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Different Reactivity of Two Brain Sialyltransferases Towards Sulfhydryl Reagents. Evidence for a Thiol Group Involved in the Nucleotide-sugar Binding Site of the NeuAcα2-3Galβ1-3GalNAc α(2-6)Sialyltransferase

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We have studied the amino-acid residues involved in the catalytic activity of two distinct brain sialyltransferases acting on fetuin and asialofetuin. These two enzymes were strongly inhibited by *N*-bromosuccinimide, a specific blocking reagent for tryptophan residues. This result suggests the involvement of such residues in the catalytic process of the two sialyltransferases. Furthermore, chemical modifications by various sulfhydryl reagents led to a strong inhibition of the fetuin sialyltransferase while the asialofetuin sialyltransferase was only slightly inhibited. For a more thorough understanding of the thiol inactivation mechanism of the fetuin sialyltransferase, we studied in more detail the reactivity of this enzyme with NEM (*N*-ethylmaleimide), an irreversible reagent. The time-dependent inactivation followed first-order kinetics and these kinetic data afforded presumptive evidence for the binding of 1 mol NEM per mol of enzyme. Only CMP-NeuAc protected the enzyme against NEM inactivation effectively. $MnCl_2$ did not enhance the protective effect of CMP-NeuAc. The modifications of the fetuin sialyltransferase kinetic parameters by NEM showed a competitive mechanism between NEM and CMP-NeuAc. The results suggest the involvement of a sulfhydryl residue in or near the nucleotide-sugar binding site of the fetuin sialyltransferase (but we could not excluded that CMP-NeuAc binding may induce a change in conformation of the protein, leading to a decreased accessibility of this thiol group located near the nucleotide-sugar binding site). This SH group is essential to the enzyme activity, which is not the case for the asialofetuin sialyltransferase.

Abbreviations: *p*-CMB, *p*-chloromercuribenzoic acid; CPDS, 6,6´-dithiodinicotinic acid carboxypyridine disulfide; DTNB, 5,5´-dithiobis-(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; DTT, dithiothreitol; Mes, 2-(*N*-morpholino)ethane sulfonic acid; NeuAc, *N*-acetylneuraminic acid.

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The substrate specificity of several sialyltransferases from different tissues has been well characterized [1], but very little is known about their molecular action mechanism, their structure and their active site. Sialyl-transferases are membrane-bound proteins and their purification is not an easy process. Information on the identity of "essential residues" is thus useful for studying their molecular mechanism of action. Some studies have been published [2-7] on the soluble galactosyltransferases from milk and colostrum. Various research groups have discussed the presence of one lysine residue at the active site of the enzyme [2] and have shown that the modification of the tyrosine [3] and tryptophan [4] residues totally abolishes the enzymatic activity. The bovine milk galactosyltransferase [5, 6] contains at least one critical sulfhydryl group. In bovine colostrum, O'Keeffe *et al*, [7] demonstrated that one of the sulfhydryl groups is in or near the active site of the enzyme. Previous studies from our laboratory on membrane-bound glycosyltransferases have shown that histidine, arginine and tyrosine residues are essential for a cerebral fucosyltransferase activity [8]. The cerebral mitochondrial mannosyl-transferase is highly sensitive to sulfhydryl reagents [9].

Previous work on rat brain gave evidence for the presence of two sialyltransferases specific for *O*-glycans. A first sialyltransferase has been previously described in our laboratory [10]. This enzyme transfers *N*-acetylneuraminic acid (NeuAc) to native fetuin. It was shown to be a NeuAcα2-3Galβ1-3GalNAc α(2-6)sialyltransferase transferring NeuAc in α(2-6)-linkage to the *N*-acetylgalactosamine residue and was designated as fetuin sialyltransferase. Its optimum activity was at 28°C. Asialo-fetuin was not an acceptor as the presence of NeuAc in α(2-3)-linkage on the galactose residue is an absolute requirement for this fetuin sialyltransferase activity. Another sialyltransferase, transferring NeuAc to asialofetuin, was also present. It was named asialofetuin sialyltransferase and was described as a Galβ1-3GalNAc α(2-3)sialyltransferase, transferring NeuAc in an α(2-3)-linkage to the galactose residue (unpublished results). Its optimum temperature was 20°C. Sialyltransferases showing similar specificities have been described by other groups [11-15] and those from porcine submaxillary glands [12, 16] and from human placenta [17] have been purified. However, their sensitivity towards sulfhydryl reagents has never been investigated.

We have recently shown [18] a different reactivity of these two brain sialyltransferases towards lysophosphatidylcholine, DIDS and trypsin, which we interpreted as being a consequence of their respective location in the endoplasmic membrane. We now report evidence for the different behaviours of these two enzymes towards sulfhydryl reagents. We have studied in more detail the reactivity of the NeuAcα2-3Galβ1-3GalNAc α(2-6)-sialyltransferase with *N*-ethylmaleimide, and shown the presence of a thiol residue essential for the enzymatic activity.

Materials and Methods

Chemicals

All reagents were of analytical grade. Fetuin (grade III), Triton X-100, CMP-NeuAc (CMP-*N*-acetylneuraminic acid), NEM (*N*-ethylmaleimide), *p*-CMP (para-chloromercuribenzoic acid), CPDS (6,6'-dithiodinicotinic acid carboxypyridine disulfide), mersalyl acid, DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] and DTT (dithiothreitol) were purchased from Sigma Chemical Co, St Louis, MO, USA. CMP-[^{14}C]NeuAc (sp. act. 305 Ci/mol:11.3 GBq/mmol) was obtained from New England Nuclear, Boston, MA USA.

Animals

Male rats, weighing 180-200 g, of OFA strain (obtained from the Sprague-Dawley strain) were purchased from IFFA-Credo (Les Oncins, France).

Enzyme Preparation

Rats were killed by decapitation without anaesthetics. The cerebral hemispheres were removed immediately after death and homogenized in a Potter-Elvejhem type homogenizer in 50 mM Tris-HCl buffer (pH 7.2), 330 mM sucrose (9 ml/g of wet tissue). The first centrifugation at 1000 x *g* for 10 min eliminated the nuclei. The resulting supernatant was centrifuged at 20 000 x *g* for 20 min, removing mitochondria, synaptosomes and lysosomes as a pellet. The centrifugation of the supernatant at 105 000 x *g* for 60 min led to the sedimentation of the microsomal fraction which was immediately frozen at -20°C. Just before using, pellets were thawed and suspended in 50 mM Tris-HCl buffer (pH 7.2), and solubilization of membrane-bound sialyltransferases was performed with 0.5% (w/v) Triton X-100, followed by a subsequent centrifugation of the microsomes at 150 000 x *g* for 60 min. The supernatant obtained was called "Triton Supernatant".

Protein Assay

Protein was determined according to the procedure of Schaffner and Weissmann [19].

Desialylation of Acceptors

The desialylation of fetuin, bovine submaxillary mucin, human serotransferrin and orosomucoid was performed by mild acid hydrolysis (0.05 M H_2SO_4, 80°C, 60 min) and checked by the resorcinol-HCl method [20].

Sialyltransferase Assays

The standard assay mixture contained, in a total volume of 250 µl, "Triton supernatant" (200 µl) containing 100-200 µg of protein, 400 µg of exogenous acceptor (fetuin or asialofetuin), 1.250 µmol $MnCl_2$, 10 µmol Mes buffer (pH 6.0) and 0.17 nmol CMP-[^{14}C]NeuAc (50 nCi). The incubations were performed at the respective optimal temperature of each enzyme for 120 min. The reaction was stopped with 2 ml of a mixture of trichloroacetic acid (10%, w/v) and phosphotungstic acid (5%, w/v). The precipitate was filtered on GF-B Whatman filters as described previously [21]. Each enzymatic assay was done in duplicate.

Chemical Modification of Thiol Groups by Reversible Sulfhydryl Reagents

p-CMB, CPDS, DTNB and mersalyl acid were added to the assay medium immediately before incubation. Sialyltransferase activities were measured as described above.

Chemical Modification of Thiol Groups by Irreversible Sulfhydryl Reagents

10 µMol Mes buffer (pH 6.0) and various NEM concentrations were added to 200 µl of "Triton supernatant". After pre-incubation at 28°C for varying periods, the reaction with

Table 1. Reactivity of the two brain sialyltransferases towards various amino-acid selective reagents.
The enzymatic preparation ("Triton supernatant" : 0.5 mg proteins/ml) was incubated as described in the Materials and Methods section for 2 h at 20°C for the asialofetuin sialyltranserase and at 28°C for the fetuin sialyltransferase, in the presence of the amino-acid selective reagents. The enzymatic activities are expressed in pmol/2 h/mg protein and, in brackets, as inhibition per cent of the control.

Reagent	Fetuin sialyltransferase 28°C		Asialofetuin sialyltransferase 20°C	
Control	98		50	-
2,3 Butanedione (5 mM)	98	(0%)	45	(-10%)
Diethylpyrocarbonate (5 mM)	74	(-25%)	34.5	(-31%)
p-CMB (0.1 mM)	3	(-97%)	38	(-24%)
Acetlimidazole (5 mM)	98	(0%)	49	(-2%)
N-Bromosuccinimide (5 mM)	2	(-98%)	1	(-98%)
Ethylacetamidate (5 mM)	78	(-20%)	46	(- 8%)

Table 2. Reactivity of two brain sialyltransferases towards various sulfhydryl reagents.
The enzymatic preparation in 50 mM Tris pH 7.2 ("Triton supernatant": 0.5 mg protein/mg) was adjusted to pH 6.0 and incubated as described in the Materials and Methods section in the presence of the sulfhydryl reagents. Results are expressed as in Table 1.

Reagent	Fetuin sialyltransferase (28°C)		Asialofetuin sialyltransferase (20°C)	
Control	98	-	50	-
p-CMB (0.1 mM)	3	(-97%)	38	(-24%)
DTNB (0.1 mM)	3	(-97%)	37	(-26%)
NEM (2 mM)	8	(-94%)	43	(-15%)

NEM was stopped by addition of 0.25 to 1.25 µmol DTT. The sialyltransferase activity was measured as described above.

Protective Effect of Substrates against Chemical Modifications by NEM

"Triton supernatant" 200 µl adjusted to pH 6.0 with 10 µmol Mes buffer (pH 6.0) was pre-incubated with different concentrations of fetuin or CMP-NeuAc (specific activities:3.1 to 305 Ci/mol) at 28°C for 15 min. Inactivation of the sialyltransferase activity by NEM was measured as described above.

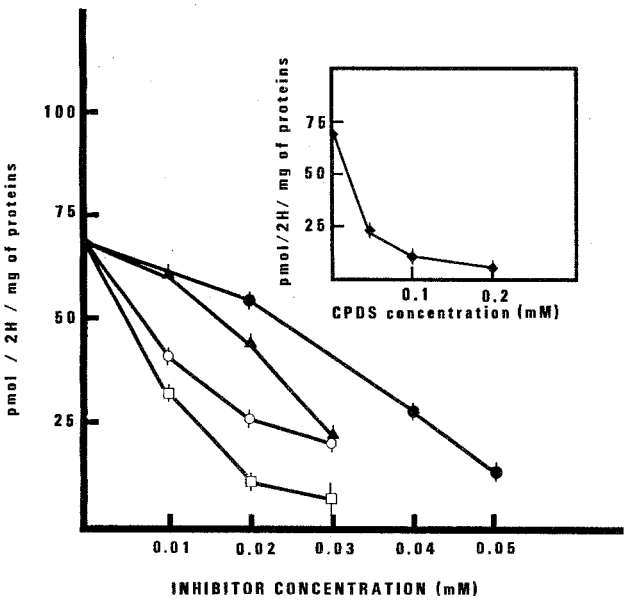

Figure 1. Inhibition of the fetuin sialyltransferase by various sulfhydryl reagents. Sulfhydryl reagents were added in the assay media at different concentrations: 0-0.05 mM for DTNB (□), NEM (○), mersalyl acid (▲), p-CMB (●)and O-0.2 mM for CPDS (inset: ◆). Enzymatic activity was measured at 28°C for 120 min as described in the Materials and Methods section.

Analysis of CMP-[^{14}C]NeuAc Breakdown

The CMP-[^{14}C]NeuAc breakdown during the incubation experiment was checked by chromatography on 3 MM Whatman paper at pH 3.8 in 1 M sodium acetate/ethanol, 30/70 by vol, for 16 h [22].

Results

Study of the Amino-acid Residues Involved in the Two Brain Sialyltransferase Activities

The extent of inactivation of the two enzymes was measured towards different blocking reagents (directly added in the incubation medium) specific for various amino-acid residues. We have used 2,3-butanedione specific for arginine residues, diethylpyrocarbonate specific for histidine residues, parachloromercuribenzoic acid (*p*-CMB) specific for cysteine residues, acetylimidazole specific for tyrosine residues, *N*-bromosuccinimide specific for tryptophan residues and ethylacetamidate specific for lysine residues. Table 1 shows that *N*-bromosuccinimide strongly inhibited the two enzymes, suggesting the involvement of tryptophan residues in the catalytic activity of the two brain sialyltransferases.

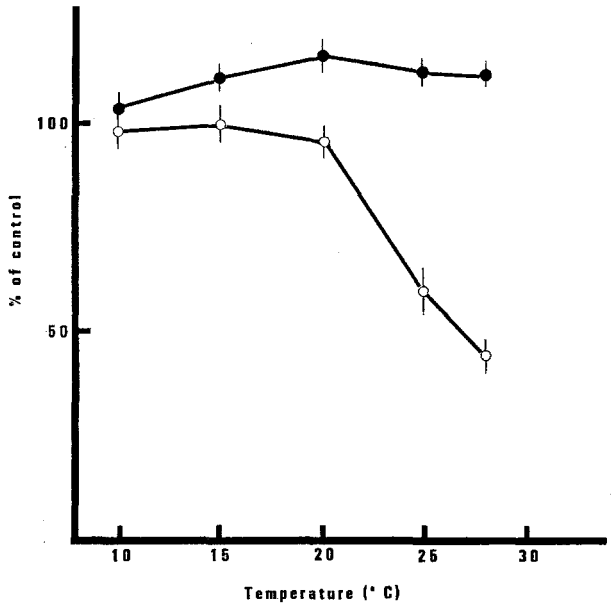

Figure 2. Influence of pre-incubation temperature on the rat brain sialyltransferases inactivation by NEM. After pre-incubation of "enzymatic preparation" (0.5 mg protein/ml) at different temperatures with 0.4 mM NEM for 15 min, the reaction was stopped by addition of 1 mM DTT. Sialyltransferase activities were measured as described in the Materials and Methods section at 28°C for fetuin sialyltransferase (O) using fetuin as acceptor, and at 20°C for asialofetuin sialyltransferase (●) using asialofetuin as acceptor. A control without NEM (100%) was performed under similar conditions.

The two enzymes interacted differently with *p*-CMB. This reagent was a strong inhibitor of the fetuin sialyltransferase, while it slightly inhibited the asialofetuin sialyltransferase.

Effect of Various Sulfhydryl Reagents on Fetuin Sialyltransferase and on Asialofetuin Sialyltransferase

Other sulfhydryl reagents were tested on the two brain sialyltransferases. Table 2 shows that DTNB and NEM as well as *p*-CMB strongly inhibited the fetuin sialyltransferase while they only slightly inhibited the asialofetuin sialyltransferase.

These results provide evidence for the involvement of thiol groups only in the activity of the fetuin sialyltransferase.

Inactivation of the Fetuin Sialyltransferase by Various Sulfhydryl Reagents

We have compared the fetuin sialyltransferase sensitivity to different concentrations of sulfhydryl reagents. Fig. 1 shows that the enzyme was strongly inhibited by all the reagents.

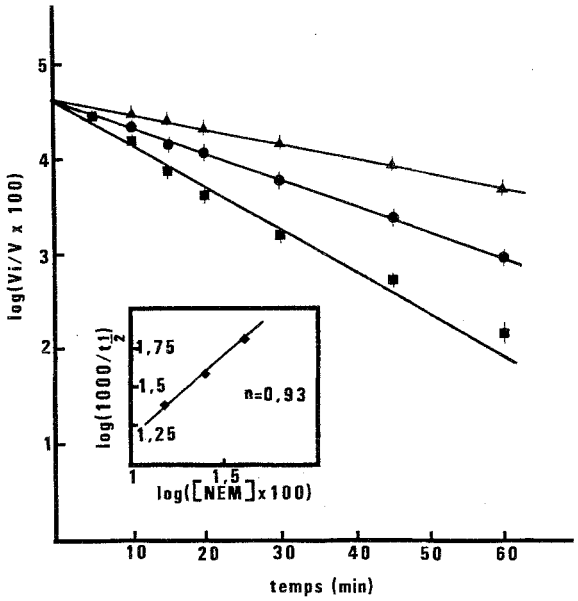

Figure 3. First-order inactivation of fetuin sialyltransferase by NEM.
Enzymatic preparation (0.5 mg proteins/ml) was pre-incubated at 28°C with various NEM concentrations. At the indicated times, the reaction was stopped by addition of 1 mM DTT, and the activity (Vi) measured as described in the Materials and Methods section. A control pre-incubated without NEM was performed under the same conditions (V). The NEM concentrations were 0.15 mM (▲); 0.25 mM (●); 0.4 mM (■). Inset: determination of the number, n, of molecules of NEM binding to one molecule of sialyltransferase.

The $I_{50\%}$ (concentration corresponding to a half inhibition of the enzymatic activity) was 0.01 mM for DTNB, 0.014 mM for NEM, 0.025 mM for mersalyl acid, 0.036 mM for *p*-CMB and 0.028 mM for CPDS. These low $I_{50\%}$ values show the very high sensitivity of the thiol groups towards these sulfhydryl reagents.

The *p*-CMB and mersalyl acid inhibition curves are different from the CPDS, DTNB and NEM inhibition curves. These results give evidence for two different mechanisms related to the structure of these compounds. *p*-CMB and mersalyl acid are organomercurial reagents while CPDS and DTNB are reagents with a dithiol bond. We speculate that they inhibit distinct thiol groups or that thiol groups do not show the same reactivity to these reagents.

For a more thorough understanding of the thiol inactivation mechanism of the fetuin sialyltransferase, we have studied in more detail the reactivity mechanism with NEM, an irreversible reagent. Kinetic experiments may not be undertaken in our conditions with the other sulfhydryl reagents, as they are reversible inhibitors.

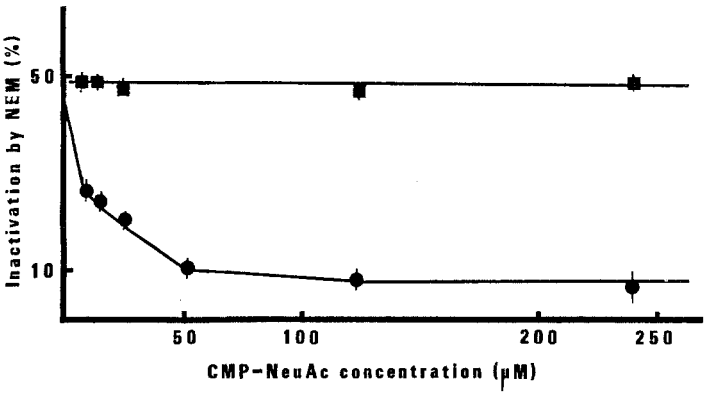

Figure 4. Protection of fetuin sialyltransferase against NEM inactivation by CMP-NeuAc.
Enzymatic preparation (0.5 mg protein/ml) was pre-incubated at 28°C for 15 min with increasing CMP-NeuAc concentrations, and a further addition of 0.4 mM NEM. After 15 min, 1 mM DTT was added and the sialyltransferase activity was measured as described in the Materials and Methods section (●). A control was performed under the same conditions except that CMP-NeuAc was added only *after* the pre-incubation with NEM (■). The extent of inactivation at each CMP-NeuAc concentrationwas calculated by comparison with the activity measured without NEM.

Influence of Temperature on the Fetuin Sialyltransferase Inactivation by NEM

As shown in Fig. 2, inactivation by NEM was temperature dependent. No significant effect occurred up to 20°C. The inhibition was very significant over 20°C. We did not investigate this effect at higher temperatures since thermal denaturation of the fetuin sialyltransferase occurred above 28°C. We have checked that no inactivation of the asialofetuin sialyltransferase was observed whatever the pre-incubation temperature. We could observed a slight activation for this latter enzyme, explained by the NEM protective effect against thermal denaturation.

The fetuin sialyltransferase inactivation by NEM was investigated with pre-incubation at 28°C.

Kinetics of Inactivation of Fetuin Sialyltransferase by NEM

A semi-logarithmic plot of V_i/V against time [23] shows a first-order rate for the enzyme inactivation (Fig. 3). The slopes gave the apparent rate constant of inactivation (k) for each NEM concentration. The plot of log $(1000/t_{1/2})$ against log(NEM x 100), with $t_{1/2} = Ln2/k$, gave a slope value close to 1 (see inset, Fig. 3). This result suggests that at least one thiol residue is involved under our conditions in the sialyltransferase activity.

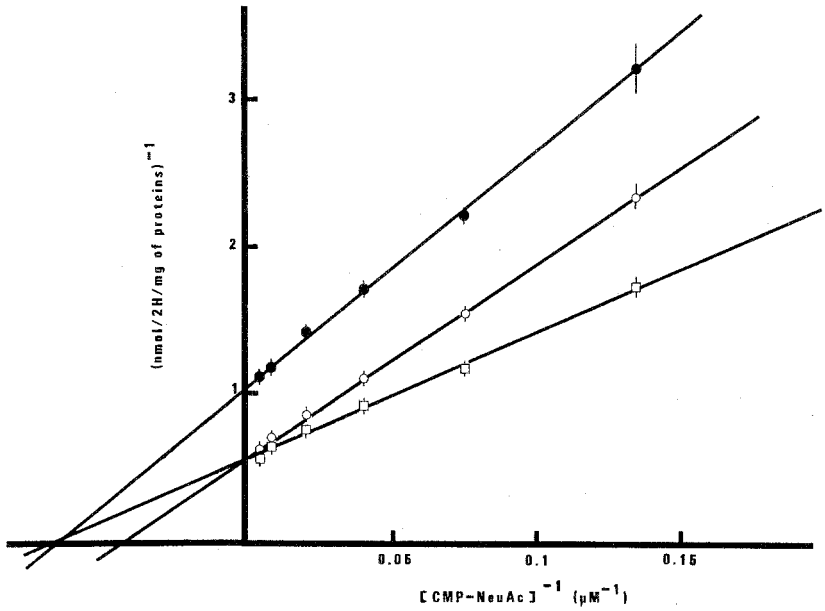

Figure 5. Effects of inactivation by NEM on kinetics parameters of sialyltransferase activity.
The enzymatic preparation (0.5 mg protein/ml) was pre-incubated with increasing CMP-NeuAc concentrations, then with (O) or without (■) 0.4 mM NEM for 15 min at 28°C. An unprotected control where CMP-NeuAc was added only *after* NEM was performed (●).

Protective Effect of Substrates against Inactivation of Fetuin Sialyltransferase by NEM

Protective effects of substrates were studied for an NEM concentration of 0.4 mM (cf. Fig. 3). The pre-incubation with NEM was performed for 15 min, corresponding to a half inactivation of the fetuin sialyltransferase. No modification was observed by pre-incubation of the enzyme with the fetuin substrate whatever its concentration (35, 52.5, 70 or 105 µM) (data not shown). On the other hand, the inactivation by NEM was lowered with increasing concentrations of the substrate CMP-NeuAc (Fig. 4). Almost complete protection was obtained at a 50 µM concentration of CMP-NeuAc. A half maximal effect was obtained at 10 µM. Total protection could not be obtained suggesting that another thiol group, not protected by CMP-NeuAc, might contribute to the fetuin sialyltransferase activity by about 10%. The addition of $MnCl_2$ did not enhance the protective effect of CMP-NeuAc; consequently, Mn^{2+} was not necessary for the protection against NEM (data not shown).

Various nucleotides and sialic acid were tested as protective agents, to indicate whether the nucleotide moiety or the sialic acid was responsible for the protective action of CMP-NeuAc. CMP, the product of the transfer reaction, is a strong inhibitor of the enzyme, even at low concentrations (50 mM). Protection experiments with CMP could not be undertaken. The other nucleotides (AMP, GMP and UMP) and sialic acid did not protect the enzyme against NEM inactivation (data not shown).

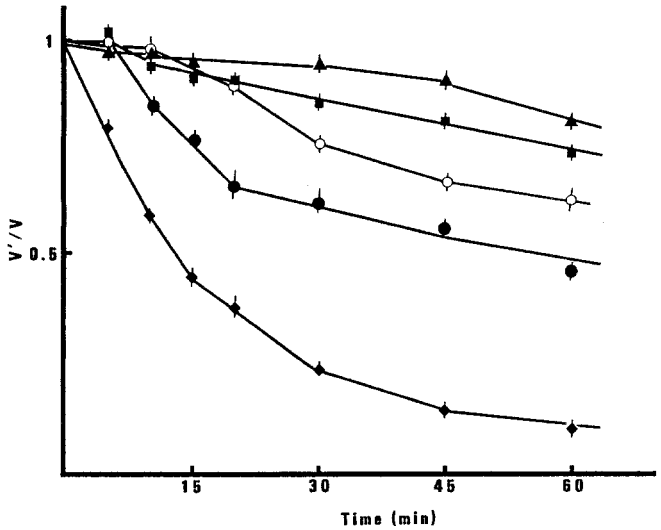

Figure 6. Effects of CMP-NeuAc concentration on the kinetics of inactivation by NEM.
The enzymatic preparation (0.5 mg protein/ml) was pre-incubated for 15 min at 28°C without (◆) or with 26 µM (●), 34 µM (O), 50 µM (■) or 120 µM CMP-NeuAc (▲). 0.4 mM NEM was then added and pre-incubated for various times at 28°C. After addition of DTT, the sialyltransferase activity was measured as described in the Materials and Methods section.
V'/V: velocity in the presence of NEM/velocity without NEM.

The modification of the sialyltransferase kinetic parameters by NEM are shown in Fig. 5. When the enzyme was not previously protected by CMP-NeuAc (closed circles compared to opened squares), NEM seemed to act as a non-competitive inhibitor. These results are in agreement with the irreversible fixation of NEM to the anionic site (nucleotide sugar binding site) of the enzyme. When the sialyltransferase was protected with CMP-NeuAc before addition of NEM (opened circles compared to opened squares), we observed an increase of the K_M value from 16 to 25 µM and no modification of the V_{max}, giving evidence for a competitive mechanism between CMP-NeuAc and NEM. This result confirms the presence of a thiol group in or near the anionic site (nucleotide-sugar binding site) of the enzyme.

When the pre-incubation time with 0.4 mM NEM was further extended, the high specific protective effect of CMP-NeuAc was still observed. As shown in Fig. 6, a very efficient protection (90%) could be obtained with 120 µM CMP-NeuAc for up to 45 min pre-incubation with 0.4 mM NEM.

When short pre-incubation times (5-10 min) with NEM were used, inhibition was no longer observed in the presence of CMP-NeuAc, at variance with the experiments shown in Fig. 4 (where the 10% inhibition observed represents the inhibition of a second thiol group not protected by CMP-NeuAc). These results suggest that the second thiol group seems to be less sensitive to NEM that the one specifically protected by CMP-NeuAc. This second thiol group appeared to be insensitive to 0.4 mM NEM for these short times.

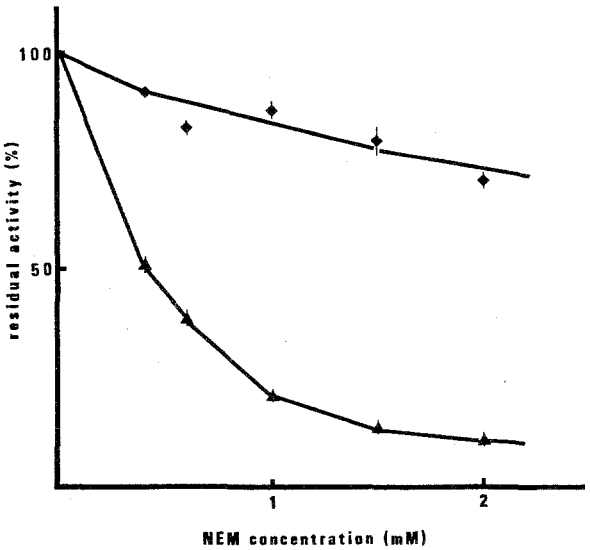

Figure 7. Protection of fetuin sialyltransferase by CMP-NeuAc against high NEM concentrations.
The enzymatic preparation (0.5 mg protein/ml) was pre-incubated with 120 µM CMP-NeuAc for 15 min at 28°C. Then increasing NEM concentrations were added. After 15 min, the reactions were stopped by addition of 5 mM DTT and sialyltransferase activity was measured (◆) compared to a control where CMP-NeuAc was added only *after* NEM (▲).

The protective effect of CMP-NeuAc was also studied against higher NEM concentrations. Fig. 7 shows that even at 2 mM NEM (corresponding to 90% inhibition of the sialyltransferase activity), 70% protection with 120 mM CMP-NeuAc was still obtained.

Discussion

The purpose of this work was to study the amino-acyl residues, in particular sulfhydryl groups, involved in the enzymatic activity of two brain sialyltransferases. Our results demonstrated the presence of one essential sulfhydryl group in or near the nucleotide-sugar binding site of the fetuin sialyltransferase. This sulfhydryl group was not involved in the catalytic activity of the asialofetuin sialyltransferase.

Among the different amino-acid selective reagents used in this work, only thiol reagents reacted differently with the two sialyltransferases. All sulfhydryl reagents strongly inhibited the fetuin sialyltransferase, suggesting that one or several thiol groups are involved in the fetuin sialyltransferase activity. The asialofetuin sialyltransferase was only slightly inhibited.

The inactivation reaction by NEM approximates to first-order kinetics and we found that at least one mol NEM binds per mol of fetuin sialyltransferase, according to the kinetic method of Levy *et al.* [23].

The high specific protection by CMP-NeuAc indicates that this essential thiol group is located in or near the anionic site (nucleotide-sugar binding site). No protection occurred with fetuin, so this thiol group is not located in the glycoprotein (fetuin) binding site. From these results, we can assume that the nucleotide-sugar binding site and the glycoprotein binding site are distinct. These results are in agreement with others previously found in our laboratory [18]. We had shown that the fetuin sialyltransferase was inhibited by DIDS, a specific anionic inhibitor, which bound to the exposed anionic site (nucleotide-sugar binding site), while, under similar conditions, the enzyme was insensitive to trypsin and pronase (whereas asialofetuin sialyltransferase was strongly inhibited by these proteases). Our conclusions agree with those of Lee *et al.* [24] who showed that α–lactalbumin does not bind to galactosyltransferase at the UDP-galactose binding domain.

The protection obtained with CMP-NeuAc agrees with the work of Magee and Ebner [5] and Clymer *et al.* [4] who protected soluble bovine milk galactosyltransferase with UDP-galactose and its analogs, against NEM or ultra-violet light inactivation. They did not observe any protection with the substrate *N*-acetylglucosamine.
In our conditions, Mn^{2+} was not required for the CMP-NeuAc protection, although it was essential to the fetuin sialyltransferase activity. It seemed that Mn^{2+}, would not be involved in the CMP-NeuAc binding. Our results concerning Mn^{2+} are at variance with those of Magee and Ebner [5] who have showed that Mn^{2+} is necessary for UDP-galactose protection through the formation of an Enzyme-Mn^{2+}-UDP-galactose complex which in turn induced a conformational change of the enzyme. On the other hand, O'Keeffe *et al.* [7] gave evidence for two binding sites of the Mn^{2+} ion, one of them being associated with the binding site of UDP-galactose. In our study, the participation of Mn^{2+} in the CMP-NeuAc binding could be excluded. However, the possibility that Mn^{2+} has another role in the nucleotide-sugar site could be expected.

Although, we demonstrated a competitive mechanism between NEM and CMP-NeuAc, we cannot assume that this thiol group is responsible for the CMP-NeuAc binding, as asialofetuin sialyltransferase did not seem to have such a group in its anionic site structure. However, we have shown [18] that this fetuin sialyltransferase is an enzyme located on the cytoplasmic face of the endoplasmic membrane. There it was directly accessible to its substrate, CMP-NeuAc. We can speculate that a carrier system for the nucleotide-sugar would not be necessary for this sialyltransferase. It was not the case for the asialofetuin sialyltransferase which is located on the luminal side of the endoplasmic membranes. We may assume that the thiol group located in or near the nucleotide-sugar binding site of the fetuin sialyltransferase might be involved in this direct binding of the CMP-NeuAc to the enzyme.

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