Detection of CMP-N-acetylneuraminic acid hydroxylase activity in fractionated mouse liver

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The finding that N-glycoloylneuraminic acid (Neu5Gc) in pig submandibular gland is synthesized by hydroxylation of the sugar nucleotide CMP-Neu5Ac [Shaw & Schauer (1988) Biol. Chem. Hoppe-Seyler 369, 477–486] prompted us to investigate further the biosynthesis of this sialic acid in mouse liver. Free [14C]Neu5Ac, CMP-[14C]Neu5Ac and [14C]Neu5Ac glycosidically bound by Galα2–3- and Galα2–6–GlcNAcβ1–4 linkages to fetuin were employed as potential substrates in experiments with fractionated mouse liver homogenates. The only substrate to be hydroxylated was the CMP-Neu5Ac glycoside. The product of the reaction was identified by chemical and enzymic methods as CMP-Neu5Gc. All of the CMP-Neu5Ac hydroxylase activity was detected in the high-speed supernatant fraction. The hydroxylase required a reduced nicotinamide nucleotide [NAD(P)H] coenzyme and molecular oxygen for activity. Furthermore, the activity of this enzyme was enhanced by exogenously added Fe2+ or Fe3+ ions, all other metal salts tested having a negligible or inhibitory influence. This hydroxylase is therefore tentatively classified as a mono-oxygenase. The cofactor requirement and CMP-Neu5Ac substrate specificity are identical to those of the enzyme in high-speed supernatants of pig submandibular gland, suggesting that this is a common route of Neu5Gc biosynthesis. The relevance of these results to the regulation of Neu5Gc expression in sialoglycoconjugates is discussed.

INTRODUCTION

Sialic acid is a generic name for a group of about 30 naturally occurring acidic carbohydrates that are essential components of a large number of glycoconjugates [1]. Although certain bacteria are known to produce glycoconjugates containing sialic acids [2], these sugars occur in largest amounts and exhibit greatest heterogeneity in the animal kingdom [1]. Their occupancy of terminal or near-terminal positions in sialoglycoconjugates underlines the vital role of sialic acids in determining the surface characteristics of cells and secreted glycoproteins [3]. The origin of the variety of sialic acids, which makes possible the fine tuning of these surface properties to fulfil specific biological functions, has been the subject of much research. Although the biosynthetic pathway of N-acetyleneuraminic acid (Neu5Ac), the simplest and most ubiquitous sialic acid, has long been established [4,5], the biochemical mechanisms involved in Neu5Ac derivatization, giving rise to the diversity within this group of sugars, are largely unresolved.

N-Glycoloylneuraminic acid (Neu5Gc), which is one of the most common modified sialic acids, formally results from the hydroxylation of the 5-N-acetyl methyl function of Neu5Ac. Neu5Gc is widely distributed throughout the animal kingdom and, according to species and tissue, often constitutes a significant proportion of glycoconjugate-bound sialic acid. Certain species such as chicken and man are exceptional, since they lack Neu5Gc in normal tissues [1,6]. Recent investigations have, however, revealed that certain human and chicken tumours may contain small amounts of antigenic, lipid-bound Neu5Gc [7,8], suggesting that an otherwise dormant Neu5Ac hydroxylase gene may be activated in oncogenesis. The genetic regulation of Neu5Gc expression is further exemplified in rat intestine where its presence in various glycoconjugates is dependent both on the stage of development and on the strain [9,10]. Clearly, the enzyme(s) involved in the biosynthesis of Neu5Gc must first be identified before any genetic investigations can be interpreted.

The results of earlier experiments on various preparations of pig submandibular gland suggest that mucin-bound Neu5Gc results from the hydroxylation of free Neu5Ac and Neu5Ac glycosidically bound to the growing mucin molecule [11–13]. Although several metabolic [14–16] and structural [17] studies are consistent with this view, recent results from this laboratory clearly indicate that the hydroxylation of Neu5Ac in fractionated porcine submandibular gland occurs exclusively at the level of the CMP-Neu5Ac glycoside [18]. Since these findings are at odds with the accepted literature, it was deemed necessary to elucidate the route of Neu5Gc biosynthesis in other tissues, in order to exclude the possibility that some peculiarity or artefact was being observed in pig submandibular gland.

We therefore present the results of investigations into Neu5Ac hydroxylation in fractionated mouse liver, a tissue responsible for the production of gangliosides and plasma glycoproteins whose sialic acid residues consist almost exclusively of Neu5Gc [19,20].

Abbreviations used: Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycoloylneuraminic acid; Neu5Ac2en, 2-deoxy-2,3-didehydro-N-acetyleneuraminic acid; Neu5en5Gc, 2-deoxy-2,3-didehydro-N-glycoconjugate acid.

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MATERIALS AND METHODS

Reagents

Unless stated otherwise, solvents, mineral acids, caco-
dyl acid, FeSO₄, ascorbic acid, Tris, phosphotungstic
acid, trichloroacetic acid and metal salts were of
analytical grade from Merck (Darmstadt, Germany).
Bovine serum albumin, fetuin (fetal calf serum type III),
Triton X-100, NADH, NADPH, 6,7-dimethyl-5,6,7,8-
tetrahydrobiopterin, EDTA, CMP-Neu5Ac, dithio-
threitol and 2-oxoglutaric acid were bought from Sigma
Chemicals Ltd. (Deisenhofen, Germany). 2-Deoxy-2,3-
didehydro-N-acetylnemuramic acid (Neu5Ac2en) was
purchased from Boehringer–Mannheim (Mannheim,
Germany). Dowex ion-exchange resins and Bio-Rad
protein assay reagent were from Bio-Rad (München,
Germany). Galα2–3- and Galα2–6-sialyltransferases
(EC 2.4.99.4 and 2.4.99.1) were obtained from the
Genzyme Corporation (Boston, MA, U.S.A.). Neu5Ac
and Neu5Gc were purified from birds’ nest substance
and pig submandibular gland mucin respectively [21].

Radiochemicals

CMP-[4,5,6,7,8,9-14C]Neu5Ac (250–280 mCi/mmol)
was obtained from New England Nuclear (Dreieich,
Germany) and [4,5,6,7,8,9-14C]Neu5Ac (260 mCi/mmol)
was supplied by Amersham (Braunschweig, Germany).

Experiment to establish the sialic acid substrate
specificity of Neu5Ac hydroxylase in fractionated
mouse liver

Preparation of particulate and supernatant fractions
from mouse liver. This method is based on that of
Chambers & Rickwood [22]. All centrifuge rotors and
buffers were pre-cooled to 4 °C. Livers from freshly
killed female Balb/c mice were homogenized in 9 vol.
(ml per g wet wt. of tissue) of 50 mm-Tris/HCl, pH 7.4,
containing 0.25 m M-sucrose using a Potter–Elvehjem
homogenizer. After filtration through gauze, the homog-
enate was centrifuged at 700 g for 5 min to sediment
large particles. Smaller organelles were sedimented
by centrifugation of the resulting supernatant at 10000 g
for 20 min. Microsomes were obtained by a subsequent
centrifugation of the previous supernatant at 120000 g
for 1 h, leaving a clear red supernatant. All membrane
fractions were washed by resuspension in 9 ml of homog-
ization buffer per g wet wt. of tissue using a Potter–Elvehjem
homogenizer, followed by centrifugation using the same
buffer and value time. The washed membrane fractions
were resuspended into 1 mL of homogenization buffer per g
wt. wt. of tissue using four strokes of a Potter–Elvehjem
homogenizer and either used immediately or frozen at
−70 °C in 1–2 ml aliquots. The average protein concentra-
tions from two preparations were: 700 g pellet, 25.0 mg/ml (s.d. 3.2 mg/ml); 10000 g pellet, 5.7 mg/ml (s.d. 0.7 mg/ml); 120000 g supernatant, 5.7 mg/ml (s.d. 0.6 mg/ml) as determined
with the BioRad protein assay reagent, using bovine
serum albumin as standard.

Preparation of fetuin-bound [14C]Neu5Ac. Fetuin from
fetal calf serum was desialylated by hydrolysis at pH 1.0
(adjusted using HCl) for 1 h at 80 °C. After thorough
dialysis against water at 4 °C, the asialofetuin was
lyophilized and resuspended to a final concentration of
20 mg/ml.

A 50 μl aliquot of asialofetuin was resialylated for
6.5 h at 37 °C with 1.0 μCi of CMP-[14C]Neu5Ac using
3.4 munits of a2–6-sialyltransferase in the presence of
0.25 mg of bovine serum albumin, 12.5 μmol of sodium
cacodylate buffer, pH 6.0, and 0.5 % Triton X-100 in a
total volume of 250 μL. A further 50 μl of the asialofetuin
solution was resialylated with 2.0 munits of a2–3-
sialyltransferase using 1.0 μCi of CMP-[14C]Neu5Ac
in the presence of 0.25 mg of bovine serum albumin,
25 μmol of sodium cacodylate buffer, pH 6.0, and
1.3 % Triton X-100 in a final volume of 190 μl.

The resialylated fetuin samples were dialysed separ-
ately at 4 °C in Perspex microdialysis chambers against
2 x 1 l of water. Non-diffusible material was lyophilized
and resuspended in 200 μl of 90 % ethanol, whereupon
precipitated protein was sedimented by centrifugation
(2000 g, 10 min), leaving the remaining traces of non-
glycosidically-bound [14C]Neu5Ac in the supernatant.
The pellets containing the resialylated fetuin were
resuspended in 200 μl of 50 mm-Tris/HCl, pH 7.4,
and used in this form.

Incubation conditions and product analysis. The fol-
lowing potential substrates, in a volume of 10 μl, were
added to 1 mL of each tissue fraction at 2 °C: [14C]Neu5Ac
(0.05 μCi; 0.166 nmol), fetuin with Galα2–3-linked
[14C]Neu5Ac (0.023 μ Ci; 0.089 nmol of [14C]Neu5Ac)
and fetuin with Galα2–6-GlcNAc β1–4-linked [14C]-
Neu5Ac (0.0175 μ Ci; 0.068 nmol of [14C]Neu5Ac).
The protein concentrations in the various fractions were
as follows: 700 g pellet, 22.7 mg/ml; 10000 g pellet,
6.3 mg/ml; 120000 g pellet, 12.9 mg/ml; 120000 g
supernatant, 5.3 mg/ml. Reactions were started by addition
of NADH and FeSO₄ in 0.1 ml to final concentrations of
1 mM and 0.5 mM respectively and warming to 37 °C. The
choice of cofactors for this experiment was based on the
specificity of the enzyme from pig submandibular gland
[18]. All reaction mixtures were incubated for 3 h in
duplicate both in the presence and absence of 1 %, Triton
X-100 which was included to allow substrates and cofactors
to gain access to any enzymes present within the
membrane vesicles of the particulate fractions. The
reactions were quenched by addition of HCl to give
pH 1.0 and heating at 80 °C for 1 h. The released
radioactive sialic acid products were extracted, partially
purified and quantified by radio t.i.c. (solvent system 1)
as previously described [18].

For reasons described in the Results section, slightly
different conditions were employed in incubations with
the CMP-[14C]Neu5Ac substrate. All incubations were
conducted at 2 °C. Triton X-100. Undiluted fractions from
the two preparations described above were tested with this
substrate. A portion of 10 μl of a mixture containing 6 mm-
FeSO₄ and 12 mm-NADH (in 25 mm-Tris/HCl, pH 7.4)
was added to 0.1 ml of each tissue fraction at 37 °C
and reactions started by addition of 0.05 μCi of CMP-
[14C]Neu5Ac (0.19 nmol) in 10 μl. Incubations with the
particulate fractions were carried out for 2 h and those
with the supernatant fractions for 30 min. All assays
were quenched by addition of 0.3 ml of ice-cold technical
grade ethanol followed by cooling on ice for 10 min.
Precipitated protein was removed by centrifugation
(2000 g, 10 min) and the yield of radioactivity in the result-
 CMP-Neu5Ac hydroxylase

ing supernatants was determined by liquid scintillation counting. The product analysis was performed by radio t.l.c. (solvent system 2) using 5 µl of 3-fold-concentrated supernatant.

Radio t.l.c. All t.l.c. separations were performed using 10 cm x 20 cm h.p.t.l.c. cellulose plates (obtained from Merck). Free sialic acids were separated using propanol/butanol/0.1 M-HCl (2:1:1, by vol.) (System 1) and CMP-sialic acid glycosides were resolved with 95% ethanol/1 M-ammonium acetate, pH 7.3 (7:3, v/v) (System 2). The latter solvent can also separate Neu5Ac and Neu5Gc. Authentic non-radioactive standards of Neu5Ac and Neu5Gc and, where appropriate, their CMP-glycosides were co-chromatographed with each sample. Radioactive compounds were identified by comparison of their migration positions with those of the non-radioactive standards stained with the orcinol/Fe³⁺ reagent [23]. Zones of radioactivity were detected using either a Berthold LB2832 linear analyser or a Berthold ‘TraceMaster’ linear analyser and quantified by computer-assisted integration.

Identification of the CMP-[14C]sialic acid glycoside product from incubation of CMP-[14C]Neu5Ac with the high-speed supernatant

Preparation of product. A 4 ml portion of the 120000 g supernatant from homogenized mouse liver was incubated in a single experiment for 3 h at 37 °C with 0.5 µCi of CMP-[14C]Neu5Ac in the presence of 1 mM-NADH and 0.5 mM-FeSO₄ in a final volume of 4.2 ml. The reaction was quenched by addition of 16 ml of cold technical grade ethanol. After centrifugation at 1500 g for 10 min, followed by further extraction of the resulting pellet with 10 ml of cold 80% ethanol, the supernatants were pooled and evaporated to dryness at room temperature under reduced pressure. The residue was resuspended in 5 ml of water and applied to a 5 ml column of Dowex 1 x 8 (HCO₃⁻ counter-ion). After washing with 40 ml of 0.1 M-triethylamine bicarbonate buffer, pH 7.8, the CMP-glycosides were eluted with 25 ml of 0.7 M-triethylamine bicarbonate, pH 7.8, and lyophilized. The lyophilized material was resuspended in 0.4 ml of 1 M-triethylamine bicarbonate, pH 7.8, to give the final preparation used in the following tests.

Tests for identification of the product. (1) Effect of acid and alkali treatment on the product. Two 50 µl portions of the glycoside preparation (22950 d.p.m.) were lyophilized to dryness. One sample was resuspended in 100 µl of 0.1 M-HCl and incubated at 20 °C for 5 min. The second sample was resuspended in 100 µl of concentrated ammonia (25%) and incubated at 56 °C for 15 h. After lyophilization, both samples were resuspended in 50 µl of water, and 10 µl of each was analysed by radio t.l.c. with Neu5Gc, Neu5Ac and Neu5Ac2en standards using systems 1 and 2. (2) Use of sialyltransferase to identify the product. A 50 µl portion of the product solution (22950 d.p.m.) was lyophilized to dryness and resuspended in 10 µl of 250 nm-sodium cacodylate, pH 6.0. This was incubated for 15 h at 37 °C with α2–6-sialyltransferase (3.4 units) in the presence of 0.6 mg of asialofetuin, 25 µmol of sodium cacodylate, pH 6.0, 0.25 mg of bovine serum albumin and 1.4% Triton X-100 in a final volume of 0.18 ml. An identical control incubation was performed using sialyltransferase denatured by heating at 96 °C for 10 min. The incubations were subsequently dialysed at 4 °C against 2 × 11 of water, lyophilized, resuspended in 100 µl of water and the protein, together with the transferred [14C]sialic acid, finally precipitated with 1 ml of 12% trichloroacetic acid and 1% phosphotungstic acid. The pellets were washed with 1 ml of diethyl ether to remove trichloroacetic acid and subsequently hydrolysed with 0.1 ml of 0.1 M-HCl at 80 °C for 1 h. After removal of HCl by lyophilization, the residues were resuspended in 25 µl of water and analysed by liquid scintillation counting and radio t.l.c. (solvent system 1).

Routine assay for CMP-[14C]Neu5Ac hydroxylase

Preparation of a sucrose-free soluble protein fraction. For routine CMP-Neu5Ac hydroxylase assays, sucrose was excluded from the homogenization buffer to allow rapid processing of the samples for chromatographic analysis. Mouse liver, either fresh or stored at −70 °C, was homogenized with a Potter–Elvehjem homogenizer into 5 vol. (ml/g wet wt. of tissue) of 50 mM-Tris/HCl, pH 7.4, and centrifuged at 120000 g for 1 h. The resulting supernatant was carefully decanted, aliquotted and stored at −70 °C until use. The protein concentration in such supernatants was generally 20 mg/ml.

Incubation conditions and product analysis. Sucrose-free 120000 g supernatant from mouse liver homogenate (0.25 ml) was added at 37 °C to 25 µl of cofactors (for amount and type as well as protein concentration, see Results section). Reactions were started by addition of 0.05 µCi of CMP-[14C]Neu5Ac (generally about 0.18 nmol, according to batch) in 25 µl of 50 mM-Tris/HCl, pH 7.4, and the mixtures were incubated at 37 °C (incubation periods given in the Results section). The reactions were stopped by addition of 1 ml of ethanol and precipitated protein was removed by centrifugation. The supernatants were evaporated to dryness in a vacuum centrifuge. The residues were resuspended in 50 µl of water and 5–10 µl was analysed by radio t.l.c. with computer-assisted integration to quantify the distribution of radioactivity in the CMP-[14C]sialic acid glycoside peaks. Incubations were generally performed in duplicate.

Effect of anaerobic conditions on activity of CMP-Neu5Ac hydroxylase

A 0.5 ml portion of sucrose-free 120000 g mouse liver supernatant (3.5 mg of protein) was sealed with a tight-fitting rubber stopper and an aluminum flange into a 3 ml glass vessel. The vessel was sparged on ice for 15 min with high grade N₂ (99.99%) (Messer Griesheim, Lübeck, Germany) which had been passed through an ‘Oxisorb’ nitrogen purifier (Messer Griesheim, Kiel, Germany), care being taken to avoid foaming. Degassing under vacuum was avoided as this causes frothing. Then, 0.1 µCi of CMP-[14C]Neu5Ac, 0.6 µmol of NADH and 0.3 µmol of FeSO₄ were injected into the protein solution in 0.1 ml using gas-tight syringes, and gassing was continued for a further 2 min on ice. In addition, two further reaction mixtures were prepared using the same amounts of protein, substrate and cofactor solutions: (1) under aerobic conditions; and (2) first deoxygenating the protein solution as above but carrying out the incubations under aerobic conditions. All mixtures were incubated in duplicate for 15 min at 37 °C. Reactions were quenched
by rapid injection of 2 ml of ethanol into the sealed vessels, whereupon they were opened and the product analyses carried out as above.

Dependence of CMP-Neu5Ac hydroxylase activity on the concentration of CMP-Neu5Ac, NADH and FeSO₄

An alternative rapid test system was used to investigate the dependence of the hydroxylase on CMP-Neu5Ac, NADH and FeSO₄ concentrations. In all experiments, 0.1 ml of sucrose-free 120000 g supernatant (2 mg of protein) was warmed to 37 °C with the required amounts of NADH or FeSO₄ added in a volume of 10 µl. To start the reactions, 10 µl of CMP-[¹⁴C]Neu5Ac was added to the desired concentration and the assay mixtures incubated at 37 °C for the required time (see Results and Figure legends for individual incubation times as well as cofactor and substrate concentrations). Assays were stopped by the addition of 15 µl of 2 M-HCl and centrifuged to remove precipitated protein. The free [¹⁴C]sialic acids in about 5–10 µl of the supernatant were analysed by radio t.l.c. using solvent system 1. The improved resolution of the HCl-released sialic acids in this solvent system compared with the separation of the substrate and product CMP-glycosides in solvent system 2 allowed a more rapid and reliable estimation of CMP-Neu5Ac hydroxylation.

Scintillation counting

¹⁴C was quantified in Beckman Readilov M.P. scintillation fluid using a Beckman LS 9000 D liquid scintillation counter with appropriate correction for quenching.

RESULTS

Substrate specificity of Neu5Ac hydroxylase and distribution of activity in fractionated mouse liver homogenates

The radio t.l.c. analyses of sialic acids extracted from incubations with free [¹⁴C]Neu5Ac and [¹⁴C]sialic acids hydrolytically released from the [¹⁴C]CMP-Neu5Ac-fetuinsubstrates revealed in all cases one peak of radioactivity coinciding with the orcinol/Fe⁴⁺ reagent-stained Neu5Ac standard. This indicates that neither free nor fetuin-bound [¹⁴C]Neu5Ac are substrates for the hydroxylase. However, with the CMP-[¹⁴C]Neu5Ac substrate, there was clear evidence of hydroxylation.

From the pattern of [¹⁴C]sialic acid-containing compounds extracted from the various incubation mixtures, it was clear that none of the particulate fractions tested contained any detectable CMP-Neu5Ac hydroxylase activity. The opportunity for sialyltransferases or CMP-sialic acid hydrolase to compound these results by consuming the CMP-[¹⁴C]Neu5Ac substrate was reduced by using smaller amounts of protein and incubating for shorter time periods than in experiments with the other [¹⁴C]sialic acid-containing substrates. The relatively minor effect of sialyltransferases was evident from the yield of ethanol-soluble radioactivity extracted from incubations with the particulate fractions: 700 g pellet, 77±7.5% (S.D.); 10000 g pellet, 87±6.4%; 120000 g pellet, 96±6% (percentage recoveries are the averages of duplicate reactions using membrane fractions from two preparations, incubated in the presence and absence of Triton X-100; this detergent had only a very minor effect on the recovery of radioactivity). The amount of free [¹⁴C]Neu5Ac relative to total extracted radioactivity, detected by radio t.l.c. analysis, was generally low: 700 g pellet, 17.6±2.6%; 10000 g pellet, 23.3±7%; 120000 g pellet, 9.4±3% (percentages are from the same number of experiments stated above), indicating a tolerable level of CMP-sialic acid hydrolase activity.

In contrast, the radio t.l.c. analyses of extracts (containing 94±4% of added radioactivity) from incubation of CMP-[¹⁴C]Neu5Ac with the high-speed supernatant fractions provided persuasive evidence for the presence of CMP-Neu5Ac hydroxylase.

The results in Fig. 1 show that a significant amount (45%) of the CMP-[¹⁴C]Neu5Ac had been transformed into a product co-migrating with the CMP-Neu5Gc standard. There were also traces of radioactive substances with the same Rₚ values as Neu5Gc and Neu5Ac, which presumably arose from the hydrolysis of their respective CMP-glycosides during incubation and work up of the samples. The percentage composition of free [¹⁴C]sialic acids in relation to total [¹⁴C]sialic acid was 6±3.1% (average of duplicate incubations of two high-speed supernatant preparations). The hydroxylase turnover observed in duplicate incubations with supernatants from two tissue preparations was 5.3 pmol of CMP-[¹⁴C]-Neu5Gc·min⁻¹·mg⁻¹ of protein⁻¹ in both preparations. Interestingly, the presence of 0.8% Triton X-100 activated the hydroxylase by at least 50%, suggesting that its presence in experiments with particulate fractions had had no adverse effects on any activity that might have been present in these preparations.

Identification of the product from incubations of mouse liver high-speed supernatant and CMP-[¹⁴C]Neu5Ac

Before undertaking any further investigations into the apparent CMP-Neu5Ac hydroxylase activity observed in the supernatant, it was essential to unequivocally identify the potential CMP-[¹⁴C]Neu5Gc product. A sufficient...
Characterization of the cofactor requirements of the soluble CMP-Neu5Ac hydroxylase

The influence of various potential cofactors on the activity of CMP-Neu5Ac hydroxylase was tested with the routine enzyme assay using a sucrose-free high-speed supernatant (see the Materials and methods section).

The course of the hydroxylation reaction with NADH (1 mM) and FeSO₄ (0.5 mM) was shown to be linear up to 30 min (Fig. 3). The reaction rate was also found to be linearly dependent on protein concentration, up to 17 mg of protein/ml.

Having established linear reaction conditions, the effect of several cofactors alone and in various combinations could be tested. The results presented in Fig. 4 show that a certain amount of activity was supported solely by the addition of a reducing cofactor, NADH and NADPH being the most effective. Addition of Fe²⁺ ions, however, stimulated the hydroxylation, most markedly in conjunction with the two reduced nicotinamide nucleotide coenzymes. Activity supported by the other reductants was only slightly increased upon addition of Fe²⁺ ions. In the case of the reduced pterin cofactor, a marginal inhibition was observed in the presence of Fe²⁺. Addition of the cofactor mixture consisting of 2-oxoglutarate, ascorbate, FeSO₄ and dithiothreitol was also ineffective in enhancing the hydroxylation activity.

Influence of various metal ions on the activity of CMP-Neu5Ac hydroxylase

The metal ion specificity of the enzyme was tested with NADH as reducing cofactor. As can be seen in Fig. 5, only Fe²⁺ and Fe³⁺ stimulated CMP-Neu5Ac hydroxylase. All other metal ions tested either had no significant effect or were inhibitory. The metal ion chelator EDTA exerted a considerable inhibitory effect on the enzyme. This may have been a result of the chelation of endogenous iron.

Fig. 2. Radio t.l.c. analysis of [¹⁴C]sialic acids released by acid (a) and alkali (b) treatment of the main radioactive product generated by incubation of CMP-[¹⁴C]Neu5Ac with mouse liver supernatant in the presence of NADH and FeSO₄.

Lettered arrows indicate migration positions of non-radioactive standard compounds: A, Neu5Gc; B, Neu5Ac; C, Neu5Gc2en. Since only a trace of Neu5Ac is visible in (a), the right hand peak in (b) must be Neu5Gc2en.

A considerable amount of [¹⁴C]product was synthesized and partially purified as described in the Materials and methods section. Acid treatment of the [¹⁴C]-labelled product yielded one peak of radioactivity co-migrating with Neu5Gc upon analysis by t.l.c. in solvent systems 1 and 2 (Fig. 2a). In contrast, long-term treatment with alkali gave rise to two main peaks co-migrating with Neu5Gc and Neu5Gc2en [21], the expected hydrolysis and elimination products of CMP-Neu5Gc [24] (Fig. 2b).

The [¹⁴C]-labelled product also served as a substrate for Galα2-6-sialyltransferase, about 70% of the added radioactivity being transferred to the asialofetuin acceptor. No radioactivity was transferred by heat-denatured enzyme. The fetuin-bound [¹⁴C]sialic acid was identified as Neu5Gc by t.l.c. of the hydrolytically released radioactivity (solvent system 1).

These results therefore indicate that CMP-Neu5Ac is hydroxylated by mouse liver supernatants to give CMP-Neu5Gc.
Effect of anaerobic conditions on CMP-Neu5Ac hydroxylase activity

Removal of oxygen from reaction mixtures by extensive gassing with O₂-free nitrogen caused a drop in the rate of hydroxylation from 3.2 (identical controls incubated under aerobic conditions) to 0.18 pmol·min⁻¹·mg of protein⁻¹ (individual values of two duplicates were 0.15 and 0.21 pmol·min⁻¹·mg of protein⁻¹). In control experiments, deoxygenated supernatant samples which were subsequently re-aerated were found to have recovered 77% of their hydroxylase activity. Molecular oxygen is therefore an essential component of the hydroxylase-catalysed reaction.

Dependence of CMP-Neu5Ac hydroxylase activity on the concentrations of NADH, FeSO₄, and CMP-Neu5Ac

The effect of NADH concentration on CMP-Neu5Ac hydroxylase activity was investigated using FeSO₄ and CMP-Neu5Ac concentrations of 500 μM and 10 μM respectively. From the results in Fig. 6, it is evident that under these conditions the enzyme activity had reached a plateau at about 150 μM-NADH. Similarly, with 1 mM-NADH and 10 μM-CMP-Neu5Ac, a maximal hydroxylase activity of 14 pmol of Neu5Gc·min⁻¹·mg of protein⁻¹ was attained at 300 μM-FeSO₄, saturation occurring at 100 μM-FeSO₄, where the rate was 12.2 pmol·min⁻¹·mg of protein⁻¹. Using these optimal NADH and FeSO₄ concentrations, the dependence of the hydroxylase activity on CMP-Neu5Ac concentration was investigated. The curve shown in Fig. 7 is the result of a computer fitting of the data to a Michaelis–Menten equation by non-linear regression, performed with the ‘Enzfitter’ program (written by R. Leatherbarrow and obtained from Elsevier Biosoft, Cambridge, U.K.). The kinetic parameters determined in this analysis were Vₘ, 15.8 ± 0.63 pmol of Neu5Gc·min⁻¹·mg of protein⁻¹; Kₘ, 1.36 ± 0.137 μM. The relatively low standard errors are indicative of a good fit of the data to the Michaelis–Menten equation.

DISCUSSION

The results presented in this work clearly establish that Neu5Gc biosynthesis in fractionated mouse liver results from the hydroxylation of CMP-Neu5Ac, with CMP-Neu5Ac as the immediate product. Neither free [¹⁴C]Neu5Ac nor [¹⁴C]Neu5Ac glycosidically bound by Galα2-3- and Galα2-6-GlcNAcβ1-4 linkages to fetuin glycan chains were hydroxylated to Neu5Gc. This suggests that the hydroxylation of CMP-Neu5Ac is the only pathway for Neu5Gc biosynthesis, as was observed in pig submandibular glands [18].

The hydroxylase catalysing this reaction is extracted as a soluble enzyme occurring in the high-speed supernatant fraction. These observations suggest that this hydroxylase is either located in the cytoplasm, or is possibly loosely bound to a subcellular structure and released during homogenization.

The enzyme’s requirement for molecular oxygen
Fig. 5. Influence of metal ions and EDTA on the activity of CMP-Neu5Ac hydroxylase in sucrose-free mouse liver supernatants

Supernatant protein (1.75 mg) was incubated for 20 min at 37 °C in a final volume of 0.3 ml. Products were extracted and analysed as described in the routine assay. The data are the results of single experiments. The base-line activity in this Figure represents a turnover of CMP-[14C]Neu5Ac in the presence of 0.5 mM-NADH of 1.65 pmol·min⁻¹·mg of protein⁻¹. EDTA or metal ions were added to a final concentration of 0.2 mM. Metal salts used were: FeSO₄, FeCl₃, CaCl₂, CuSO₄, ZnSO₄, NaCl, KCl, MgCl₂, NiCl₂, CoCl₂ and MnCl₂. Bars on the ‘minus’-side of the ordinate represent enzyme inhibition, bars on the ‘plus’-side, activation. The absolute activity values were as follows (pmol·min⁻¹·mg of protein⁻¹): no added metal, 1.65; Fe²⁺, 3.42; Fe³⁺, 3.1; Ca²⁺, 1.78; Cu²⁺, 0.32; Zn²⁺, 1.2; Na⁺, 1.62; K⁺, 1.50; Mg²⁺, 1.36; Mn²⁺, 0.99; Ni²⁺, 0.85; Co²⁺, 0.93.

Fig. 6. Dependence of CMP-Neu5Ac hydroxylase activity on NADH concentration

A 0.1 ml portion of the 120000 g supernatant (2 mg of protein) was incubated at 37 °C for 20 min with 10 μM-CMP-[14C]Neu5Ac (0.05 μCi), 0.5 mM-FeSO₄ and various NADH concentrations up to 1000 μM in a final volume of 0.12 ml. After stopping the reactions with 15 μl of 2 m-HCl suggests that this hydroxylase is a mono-oxygenase, as was originally proposed for the pig enzyme [25]. This conclusion is corroborated by its specificity for a reduced nicotinamide nucleotide coenzyme. Although the mouse liver enzyme was not completely dependent on the addition of iron salts, the CMP-Neu5Ac hydroxylase from porcine submandibular glands had an almost absolute requirement for Fe²⁺ ions [18]. This requirement for exogenous iron is unusual since several NAD(P)H-dependent mono-oxygenases, for example methane mono-oxygenase [26], possess a firmly bound non-haem, non-iron-sulphur iron-based cofactor. However, some exogenous iron-requiring NAD(P)H-dependent dioxygenases are known, for example toluene dioxygenase from Pseudomonas putida [27]. Despite the fact that these studies with unfractionated high-speed supernatants do not allow an unequivocal assignment of the enzyme type, it is clear that this hydroxylase is not a cytochrome P-450, since this class of haemoproteins is membrane-bound in higher organisms and is not activated by addition of exogenous iron [28]. Further-

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Fig. 7. Dependence of CMP-Neu5Ac hydroxylase activity on CMP-Neu5Ac concentration

A 0.1 ml portion of the high-speed supernatant (2 mg of protein) was incubated at 37 °C with 1 mm-NADH, 0.5 mm-FeSO₄ and various CMP-[¹⁴C]Neu5Ac concentrations. Incubation times were varied according to CMP-[¹⁴C]Neu5Ac concentration in order to keep measurements within the linear portion of the reaction time course: 0.2–0.75 μM, 3 min; 1.0–1.5 μM, 5 min; 2.0–10.0 μM, 10 min. Reactions were stopped and product analysis was performed as described in the legend to Fig. 6.

Moreover, the addition of 2-oxoglutarate together with ascorbate, Fe²⁺ ions and dithiothreitol had little effect on the enzyme activity, suggesting that it is not a 2-oxoglutarate-dependent hydroxylase, such as prolyl hydroxylase [29]. The inability of 6,7-dimethyl-5,6,7,8-tetrahydrobiopterin to support the hydroxylation of CMP-Neu5Ac indicates that this enzyme is not a pterin hydroxylase such as tyrosine or phenylalanine hydroxylase [30].

It is, however, evident from these results that the CMP-Neu5Ac hydroxylases from mouse liver and pig submandibular gland are very similar with regard to solubility and substrate and cofactor specificity [18], suggesting that this step in Neu5Gc biosynthesis may be common to all organisms expressing this sialic acid. In support of this hypothesis, we have obtained evidence for the presence of NADH- and iron-dependent CMP-Neu5Ac hydroxylase activity in high-speed supernatants of rat liver, ox spleen and frog liver with 1 mm-NADH and 0.5 mm-FeSO₄ (specific activities were 0.5, 37 and 23 pmol of CMP-Neu5Gc · h⁻¹ · mg of protein⁻¹, measured as in the routine assay described in the Materials and methods section: L. Shaw, unpublished work). In the light of these results, the following modified name is suggested for this enzyme: CMP-N-acetylneuraminic: NAD[P]H oxidoreductase (N-acetyl hydroxylating) (EC 1.14.99.18) in accordance with the nomenclature suggested by the International Union of Biochemistry [31].

The low apparent Kₘ for CMP-Neu5Ac exhibited by this hydroxylase (1.36 μM) ties in well with the high affinity of the Golgi membrane-bound CMP-Neu5Ac/CMP antiport for cytoplasmic CMP-Neu5Ac (apparent Kₘ = 2.4 μM [32]) in rat liver. This suggests that the cytoplasmic concentration of CMP-Neu5Ac could lie in the micromolar region. This conclusion is consistent with the fact that only small amounts of CMP-sialic acid glycosides can be isolated from rat liver (34 nmol/g of tissue [33]) and pig submandibular gland (approx. 6 nmol/g of tissue [16]).

These results also raise a number of intriguing questions regarding the regulation of Neu5Gc expression in sialylglycoconjugates. The relative amounts of Neu5Ac and Neu5Gc characteristic for a certain cell type [1] could originate from metabolic control at several points along the pathway of sialic acid incorporation into glycoconjugates. The possibility that the CMP-glycoside specificity of sialyltransferases might be a deciding factor in regulating Neu5Gc incorporation has been to some extent excluded by kinetic measurements on several of these purified enzymes [34] with CMP-Neu5Ac and CMP-Neu5Gc substrates. Differential breakdown of CMP-Neu5Ac and CMP-Neu5Gc by CMP-sialate hydrolase (EC 3.1.4.40) is also an unlikely regulatory mechanism, since this enzyme is predominantly plasma-membrane bound [35].

The relative amounts of CMP-Neu5Gc and CMP-Neu5Ac available to the sialyltransferases within the Golgi stacks must therefore be a key factor in determining the ratio of Neu5Gc to Neu5Ac in the resulting glycoconjugates. The required levels of CMP-Neu5Gc might thus be maintained by the activity of the CMP-Neu5Ac hydroxylase. Alternatively, an enrichment of CMP-Neu5Gc within the Golgi apparatus may be brought about by means of a CMP-Neu5Gc-selective CMP-glycoside transporter which exchanges CMP released by sialyltransferases on the luminal side of a Golgi vesicle for a cytoplasmic CMP-sialic acid glycoside [36–38]. Although most investigations on this antiport system have concentrated on the transport of CMP-Neu5Ac [36,37], recent experiments with mouse-liver Golgi vesicles suggest that this translocation system does not exhibit any significant preference towards either CMP-Neu5Ac or CMP-Neu5Gc [39].

Clearly, there remain many open questions regarding the control of Neu5Gc incorporation into sialoglycoconjugates. Nevertheless, this determination of the substrate specificity of ‘sialic acid hydroxylase’ provides a basis for further research into the expression and role of Neu5Gc in important biological processes including differentiation and oncogenesis.

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