Evidence for a free *N*-acetylneuraminic acid-hydroxylating enzyme in pig mandibular gland soluble fraction

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The activity of a free *N*-acetylneuraminic acid (Neu5Ac)-hydroxylating enzyme which converted Neu5Ac into *N*-glycolylneuraminic acid (Neu5Gc) was demonstrated in the soluble fraction of pig mandibular gland. The hydroxylation was possible only with NADPH as the electron donor. The apparent K_m was 4.5 mM Neu5Ac. At 0.5 mM monovalent cations had no effect on the hydroxylation of Neu5Ac whereas bivalent cations gave varied inhibition capacities ranging from 14 to 75%. EDTA gave a time-dependent enhancement of activity. It was concluded that

the enzyme does not require an exogenously added inorganic cofactor. Results from salt fractionation of the soluble fraction and the use of inhibitors such as mercurials suggested that the hydroxylation of Neu5Ac to Neu5Gc may involve other, as yet unknown, component(s) and the possibility of electrons donated by NADPH being transferred to activated molecular oxygen (second substrate). We propose to name this enzyme *N*-acetylneuraminic acid hydroxylase.

INTRODUCTION

Sialic acids are components of glycoproteins and glycolipids [1,2], the so-called sialoglycoconjugates that range throughout the animal kingdom. The simplest sialic acid is *N*-acetylneuraminic acid (Neu5Ac), and it can be modified into more complex sialic acids [3]. *N*-Glycolylneuraminic acid (Neu5Gc) is made from the CMP-glycoside of Neu5Ac [4] by the enzyme *N*-acetylneuraminic acid mono-oxygenase [EC 1.14.99.18; CMP-*N*-acetylneuraminic acid hydroxylase]. Neu5Gc is a common sialic acid occurring in animals, except for humans and chickens who possess only Neu5Ac [1,5,6]. Although Neu5Gc is absent from human tissues and fluids, small amounts are known to be present in sialoglycoconjugates from several human cancers [7,8]. They are called Hanganutziu and Deicher (HD) antigens. Cancer patients occasionally generate high-titre antibodies against HD antigens [9–11]. Chickens with Marek's disease lymphoma also express Neu5Gc [12] and the fact that chickens lack Neu5Gc was confirmed by the production of high-titre antibody in chickens immunized with Neu5Gc-containing gangliosides [13].

Using soluble fractions of pig mandibular gland, it has been established that Neu5Gc is synthesized from CMP-Neu5Ac to give CMP-Neu5Gc [4]. This recent discovery goes against one made earlier showing free Neu5Ac to be the authentic substrate for the pig mandibular gland enzyme [14]. Due to our interest in the expression of Neu5Gc in malignancy, we have embarked on the study of Neu5Gc biosynthesis in pig mandibular gland. We report here that a free Neu5Ac-hydroxylating enzyme exists in the soluble fraction of the pig mandibular gland and propose that the previous work on the metabolism of Neu5Gc should be re-investigated in the light of the findings described in this paper.

In this study, we have used a spectrophotometric method that produces fast results. The hydroxylation of free Neu5Ac was

confirmed by use of [14 C]Neu5Ac and gas chromatography where the product was identified to be free Neu5Gc.

EXPERIMENTAL

Materials and chemicals

Mandibular glands were obtained from male pigs aged about 6 months. NADH, NADPH, GSH, phenylmethanesulphonyl fluoride (PMSF), *p*-chloromercuriphenylsulphonic acid (PCMS), *p*-hydroxymercuribenzoate (PHMB), HgCl₂, EDTA and cetylpyridinium chloride were from Sigma, U.S.A. Tiron and *o*-phenanthroline were from Wako, Japan. Benzamidine hydrochloride was from Nakarai Chemical Co., Japan.

Radiochemical [4,5,6,7,8,9- 14 C]Neu5Ac (311 mCi/mmol) was bought from Amersham, U.K. Neu5Ac was from MECT Co., Japan and Neu5Gc was purified from G_{M3} (Neu5Gc) following neuraminidase treatment. Other chemicals were of analytical grade.

Preparation of mandibular gland fractions for assay of enzyme activity

All procedures were carried out at 4 °C using precooled buffer and centrifuge rotors. Tissues were passed through a meat mincer. For each gram of tissue was added 5 ml of buffer (0.05 M Tris/HCl, pH 7.4, containing 0.15 M KCl, 5 mM GSH, 1 mM PMSF and 1 mM benzamidine). The mixture was thoroughly homogenized using a Teflon–glass homogenizer. The slurry was centrifuged at 10000 *g* for 20 min. The microsomal pellet obtained was kept for determination of enzyme activity. The resultant supernatant was again centrifuged at 120000 *g* for 1 h to obtain a microsomal pellet and a high-speed supernatant. The

Abbreviations used: GSH, glutathione (reduced); Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PMSF, phenylmethanesulphonyl fluoride; PCMS, *p*-chloromercuriphenylsulphonic acid; PHMB, *p*-hydroxymercuribenzoate.

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highly viscous supernatant was treated with 5% cetylpyridinium chloride [15] to remove mucin and the mixture spun again at 120000 *g* for 1 h. Most of the 'mucin-free' supernatant was sequentially subjected to 30%, 50%, 70% and 100% satd. ammonium sulphate precipitations. For later experiments, fractions 50%, 70% and 100% were repooled and further salted out to determine the location of the enzyme (see Results). All fractions were determined for protein content using BSA as standard [16].

Enzyme assay (spectrophotometry)

The activity of Neu5Ac-hydroxylating enzyme was monitored at 340 nm using a double-beam spectrophotometer (model U3210, Hitachi Corp., Japan) equipped with on-screen time-scan and expansion capabilities. The machine also has a chart recorder. The procedure was carried out at 25 °C. As both NADH and NADPH have a characteristic absorbance at 340 nm (molar absorption coefficient being 6.2 M⁻¹·cm⁻¹), the decrease in absorbance at this wavelength is a direct measure of enzyme activity [17].

For the endogenous substrate assay, 0.2 ml of enzyme fraction was added into a buffer containing 1 mM GSH (minus others) in a total volume of approx. 2.3 ml. The incubation time intended to exhaust the endogenous activity varied from one enzyme fraction to another. The amount of NAD(P)H was adjusted so as not to give an absorbance reading of or less than 1.0 at the time of adding the substrate. The other details are as described in the Results section. The assay system with exogenous substrate (Neu5Ac) was the same as the one above except various concentrations of Neu5Ac were used. The time course of the enzyme reaction was monitored for 10 min against decrease in absorbance, the curve expanded and the activity was calculated by first drawing a tangent to the early part of the progress curve, thereby measuring the initial rate of reaction [18]. The enzyme activity (an average of three assays) was expressed as nmol/min per mg of protein [17,19].

Confirmation of reaction product by radiometry

This was done essentially according to Shaw and Schauer [4]. Into a 2 ml plastic vial was added in succession; 60 µl of buffer, 2.82 µl of [¹⁴C]Neu5Ac (0.2 nmol), 10 µl of unlabelled Neu5Ac (180 mM) and 50 µl (60 mM) of NADH or NADPH. The reaction was started by addition of 0.2 ml of enzyme fraction (P60) and the reaction mix incubated at 25 °C for 2 h. The reaction was stopped by addition of 1 ml of ice-cold ethanol. The precipitated protein was removed by centrifugation at 1500 *g* for 10 min. The supernatants were evaporated to dryness in a vacuum centrifuge. The residues were resuspended in 50 µl of water and 3 µl was analysed by radio-t.l.c. on 10 cm × 20 cm glass-backed cellulose high-performance t.l.c. plates (Merck, Germany) in a solvent system consisting of n-propanol, n-butanol and 0.1 M HCl (2:1:1, by vol.). Non-radioactive Neu5Ac and Neu5Gc standards were run in parallel and visualized with resorcinol/HCl reagent.

Confirmation of reaction product by g.c.

In a separate experiment, 1 ml of P60 fraction (6 mg/ml) was incubated with either 50 µl of unlabelled Neu5Ac (180 mM) or with 50 µl of unlabelled Neu5Ac and 1 nmol of [¹⁴C]Neu5Ac at 25 °C for 8 h. It was felt necessary to make a product that is non-radioactive to avoid contamination of the g.c. column. The reactions were started by addition of NAD(P)H (10 mM final

concentration) and terminated by addition of 5 ml of ice-cold ethanol. The contents were precipitated at 3000 *g* for 10 min and the supernatants evaporated. The residues containing the radio-isotope were resuspended in 200 µl of water and 2 µl aliquots were analysed by radio-t.l.c. as described above (see Results section).

For the analysis of unlabelled product, the free neuraminic acids were first passed through a Dowex 1X8 column (200–400 mesh, chloride form) and then analysed as their per(trimethylsilyl) derivatives [20] on a ultra-performance capillary column (25 m × 0.32 mm) packed with cross-linked methyl silicone with a 0.17 µm film thickness (Hewlett Packard Co., U.S.A.) at a rate of temperature increase of 4 °C/min from 160 °C to 240 °C (Shimadzu GC-7A). Derivatives of an equal mixture of pure Neu5Ac and Neu5Gc were used for identification of substrate and product respectively.

RESULTS

At first, the distribution of enzyme activity in all (three) tissue fractions (mitochondrial, microsomal and cytosolic) was determined. The enzyme assays in mitochondrial and microsomal fractions were done by use of [¹⁴C]Neu5Ac and not by spectrophotometry because it was not possible to exhaust the endogenous substrate that utilized NAD(P)H. We could not detect any enzyme activity in either mitochondrial or microsomal fractions. Preliminary studies had shown that the nuclear fraction also lacks this enzyme. A rigorous experiment (based on that for CMP-Neu5Ac hydroxylation [21]), aimed at determining the effect of the microsomal fraction on the activity of the soluble fractions with or without a detergent such as Triton X-100, was performed (not shown). No enhancement of specific activity was observed.

For the assay of enzyme in the supernatant (soluble) fractions,

Table 1 Enzyme activity of original supernatant (PO) and salted out fractions

A 0.2 ml aliquot of each of the 12 fractions (0.5–1.6 mg of protein), 20–30 µl of NADPH (60 mM) and 2 ml of buffer were incubated at 25 °C for various times (10–20 min) to exhaust the endogenous activity before 7.75 mM Neu5Ac was added to start the exogenous activity. The progress curve of the exogenous reaction was monitored for 10 min. The original (PO) supernatant was salted out by addition of solid ammonium sulphate in 30%, 50%, 70% and 100% satd. fractions (a). Fractions P50T, P70T and P100T (a) were repooled and further fractionated into six fractions (b). An equal volume of P50, P60 and P70 (b) were mixed (c).

Fraction	Activity (nmol/mg of protein per min)
(a)	
PO (0–100)	14.56
P30T (0–30)	0.00
P50T (31–50)	27.93
P70T (51–70)	71.71
P100T (71–100)	5.06
(b)	
P40 (31–40)	12.27
P50 (41–50)	20.42
P60 (51–60)	26.72
P70 (61–70)	35.18
P80 (71–80)	8.61
P90 (81–90)	0.00
(c)	
P50 + P60 + P70 (equal vol. mixture)	56.43

Figure 1 Effect of protein concentration on the activity of Neu5Ac hydroxylase

Into 2 ml of assay buffer was added 0.2 ml of 2-fold serial dilutions of the original P60 fraction (4 mg/ml) and 20 μ l of 60 mM NADPH. After about 20 min of incubation to exhaust endogenous activity, 100 μ l of 180 mM Neu5Ac (7.75 mM) was added and the progress curve monitored for 10 min. The activity was calculated as described in the Experimental section.

the reaction mixture contained buffer, enzyme fraction and a coenzyme (usually NADPH). No exogenous reaction was found with NADH. At first, the optimal conditions for the activity of enzyme were investigated. The pH range was from 7.2 to 7.4 with a 50 mM buffer. Assuming that the hydroxylation of free Neu5Ac requires an inorganic cofactor such as Fe^{2+} in CMP-Neu5Ac hydroxylation [4], the effect of several metals on the activity of the enzyme was studied. The metal salts used were: FeSO_4 , CaCl_2 , CuSO_4 , ZnCl_2 , MgCl_2 , MnCl_2 , NaCl and KCl (all at 0.5 mM final concentration). K^+ and Na^+ had no effect while the rest had a varying inhibitory effect ranging between 14% and 75%. However, Fe^{2+} , Mg^{2+} and Ca^{2+} gave an enhanced endogenous reaction (above results not shown).

The original (PO) supernatant was salted out by ammonium sulphate into 30%, 50%, 70% and 100% fractions (Table 1a). Fraction P30T had no enzyme activity while fractions P50T and P70T contained almost all the activity. In order to locate the enzyme more precisely, fractions P50T, P70T and P100T were repooled and further fractionated into six fractions (Table 1b). Five of the fractions expressed enzyme activity with P70 having the highest. Fraction P60 had the highest amount of protein (results not shown) and was therefore used in further experiments. When equal volumes of fractions P50, P60 and P70 were mixed, the combined fraction gave a much higher activity than any of the individual fractions (Table 1c).

An experiment for the dependence of initial velocity on the enzyme concentration (Figure 1) showed that the velocity was proportional to the concentration of the enzyme up to a low protein concentration (0.225 mg/ml). The effect of Neu5Ac concentration on the initial velocity gave a hyperbolic curve (Figure 2) giving an apparent K_m of 4.5 mM Neu5Ac.

It was felt necessary to confirm the product of hydroxylation of free Neu5Ac by the enzyme in the P60 fraction. Therefore,

Figure 2 Dependence of Neu5Ac hydroxylase activity on Neu5Ac concentration

A 0.2 ml portion of P60 fraction (0.4 mg of protein) was incubated with 0.5 mM NADPH and various volumes of Neu5Ac as described for Figure 1 and in the Experimental section. This hyperbolic curve is a result of fitting the data to the Michaelis–Menten equation.

Figure 3 Confirmation of hydroxylation of Neu5Ac to Neu5Gc with NADPH but not with NADH

The plate was developed as described in the Experimental section. (a) Autoradiograph; (b) the same plate as in (a) was sprayed with resorcinol/HCl reagent, heated at 120 °C for 15 min to reveal purple-coloured spots; (c) a photocopy of (b) (see the Results section). Lanes 1, unlabelled Neu5Gc; lanes 2, with NADPH; lanes 3, with NADH; lanes 4, without NAD(P)H; lanes 5, unlabelled Neu5Ac.

[^{14}C]Neu5Ac was used as the substrate and autoradiography performed (Figure 3a). The product was identified as [^{14}C]Neu5Gc as it moved to the same position as the co-chromatographed unlabelled Neu5Gc which was detected by resorcinol/HCl reagent [Figure 3(a), lane 2 and Figure 3(b), lane 1]. As was found earlier by spectrophotometry, no activity was detectable with NADH as coenzyme [Figure 3(a), lane 3 and Figure 3(b), lane 3]. Figure 3(c) was obtained by taking a photocopy of Figure 3(b) with the intention of showing a clearer separation of spots in lanes 2, 3 and 4. Consequently, spots in lanes 1 and 5 disappeared. The spot without NAD(P)H (lane 4) moved faster on t.l.c., suggesting the possible formation of a complex between NAD(P)H and substrate and/or product. Spots on lanes 2, 3 and 4 in Figure 3(b) gave a purple colour with resorcinol/HCl, thereby confirming the hydroxylation of Neu5Ac with NADPH and not with NADH.

In order to provide clearer evidence of the identity of the product of the reaction, a large-scale enzyme reaction was carried out as described in the Experimental section using both labelled and unlabelled Neu5Ac. The reaction with the unlabelled substrate was analysed by g.c. and a new peak representing free Neu5Gc was detected in a reaction involving NADPH (Figure

Figure 4 Analysis of reaction product by g.c.

(a) A chromatogram of TMS derivatives of a standard mixture of pure Neu5Ac and Neu5Gc. The retention time of the Neu5Ac peak (horizontal arrow) was 23.19 min while that of Neu5Gc (vertical arrow) was 26.35 min. (b) Neu5Gc peak in the reaction with NADPH, and (c) absence of Neu5Gc peak with NADH.

4b) but not NADH (Figure 4c). The enzyme reaction containing [^{14}C]Neu5Ac was analysed by h.p.t.l.c. followed by autoradiography and resorcinol/HCl spray (Figure 5). A reaction product co-migrating with free Neu5Gc in the reaction with NADPH (Figures 5a and 5b, lanes 2) and not with NADH (Figures 5a and 5b, lanes 3) was observed. The results of Figures 3, 4 and 5

Figure 5 Hydroxylation of Neu5Ac to Neu5Gc with NADPH but not with NADH

The plate was treated as in Figure 3. (a) Autoradiograph; (b) resorcinol/HCl spray. Lanes 1, unlabelled Neu5Gc; lanes 2, with NADPH; lanes 3, with NADH; lanes 4, [^{14}C]Neu5Ac; lanes 5, unlabelled Neu5Ac.

Table 2 Effect of inhibitors on the hydroxylation of Neu5Ac

An aliquot (0.2 ml) of P60 (0.72 mg), 20 μl of NADPH and 2 ml of buffer were incubated for 15 min at 25 $^{\circ}\text{C}$ to exhaust the endogenous activity. The inhibitor was then added and the mixture stood for another 10 min before Neu5Ac (3.96 mM) was added to start the exogenous reaction. The activity is expressed as a percentage of that without inhibitor (100%). The above data is taken from an average of three consecutive experiments.

Inhibitor	Enzyme activity (%)
Sodium azide (1 mM)	109
Potassium cyanide (1 mM)	108
Potassium chloride (100 mM)	87
Tiron (1 mM)	90
Phenanthroline (1 mM)	106
Mercuric chloride (1 mM)	0
<i>p</i> -Hydroxymercuribenzoate (1 mM)	0
<i>p</i> -Chloromercuriphenylsulphonic acid (1 mM)	0
EDTA (disodium salt) (1 mM)	133

provided unequivocal identification of the reaction product to be free Neu5Gc with NADPH as the electron donor, a result obtained earlier by spectrophotometry.

The effect of several compounds (as inhibitors) on the hydroxylation of Neu5Ac was investigated (see Discussion). This is shown in Table 2. Azide and cyanide had the same enhancing effect. Concentrated KCl inhibited 13% of the enzyme activity. Tiron and phenanthroline had a slight inhibitory and an enhancing effect respectively. Mercuric chloride, PHMB and PCMS completely inhibited the hydroxylation. EDTA gave the highest enhancing effect of 33%.

DISCUSSION

The present study demonstrates that a free Neu5Ac-hydroxylating enzyme exists in the soluble fraction of pig mandibular gland. Its activity is solely NADPH-dependent as NADH could not support any hydroxylation. We may at this juncture, therefore, call this enzyme NADPH-specific. The soluble fraction was fractionated by ammonium sulphate in order to locate the enzyme more precisely. The enzyme is salted out by 30–70% saturation of salt. An attempt to fractionate the enzyme further did not produce a higher activity as was expected. This led us to presume that the enzyme is deprived of its other essential components by further fractionation and therefore the hydroxylation of Neu5Ac involves other factors whose identity are as yet unknown. Our presumption was supported by the

results of mixing three fractions (P50, P60 and P70). The activity obtained was much higher than those of individual fractions. The hydroxylation of free Neu5Ac could be similar to that of CMP-Neu5Ac in mouse liver whose hydroxylation involves other factors such as cytochrome *b*₅ [22]. However, the hydroxylation of Neu5Ac (present study) in pig mandibular gland differs from that of CMP-Neu5Ac in the same tissue as regards coenzymes. It has been reported that CMP-Neu5Ac hydroxylation may be supported by either NADH or NADPH [4]. Our studies, so far, show NADPH to be the only electron donor.

A plot of the reaction velocity as a function of the Neu5Ac concentration gave an apparently high K_m (4.5 mM). The curve was hyperbolic, meaning that the enzyme obeys Michaelis-Menten kinetics. In an attempt to look for similarities between the hydroxylations of free Neu5Ac and CMP-Neu5Ac, we used several compounds known to inhibit CMP-Neu5Ac hydroxylation [22,23] or to inhibit the enzymes involved in mitochondrial and microsomal electron transport systems. Azide and cyanide inhibit by reacting with the ferric forms of cytochromes. The two compounds did not inhibit the hydroxylation of Neu5Ac and, therefore, the electron transport system that may be involved [22,23] is not dependent on an accessible ferric form of cytochrome. Tiron and phenanthroline are iron chelators. Tiron inhibited 10% of the enzyme activity, while phenanthroline did not. Again, hydroxylation of Neu5Ac differs from that of CMP-Neu5Ac [4] with regards to the requirement for Fe^{2+} . A concentration of 0.5 mM Fe^{2+} gave rise to major inhibition (75%, results not shown). Perhaps the most convincing result was the effect of EDTA. Addition of EDTA, a bivalent cation chelator, enhanced the enzyme activity. Addition of EDTA at the start of the endogenous activity enhanced the activity even further (152%, results not shown). This indicates that hydroxylation of Neu5Ac is not dependent on any exogenously added bivalent cation as an inorganic cofactor. Both $HgCl_2$ and PCMS inhibits both NADPH-cytochrome *P*-450 and NADH-cytochrome *b*₅ reductases [24]. Both completely inhibited the hydroxylation of Neu5Ac. Their inhibition strongly suggests the existence of an electron transport system, involving NADPH and possibly NADPH-cytochrome *P*-450 reductase (EC 1.6.2.4) which requires no exogenous Fe^{2+} . PHMB also inhibits both reductases [25] by reacting with the sulphhydryl groups. Because of its requirement for a reduced coenzyme (NADPH) and NADPH-cytochrome *P*-450 reductase (from studies on inhibitors), the enzyme involved in Neu5Ac hydroxylation is most likely a mono-oxygenase, as proposed for CMP-Neu5Ac hydroxylase [4], but since we found no reaction with NADH, it is likely that if cytochrome *b*₅ is involved at all, as is the case with CMP-Neu5Ac hydroxylation in mouse liver [22,23], the electrons from a reduced NADPH are transferred via soluble NADPH-cytochrome *P*-450 reductase and cytochrome *b*₅ to ultimately activate molecular oxygen [26,27]. Surprisingly, we could not demonstrate the presence of soluble NADPH-cytochrome *P*-450 reductase in our enzyme fraction by immunoblotting using rabbit anti-(rat liver microsomal NADPH-cytochrome *P*-450 reductase). This phenomenon may be similar to that of participation of cytochrome *b*₅ in CMP-Neu5Ac hydroxylation, where its presence has not been unequivocally demonstrated in the mouse liver soluble fraction [22].

Interactions between electron carriers may be mediated by charges on their surfaces and this could have led to a considerable inhibition of Neu5Ac hydroxylation by a high concentration of KCl. However, unlike for CMP-Neu5Ac hydroxylation [22], 100 mM of KCl did not completely inhibit Neu5Ac hydroxylation.

The study of Neu5Ac and CMP-Neu5Ac hydroxylation may be facilitated by a simpler and more reliable assay, as the ones published [4,22] are discontinuous indirect assays that are technically tedious. The spectrophotometric method developed here is much easier and can be used in any laboratory. This direct continuous assay allows observation of the progress curves, simplifies the estimation of initial rates and allows detection of any anomalous behaviour.

Since free Neu5Ac may be hydroxylated in some tissues, the studies on biosynthesis and re-utilization of Neu5Gc [28] should be re-investigated as studies have suggested that another hydroxylase which synthesizes glycoprotein-committed Neu5Gc specifically also exists and that CMP-Neu5Ac is not its substrate [29]. It appears that there exist several types of hydroxylases. Attempts are being made to purify this enzyme and the components supposedly involved.

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