was reported in man [11]. Our present work poses the interesting question of whether or not the expression of the terminal enzyme regulates the oncofetal expression of NeuGc in man. If so, the detection of the activity provides a new method for studies on the expression of NeuGc in cancer tissues.

At present, the biological relevance of NeuGc is unclear. It was demonstrated that haemagglutinins of influenza virus can discriminate NeuAc and NeuGc on the host cell surface [5]. Recently, it was suggested that a sialic acid species

difference may determine the species specificity during fertilization in salmonid fish [25]. There is the possibility that the sialic acid species play some role in development, differentiation and oncogenesis as recognition molecules, but few data are available. Biochemical analysis of the terminal enzyme may provide a basis for answering these questions.

The hydroxylation is a monooxygenase type of reaction, and the hydroxylaton of lipid substrates, such as fatty acids, and steroids is carried out by an electron transport system consisting of cytochrome P450, cytochrome b_5 and NADH and/or NADPH dependent cytochrome b_5 reductases [26]. It would be quite interesting to determine whether or not the terminal enzyme for CMP-NeuAc hydroxylation exhibits any structural similarity to cytochrome P450s.

In the present study, the assay conditions for the quantitative determination of the terminal enzyme in CMP-NeuAc hydroxylation were established, which are required for the purification of the terminal enzyme.

Acknowledgements

We would like to thank Dr Kentaro Iwasaki for the helpful discussions and Professor Tamio Yamakawa for much encouragement. This work was partly supported by Grants-in-Aid for Scientific Research in Priority Areas, No. 04250105 and 04206102, from the Ministry of Education, Science and Culture of Japan.

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