Glycoconjugate Journal 15, 885–893 (1998)

The role of CMP-*N*-acetylneuraminic acid hydroxylase in determining the level of *N*-glycolylneuraminic acid in porcine tissues

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The biosynthesis of the sialic acid *N*-glycolylneuraminic acid (Neu5Gc) occurs by the action of cytidine monophosphate-*N*-acetylneuraminate (CMP-Neu5Ac) hydroxylase. Previous investigations on a limited number of tissues suggest that the activity of this enzyme governs the extent of glycoconjugate sialylation with Neu5Gc. Using improved analytical procedures and a panel of nine porcine tissues, each expressing different amounts of Neu5Gc, we have readdressed the issue of the regulation of Neu5Gc incorporation into glycoconjugates. The following parameters were measured for each tissue: the molar ratio Neu5Gc/Neu5Ac, the activity of the hydroxylase, and the relative amount of hydroxylase protein, as determined by enzyme-linked immunosorbent assay (ELISA). A positive correlation between the activity of the hydroxylase and the molar ratio Neu5Gc/Neu5Ac was observed for each tissue. In addition, the hydroxylase activity correlated with the amount of enzyme protein, though in heart and lung disproportionately large amounts of immunoreactive protein were detected. Taken together, the results suggest that the incorporation of Neu5Gc into glycoconjugates is generally controlled by the amount of hydroxylase protein expressed in a tissue.

***Keywords*: hydroxylase, sialic acids, *N*-glycolylneuraminic acid, CMP-*N*-acetylneuraminic acid, porcine tissues, ELISA, regulation**

Introduction

Sialic acids constitute a family of about forty structurally related acidic sugars which occur in a large number of animal glycoconjugates [1]. Their predominantly terminal position on the oligosaccharide chains enables sialic acids to play vital roles in cellular recognition processes, mediating important cell-cell interactions in development, the immune response, inflammatory reactions, and in a number of adhesion processes [2]. The structural diversity of the sialic acids arises from a variety of biosynthetic modifications of *N*-acetylneuraminic acid (Neu5Ac), the simplest and most ubiquitous sialic acid. One of the most common derivatization reactions is the hydroxylation of the *N*-acetyl group bound to the C-5 position of Neu5Ac, which gives rise to *N*-glycolylneuraminic acid (Neu5Gc). The extent of glycoconjugate sialylation with Neu5Gc is very variable and dependent on the species, tissue, and stage in development [3–5]. Although Neu5Gc occurs in practically all animal groups possessing sialylated glycoconjugates, its presence in normal human tissues has not been demonstrated conclusively [1, 3, 6].

It is now generally accepted that the biosynthesis of Neu5Gc occurs by the hydroxylation of the sugar-nucleotide cytidine-5′-monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac), giving rise to CMP-Neu5Gc [7, 8]. The CMP-Neu5Ac hydroxylase (EC 1.14.13.45) was purified from pig submandibular glands and mouse liver and found to be a soluble monomeric protein with a molecular mass of 65 kDa [9, 10]. The enzyme is an NAD(P)H-dependent monooxygenase, which possesses an iron-containing, Rieske-type prosthetic group [11] and requires the ubiquitous electron transport proteins cytochrome b_5 reductase and cytochrome b_5 for activity [12, 13].

Several lines of evidence indicate that CMP-Neu5Ac hydroxylase is a key factor in determining the level of Neu5Gc characteristic for glycoconjugates produced by a particular tissue. First, this is the only enzyme shown to be involved in Neu5Ac hydroxylation [7, 8, 14, 15]. Furthermore, none of the proteins involved in the subsequent proc-

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essing of CMP-sialic acids, i.e., the CMP-sialate transporter, sialyltransferases, or CMP-sialate hydrolase, exhibit any pronounced preference for CMP-Neu5Ac or CMP-Neu5Gc [14, 16, 17]. The qualitative correlation found between the hydroxylase activity and the molar ratio Neu5Gc/Neu5Ac in comparative studies on mouse and rat liver [16] and with mouse lymphoma cell lines [18] provides further proof for the regulatory role of the hydroxylase. The CMP-Neu5Ac hydroxylase activity also paralleled the postnatal increase in the level of G_{M3} (Neu5Gc) in rat small intestine, though no such correlation was found for the Neu5Gc-containing glycoproteins in the same organ [14]. By contrast, no relationship between hydroxylase activity and developmental changes in Neu5Gc in other rat tissues was observed [19]. Subsequent to these investigations, it was discovered that CMP-Neu5Ac hydroxylase is dependent on the cytochrome b_5 system [12]. Since the regulatory studies were performed with high-speed supernatants in which the level of cytochrome b_5 and the respective reductase severely limited the rate of the hydroxylase reaction [13], the observed activities may not have reflected the true level of enzyme in the tissues.

It has also not been unequivocally established how the level of the hydroxylase activity characteristic for a particular cell type is achieved. Preliminary data suggest a qualitative correlation between the amount of immunoreactive hydroxylase protein and the hydroxylase activity in extracts of pig, rat, and mouse liver [9]. Qualitative data, obtained by Northern blot analysis of CMP-Neu5Ac hydroxylase from some mouse organs [20], support this hypothesis. These limited studies do not allow us to exclude other mechanisms regulating the hydroxylase activity, e.g., posttranslational modifications or modulation of the apparently rather weak interaction with membrane-bound cytochrome b_5 [13]. The aim of the work presented here is to shed more light on the regulation of Neu5Gc biosynthesis using an improved enzyme test and a more accurate method of sialic acid analysis. To this end, a panel of tissues from one species was used to study the relationship between the quantity of Neu5Gc, the activity of CMP-Neu5Ac hydroxylase, and the relative amount of the hydroxylase protein, as determined by an ELISA whose development is described in this work.

Materials and methods

Materials

Analytical grade reagents from Sigma Chemicals Ltd. (Deisenhofen, Germany), Merck (Darmstadt, Germany), and Boehringer Mannheim (Mannheim, Germany) were used throughout this study. CMP-[4, 5,6,7,8,9 - ^{14}C]Neu5Ac (250 mCi/mmol) was obtained from Amersham (Braunschweig, Germany). Chromatographic materials were purchased from the following companies: DEAE-Sephadex A-50 and Superose S.12 column from Pharmacia Biotech (Freiburg, Germany) and Protein A-Sepharose, Cibacron Blue 3GA-Agarose (type 3000-CL-L) and Reactive Brown 10-Agarose from Sigma Chemicals Ltd.

High-speed supernatants from various tissues

The pig tissues were collected fresh from the local slaughterhouse and were processed immediately for the enzyme assays. In addition, small samples of the respective tissues were stored at −20°C for the sialic acid analyses. All procedures were performed at 4°C using precooled buffers and centrifuges. The tissues were homogenized with an Ultraturrax and/or a Potter-Elvehjem homogenizer into 50 mM HEPES/NaOH (pH 7.4; 1g tissue to 5 ml buffer). High-speed supernatants were produced by centrifugation at 100 000 × **g** for 1 hr. The hydroxylase activity in each high-speed supernatant was determined immediately, and the remaining supernatant was frozen in aliquots and stored at −20°C for the subsequent ELISA assay and protein determination. In some experiments, the resulting pellet was resuspended in the initial volume of 50 mM HEPES/NaOH (pH 7.4) and centrifuged at 100 000 × **g** for 1 hr. The pellet was finally resuspended in the initial volume of the same buffer to give a known volume and the hydroxylase activity was measured.

Enzyme assay

The activity of the CMP-Neu5Ac hydroxylase in the high-speed supernatants of various pig tissues and in different fractions from the purification of the hydroxylase from pig submandibular glands was measured [9]. Briefly, the enzyme assay was performed in 50 mM HEPES/NaOH (pH 7.4) at 37°C in the presence of 1 mM NADH, 0.5 mM $FeSO_4$, 10 μM CMP-[4,5,6,7,8,9-^{14}C]Neu5Ac (12.5 nCi in the test), and pig liver microsomes (30 μg protein in the test) solubilized in Triton X-100 (2.5% by mass), the final volume of the assay being 25 μl. The assays, performed in duplicate, were stopped by the addition of 5 μl 1 M trichloroacetic acid. The precipitated material was removed by centrifugation for 4 min at 14 000 × **g** and released [^{14}C]–sialic acids were analyzed by radio thin layer chromatography [8]. The accuracy of the enzyme assays was within 6%.

Enzyme inhibition tests were performed by preincubation of purified antibodies with the enzyme for 40 min at room temperature before starting the assay.

Sialic acid analysis

Fluorimetric high-performance liquid chromatography-analysis of various sialic acid forms

The small samples taken from the tissues used to determine hydroxylase activity were thawed and homogenized in 1–2 volumes water (ml/g wet mass) with a Potter-Elvehjem homogenizer at 4°C. Each homogenate (0.2 ml) was mixed with the same volume of 4 M propionic acid (final pH 2.3),

and sialic acids were released by incubation at 80°C for 4 h [21]. The samples were cooled and centrifuged for 10 min at 14 000 × **g**. The resulting pellets were resuspended in 0.5 ml 0.1 M HCl, incubated for 50 min at 80°C to completely release sialic acids from glycoconjugates, and centrifuged as above. The supernatants after both hydrolyses were lyophilized, dissolved in water, and united, giving a final volume 0.6 ml. A 0.1 ml aliquot of this solution, containing released sialic acids, was taken for saponification of potential *O*-acetylated sialic acids, and was carried out by incubation with a final concentration of 0.05 M NaOH for 30 min at room temperature, followed by neutralization with 0.1 M HCl. The sialic acids in saponified and nonsaponified samples were analyzed as follows by the method of Hara *et al.* [22] with some modifications. Aliquots (0.1 ml) of the total released sialic acids or the saponified samples were lyophilized, resuspended in 10 µl 2 M acetic acid, and incubated for 1 h at 56°C in the dark with 49 µl of a reagent consisting of 7.0 mM 1,2-diamino-4,5-methylene dioxybenzene (DMB), 0.75 M β-mercaptoethanol, and 18 mM $NaHSO_3$ dissolved in water. After derivatization, samples were cooled and 5–10 µl aliquots were analyzed by high-performance liquid chromatography (HPLC) on a reversed-phase column, RP18 (250 × 4 mm, particle size 5 µm; Merck) with the mobile phase acetonitrile-methanol-water (9:7:84 by volume). The flow rate was 1 ml/min, and the derivatized sialic acids were detected with a flow-through fluorimeter (emission and excitation wavelengths 448 nm and 373 nm, respectively). The column was calibrated using authentic sialic acids derivatized as described above.

Quantification of total sialic acid

To quantify total sialic acids, the tissues were homogenized, treated with propionic acid, and centrifuged as described above. The supernatant was filtered with a Centricon 10 ultrafiltration unit (10 kDa cut off; Amicon, Eschborn, Germany). The retained material was removed from the filter and united with the pellet from the previous centrifugation, resuspended in 0.5 ml 0.1 M HCl, and hydrolyzed for 50 min at 80°C. The resulting hydrolysate was centrifuged for 10 min at 14 000 × **g** and the supernatant was ultrafiltered with a Centricon 10. The retained material was washed free of released sialic acids by the addition of 2 lots of 0.5 ml water, followed by ultrafiltration. The filtrates from the propionic and hydrochloric acid hydrolyses were lyophilized, dissolved in water, and united (total volume 0.7 ml). The released sialic acids were purified by chromatography on cation- and anion-exchange resins as previously described [23]. Adsorbed sialic acids were eluted from the latter resin with 1 M formic acid. The sialic acid-containing eluate was lyophilized. The quantification of the enriched sialic acids was carried out in triplicate with the orcinol-Fe^{3+}-HCl test [23], using Neu5Ac as standard.

Purification of the CMP-Neu5Ac hydroxylase

The purification of the hydroxylase from pig submandibular glands was carried out according to the suggested protocol [9], with some modifications. In brief, the tissue was homogenized in 50 mM HEPES/NaOH (pH 7.4). The resulting homogenate was centrifuged at 10 000 × **g** and the mucin, together with the remaining particulate material, was precipitated from the 10 000 × **g** supernatant with *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide, followed by centrifugation at 100 000 × **g**.

The mucin-depleted, high-speed supernatant was fractionated in a batch procedure by addition of an equal volume of DEAE-Sephadex A-50 slurry equilibrated with 50 mM HEPES/NaOH (pH 7.4). Nonbound protein was removed from the DEAE-Sephadex with 2 volumes of the equilibration buffer, and bound protein, including the hydroxylase, was eluted with 1 volume of 50 mM HEPES/NaOH (pH 7.4), containing 0.4 M NaCl. After a 1.5-fold dilution with 50 mM HEPES/NaOH (pH 7.4), the DEAE-Sephadex eluate was applied to a column of Cibacron Blue 3GA-Agarose, and elution was performed as described previously [9]. The following variation of the fractionation on Reactive Brown 10-Agarose [9] was employed. The column of Reactive Brown 10-Agarose (2.6 × 7 cm) was equilibrated with 50 mM HEPES/NaOH, containing 0.1 M NaCl, and, after applying the pooled and desalted fractions from Blue Agarose, was washed with 50 mM HEPES/NaOH (pH 7.4), containing 0.4 M NaCl. An affinity elution was performed with 70 ml 50 mM HEPES/NaOH (pH 7.4), containing 130 µM CMP-Neu5Ac and 0.4 NaCl at a flow rate of 0.1 ml/min. After pooling, concentration, and desalting of the most active fractions, the final purification was performed on a Superose S.12 columns as previously described [9].

Production of antibodies specific for CMP-Neu5Ac hydroxylase

Two types of antigen were used to produce the antihydroxylase antibodies: purified CMP-Neu5Ac hydroxylase from pig submadibular glands and synthetic decapeptides. The appropriate peptide sequences were chosen by a computer analysis (GENMON program, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany) of the amino acid sequence of the hydroxylase from pig submandibular glands [11] for regions of optimal hydrophilicity, antigenic values, and flexibility. The three peptides, having the highest rank according to the above criteria, *Cys* Glu His Pro Tyr Glu Glu Ile Arg Ser Arg (p-1), Leu Ala Glu Arg Arg Pro Asp Val Pro Ile *Cys* (p-2), *Cys* Ile Thr Glu Arg Lys Lys Leu Leu Asn (p-3) were synthesized by Neosystem Laboratoire (Strasbourg, France). The peptides were bound through an additional terminal cysteine residue, shown in italics in the above sequences, to maleimide-activated keyhole limpet hemocyanin (KLH) (Pierce, Sankt,

Augustin, Germany). Peptide (0.3-0.8 mg) were bound to 1 mg carrier protein and used for the immunizations.

Immunization

The immunization of a rabbit with the hydroxylase protein was performed as in Schlenzka *et al.* [9]. Antibodies against the KLH-coupled peptides were raised according to the same protocol, with 500 μg of the corresponding protein (final volume 0.7 ml) for the first immunization and first boost and 300 μg for the second boost (final volume 0.4 ml). The sera obtained after the third boost for the immunization with the hydroxylase (h-3) and the second boost for the immunization with peptides (p-1, p-2, p-3), exhibited the highest titer for the corresponding antigens and were used in further investigations.

Purification of antibodies

Immunoglobulin G (IgG) fractions from all antisera were isolated by chromatography on Protein A-Sepharose [24]. The eluted IgG was equilibrated in phosphate-buffered saline (PBS; pH 7.4) and further affinity-purified on a 1 ml HiTrap column consisting of the purified hydroxylase (100 μg) immobilized to *N*-hydroxysuccinimide-activated agarose, according to the manufacturer's protocol (Pharmacia Biotech, Germany). The column was equilibrated with PBS, and bound antibodies were eluted with 8 ml of 100 mM glycine/HCl (pH 3.0). Eluate fractions were neutralized immediately with 1 M TRIS buffer (pH 8.0).

The IgG fractions of the sera raised against the peptides were equilibrated with PBS and applied to affinity columns consisting of the corresponding peptide coupled through the cysteine residues to an iodoacetyl group immobilized on cross-linked agarose (Pierce, Germany). The elution of the affinity-purified IgG was carried out as described above for the antihydroxylase antibodies.

Labeling antibodies

Antibodies were labeled with biotin using biotinyl-X-*N*-hydroxysuccinimide ester (Calbiochem-Novabiochem GmbH, Bad Soden, Germany) [25].

Immuno- and dot-blotting

The high-speed supernatants from various tissues and purified CMP-Neu5Ac hydroxylase were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a cellulose nitrate membrane as described in Schlenzka *et al.* [9]. Immununostaining was performed with affinity-purified primary antibodies raised against the hydroxylase and the peptides using secondary alkaline phosphatase-labeled antibodies with the BM chromogenic Western blotting kit (Boehringer Mannheim, Germany) according to the manufacturer's protocol.

For dot-blot analysis, 0.4 μg of purified hydroxylase was applied to a cellulose nitrate membrane and the immunostaining was performed as described for the Western blot.

Enzyme-linked immunosorbent assay

The ELISA was developed according to the methodology given in Harlow and Lane [26]. The following two procedures were tested.

Antibody capture assay

The peptides, coupled to maleimide-activated bovine serum albumin (BSA), or the partially purified hydroxylase were diluted in PBS (20 μg/ml) and bound to a Titertek polyvinylchloride microplate (Flow Laboratories, Meckenheim bei Bonn, Germany). The plate was washed with 0.154 M NaCl containing 0.1% (v/v) Tween 20; the remaining binding sites were blocked using "Blocking reagent for ELISA" (Boehringer Mannheim, Germany), and affinity-purified IgG fractions diluted in blocking reagent were bound to the immobilized antigen. The plate was washed as described above, incubated with the goat antirabbit IgG labeled with horseradish peroxidase (Sigma Chemicals Ltd., Germany), and diluted in blocking reagent, and bound peroxidase was visualized with ABTS substrate (Boehringer Mannheim, Germany).

Sandwich assay

The IgG fraction of antibodies against the hydroxylase (primary antibodies) was diluted to 20 μg/ml in 0.2 M carbonate buffer (pH 10) and bound to the MaxiSorp plate (Nalge Nunc International, Wiesbaden, Germany). After washing the plate and blocking the remaining binding sites (see above), the hydroxylase in the high-speed supernatants was titrated in the blocking reagent and allowed to bind to the primary antibodies. After removal of unbound proteins, immunopurified polyclonal antibodies labeled with biotin (secondary antibodies) and diluted in blocking reagent were bound to the hydroxylase. The plate was washed and incubated with streptavidin-polymerised horseradish peroxidase complex (Sigma Chemicals Ltd., Germany), and, after washing, the bound peroxidase was detected with ABTS. The test was performed in duplicate and the relative amount of the hydroxylase protein was determined by comparison of the midpoints of the titration curves.

Protein concentration

Protein concentrations were measured using the BCA protein assay reagent (Pierce, Germany) or by the method of Bradford [27], using the Bio-Rad reagent (Bio-Rad, Mu-

Table 1. Activity of CMP-Neu5Ac hydroxylase and the quantity of sialic acids in various tissues

Tissue	*Specific enzyme activity (pmol Neu5Gc/ min × mg protein)*	*% Neu5Gc of total sialic acids**	*Total amount of sialic acids (mg/ g protein)*	*Neu5Gc (mg/ g protein)***
Submandibular gland	105.7	86	94.5	81.3
Lymph node	49.0	75	3.4	2.6
Small intestine	46.6	62	5.1	3.2
Spleen	37.8	71	4.3	3.1
Thymus	20.6	77	3.3	2.5
Lung	7.2	40	5.7	2.3
Liver	2.3	29	1.0	0.3
Kidney	2.0	19	3.2	0.6
Heart	1.3	28	2.8	0.8

*This was calculated from HPLC analysis as Neu5Gc/(Neu5Gc+Neu5Ac);mean of two experiments.
**These values were calculated from the data in the previous two columns.

nich, Germany). Bovine serum albumin was used as a standard throughout.

Results

Relationship between Neu5Gc content and activity of CMP-Neu5Ac hydroxylase in various tissues

Sialic acid analysis

The data from the HPLC-analysis of the fluorescent derivatives of total tissue sialic acids are shown in Table 1. Although sialic acids were analysed from crude homogenates, the chromatograms exhibited well-resolved peaks and showed no evidence of interfering substances.

The sialic acid analyses also revealed that only Neu5Gc and Neu5Ac were present in all tissues studied. No evidence for *O*-acetylation of the sialic acids was found, since no difference in the amount and type of sialic acids on HPLC analyses before or after saponification was observed.

The data presented in Table 1 show that the total content of Neu5Gc in each tissue was variable. Furthermore, the amount of Neu5Gc relative to Neu5Ac also differed significantly according to the tissue.

CMP-Neu5Ac hydroxylase activity

In accordance with the cytosolic location of the hydroxylase reported in mammals [7, 10, 20], 93% to 96% of the total enzyme activity was detected in the high-speed supernatants of the tissues studied in this work. For this reason, all investigations on the enzyme were performed with these supernatants (Table 1).

A graphical comparison of the Neu5Gc/Neu5Ac ratio in each tissue with the respective specific CMP-Neu5Ac hydroxylase activity summarized in Table 1 revealed a positive correlation between these two parameters (Figure 1).

Immunochemical quantification of the CMP-Neu5Ac hydroxylase protein

Characterization of the antibodies

The antibodies were characterized in an enzyme inhibition assay, antibody capture ELISA, a dot blot, and a Western blot analysis. Antibodies against the CMP-Neu5Ac hydroxylase itself and the various peptide antigens studied were potent inhibitors of this enzyme (Figure 2). In a West-

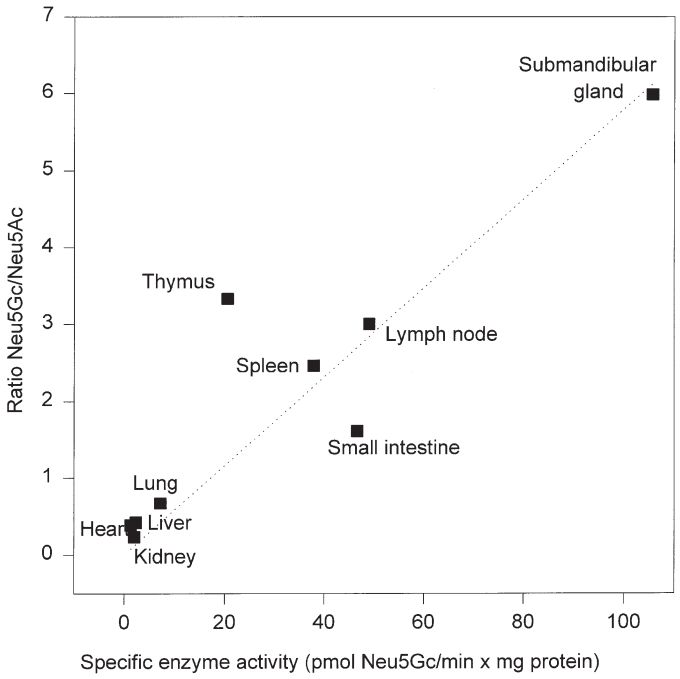

Figure 1. Correlation between the activity of the CMP-Neu5Ac hydroxylase in the high-speed supernatants of several porcine tissues and the respective molar ratio of Neu5Gc/Neu5Ac.

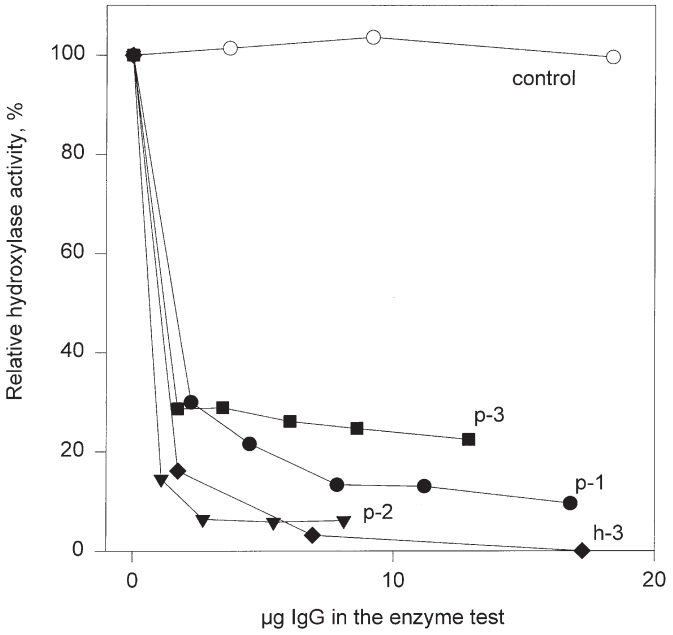

Figure 2. Inhibition of the CMP-Neu5Ac hydroxylase activity by various antibodies. Purified hydroxylase 0.1 µg from submandibular glands was preincubated with the different amounts of affinity-purified antibodies against peptides (p-1, p-2, p-3) and IgG fractions of serum against hydroxylase (h-3) and nonimmune serum (control). The enzyme test was carried as described in Materials and Methods.

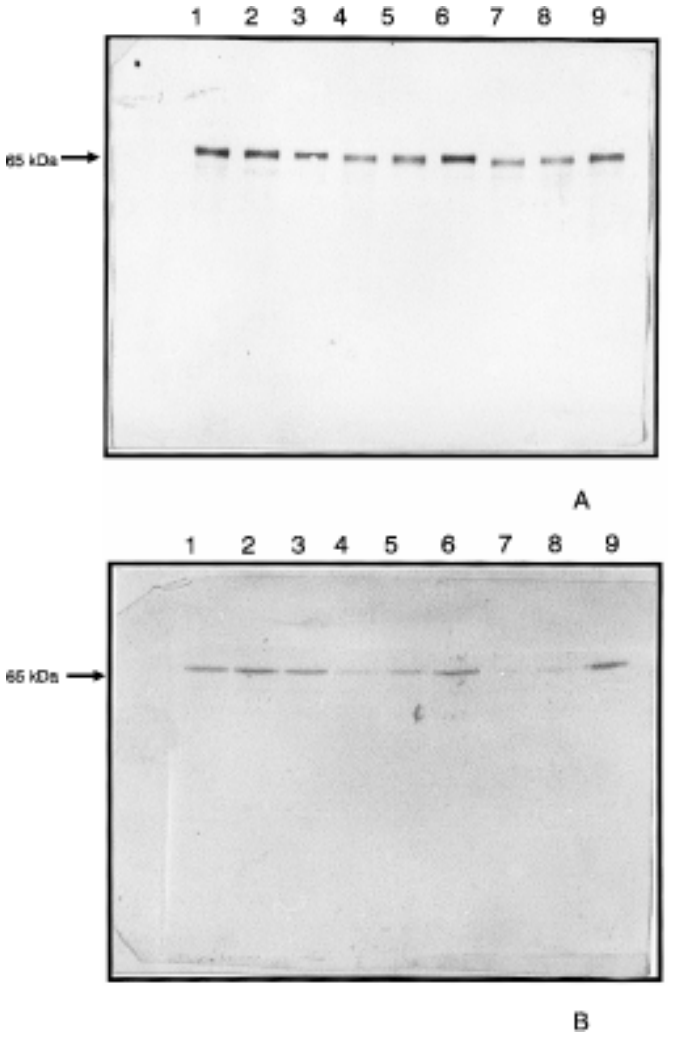

Figure 3. Immunostaining of CMP-Neu5Ac hydroxylase in supernatants from various porcine tissues. SDS-gel electrophoresis and transfer to nitrocellulose membrane was carried out as described in Materials and Methods. High-speed supernatants (1 µg protein per track for gel A and 5 µg protein per track for gel B) were applied in the following lanes: 1-submandibular gland, 2-lung, 3-small intestine, 4-kidney, 5-thymus, 6-lymph node, 7-liver, 8-spleen, 9-heart. The membranes were incubated with affinity-purified antibodies against the hydroxylase (h-3, 0.04 µg/ml) (A) and affinity-purified antibodies against peptide 1 (p-1, 3 µg/ml) (B). The bands were visualized using alkaline-phosphatase-labeled secondary antibodies.

ern blot analysis, using high-speed supernatants from various tissues, only the antibodies raised against the complete protein and peptide 1 specifically stained the hydroxylase, migrating with an apparent molecular weight of 65 kDa (Figure 3). Antibodies against peptides 2 and 3 cross-reacted with a number of other proteins as was observed for high-speed supernatants of pig submandibular glands (data not shown). In the dot blot assay, the titer of the immunopurified antihydroxylase and antipeptide 1 antibodies was about 1:50,000. These two antibodies were employed in further investigations.

Quantification of the hydroxylase protein

For the immunodetection of the hydroxylase protein, a sandwich ELISA was developed, as described in Materials and Methods. The sandwich assay proved to be more specific and an order of magnitude more sensitive than an antibody capture ELISA, allowing the detection of about 1 ng of the hydroxylase protein. The titration curves from the sandwich ELISA analyses of the high-speed supernatants of various porcine tissues are shown in Figure 4. The quantities of the hydroxylase protein calculated from these data and related to the hydroxylase amount in a high-speed supernatant of pig submandibular gland are shown in Figure 5.

The relative intensities of the hydroxylase band stained in the Western blot analysis of the same high-speed supernatants using antibodies against the hydroxylase and peptide 1 (Figure 3) are in agreement with the results in Figures 4B and 5. A comparison of the amount of the hydroxylase protein with the hydroxylase activity expressed as enzyme activity per ml of high-speed supernatant revealed a correlation between these two parameters for most of the tissues studied (Figure 5). However, in the high-speed supernatants from lung and heart, a large amount of the hydroxylase protein was detected (Figures 3 and 4B), though the specific hydroxylase activity and the ratio Neu5Gc/Neu5Ac in these tissues were low (Table 1; Figure 1).

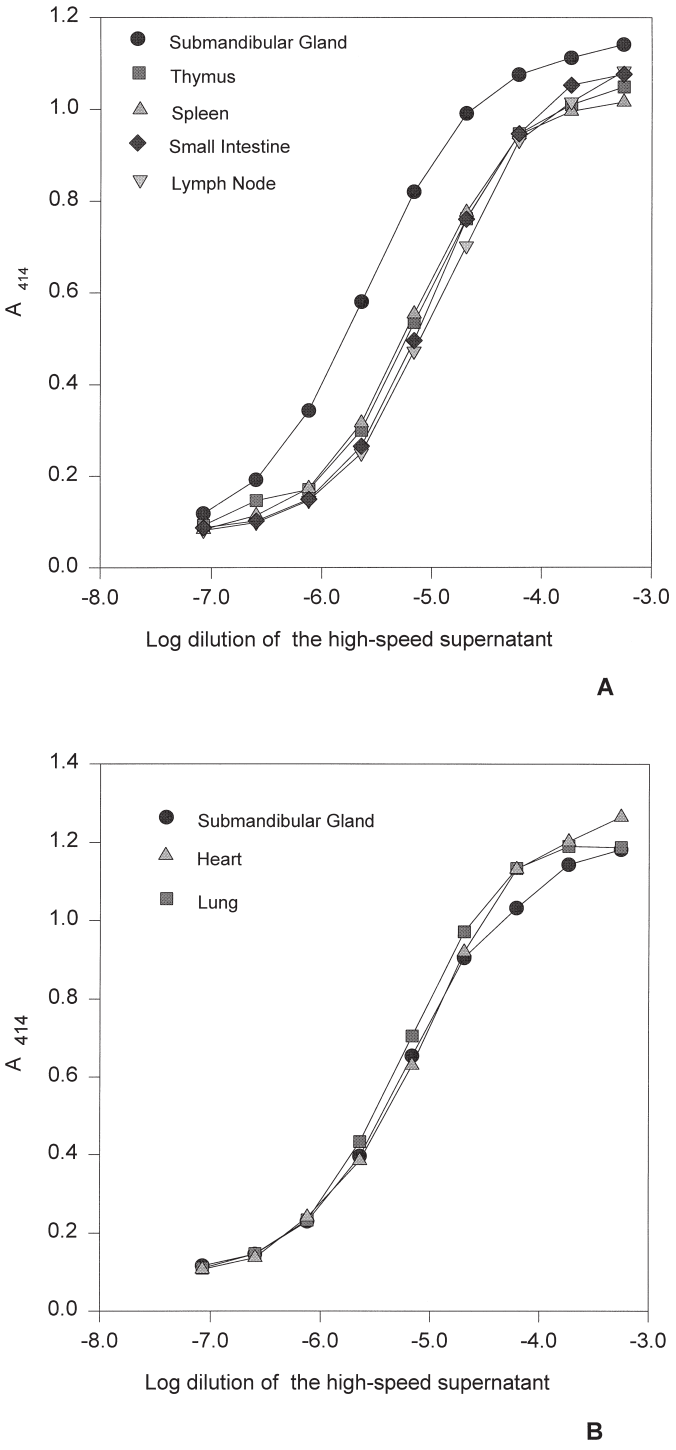

Figure 4. Sandwich ELISA to detect CMP-Neu5Ac hydroxylase in high-speed supernatants of different porcine tissues (A and B). The assays were performed as described in Materials and Methods. High-speed supernatants were applied in different dilutions to a microtiter plate with immobilized antibodies against the CMP-Neu5Ac hydroxylase (h-3). The bound hydroxylase was detected after incubation with biotinylated anti-hydroxylase antibodies (h-3), followed by incubation with streptavidin-polymerized horseradish peroxidase complex and ABTS substrate. The points on the titration curves are means of duplicates.

Discussion

The biosynthesis of Neu5Gc has been examined using improved analytical techniques and a quantitative assay for CMP-Neu5Ac hydroxylase. The approach taken in this study involved comparing the content of Neu5Gc in a panel of porcine tissues, with the respective hydroxylase activities and relative amounts of enzyme protein determined in an ELISA.

Although one might expect the amount of Neu5Gc in a tissue to be reflected in the activity of the hydroxylase, no clear correlation between these parameters was observed (Table 1). This was particularly apparent for submandibular gland in which stored, highly sialylated mucin accounted for the very large amounts of Neu5Gc detected. The storage of secreted glycoconjugates may well have contributed, in various degrees, to the amount of Neu5Gc determined in other tissues, particularly of secretory ones such as intestine or lung. Since the biosynthesis of Neu5Gc occurs by the hydroxylation of CMP-Neu5Ac, it was considered more appropriate to compare the molar ratio Neu5Gc/Neu5Ac in total extracted sialic acids with the respective hydroxylase activity. The results in Figure 1 show a positive correlation between the specific activity of the hydroxylase and the ratio Neu5Gc/Neu5Ac, demonstrating that the activity of this enzyme has a direct influence on the extent of glycoconjugate sialylation with Neu5Gc. This corroborates and extends the observations made in earlier studies [16, 18].

Since secretory organs produce sialic acids more rapidly than other tissues, as shown, for example, by their increased UDP-*N*-acetylglucosamine-2-epimerase / *N*-acetylmannosamine kinase activity [28], a greater hydroxylase activity might have been expected in submandibular glands. The metabolic state of the animals prior to slaughtering may, however, have influenced the enzyme activity.

ELISA and Western blot techniques were employed to assess to what extent the activity of the hydroxylase was governed by the amount of enzyme protein in the high-speed supernatants. The results presented in Figures 3–5 show that in most of the tissues, the catalytic activity of the hydroxylase correlated well with the amount of enzyme protein, suggesting that the expression of CMP-Neu5Ac hydroxylase is a dominant factor in regulating the formation of glycoconjugate-bound Neu5Gc. However, in heart and lung the amount of immunoreactive protein was disproportionately high, considering the very low enzyme activities measured and their ratios of Neu5Gc/Neu5Ac (Figure 1). These tissues were not further investigated in this work, though the results suggest that mechanisms to control the activity of preformed enzyme may exist.

Previous studies have demonstrated that none of the main intermediates of sialic acid metabolism, including CMP-Neu5Gc, the product of the hydroxylase reaction, has any influence on the activity of this enzyme [29]. Moreover,

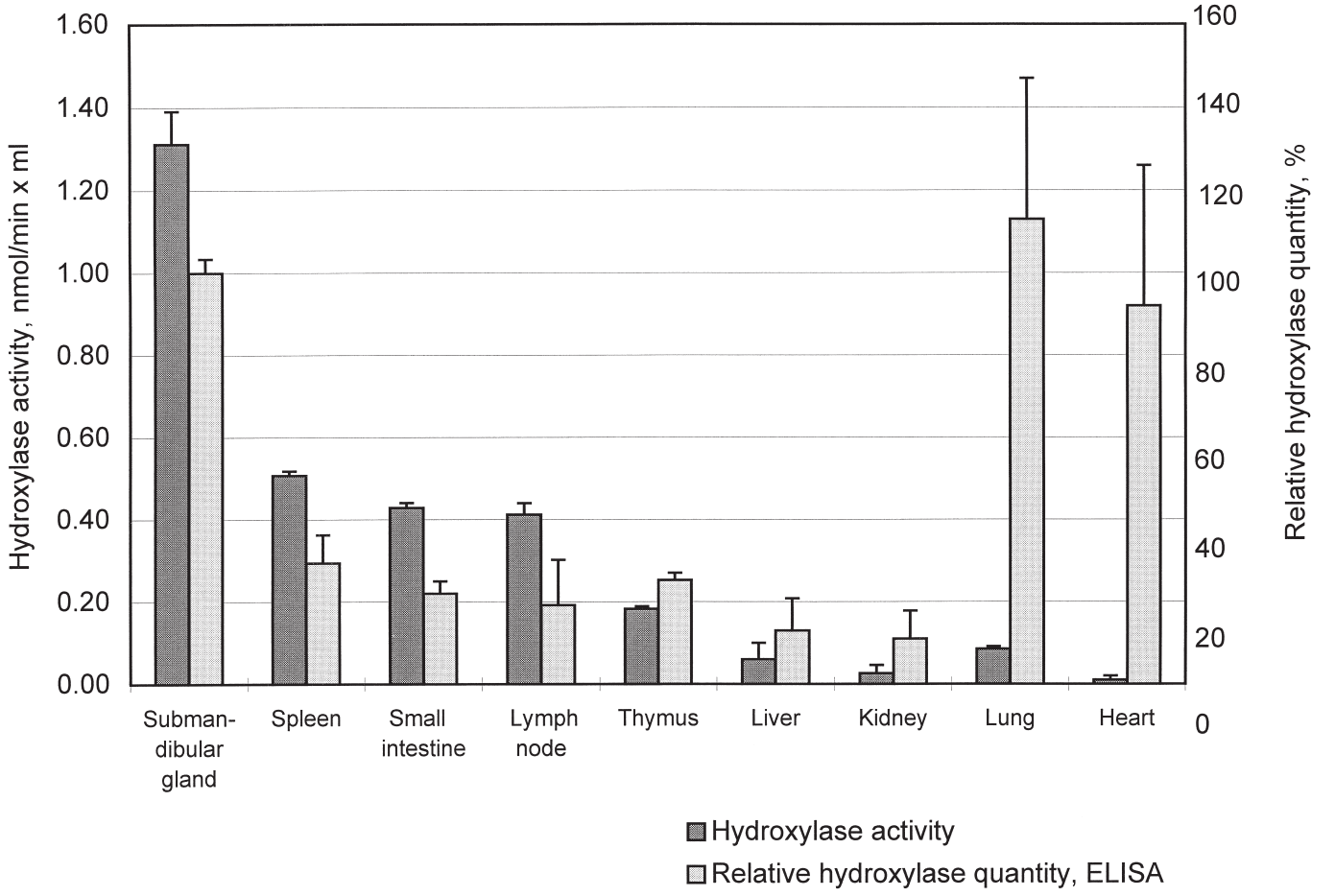

Figure 5. The correlation between the CMP-Neu5Ac hydroxylase activity and the quantity of enzyme protein, determined by ELISA, in high-speed supernatants of different porcine tissues. For calculation of the ELISA data, the quantity of hydroxylase in the supernatant from submandibular gland was taken as 100%. The linear portions of the titration curves were used and compared at the point $A_{414} = 0.5$. The error was calculated by the addition of the 95% confidence intervals determined at this point for submandibular gland and the corresponding tissue. To allow a comparison with the relative concentration of enzyme protein, the activity is expressed in nmol/(min $\times$ ml) high-speed supernatant. The means of two experiments are given.

it is unlikely that the amount of cytochrome b_5 and the respective reductase in the cell specifically limits the catalytic turnover of the hydroxylase, since these electron transfer proteins are involved in many enzymatic processes [30]. It is also improbable that the concentration of CMP-Neu5Ac substrate restricts the activity of the hydroxylase. The size of the CMP-Neu5Ac pool has been estimated to lie in the range of 1.6–3.4 nmol/mg protein [31, 32] for cultured cells and about 40 nmol/g tissue in rat and mouse liver [31]. Taking the values for cultured cells and assuming an average of 10 mg protein/ml high-speed supernatant in the tissues studied here, as well as a fivefold increase in tissue volume due to the addition of homogenization buffer, a minimum CMP-Neu5Ac concentration of 80–170 μM could be estimated for each organ. From the data for rat and mouse liver, a tissue concentration of about 40 μM CMP-sialic acid could be calculated. Since these values do not take compartmentalization into account, the concentration of CMP-sialic acid to which the hydroxylase is exposed *in vivo* could be significantly higher, giving values of more than an order of magnitude greater than the apparent Km of 3 μM exhibited by this enzyme for CMP-Neu5Ac [9]. The existence of low–molecular-weight cytosolic inhibitors of the hydroxylase in CHO and MDAY-D2 cell lines indicates that the level of these substances may regulate the activity of the hydroxylase *in vivo* [33]. Other regulatory mechanisms, for example, phosphorylation, must also be considered.

Despite these unusual results for the hydroxylase in heart and lung, Neu5Gc biosynthesis appears to be largely regulated by the amount of CMP-Neu5Ac hydroxylase in a particular tissue, suggesting that the expression of the enzyme is under transcriptional control. A Northern blot analysis of several mouse organs revealed that no messen-

ger ribonucleic acid (mRNA) coding for CMP-Neu5Ac hydroxylase was present in the brain, consistent with the complete absence of this sialic acid in this tissue [20]. Although a signal for hydroxylase-coding mRNA was detected in other tissues, no attempt was made to correlate the level of mRNA with the respective amount of enzyme protein or the content of Neu5Gc. The recent observation that an inactive, truncated form of CMP-Neu5Ac hydroxylase is formed in mouse liver [34] raises the possibility that alternative mRNA splicing might be a regulatory mechanism. However, in none of the Western blots presented here (Figure 3) was there any evidence for the presence of such a form of the enzyme.

Since sialic acids in glycoconjugates play a number of important roles in the functioning of an organism as a whole, it is reasonable to assume that the formation and enzymatic modification of sialic acids characteristic for particular cell types in mature and developing tissues is under strict control [1]. The regulatory mechanisms ensuring that the correct sialic pattern is formed are likely to be under the influence of many intracellular and extracellular factors. The work in this article therefore represents a starting point in our investigations on the regulation of Neu5Gc biosynthesis.

Acknowledgments

The financial assistance of the Volkswagen-Stiftung, Hannover, the Sialic Acids Society, Kiel, and the Fonds der Chemischen Industrie, Frankfurt, is gratefully acknowledged.

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