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Comparative Effects of Sulfhydryl Reagents on Membrane-Bound and Solubilized UDP-Glucose:ceramide Glucosyltransferase from Golgi Membranes. Evidence for Partial Involvement of a Thiol Group in the Nucleotide Sugar Binding Site of the Solubilized Enzyme

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We have studied the inactivation of membrane-bound and solubilized UDP-glucose:ceramide glucosyltransferase from Golgi membranes by various types of sulfhydryl reagents. The strong inhibition of the membrane-bound form by the non-penetrant mercurial-type reagents clearly corroborated the fact that in sealed and "right-side-out" Golgi vesicles the ceramide glucosyltransferase is located on the cytoplasmic face. No significant differences in the susceptibility to the various sulfhydryl reagents were noted when solubilized enzyme was assayed, showing that solubilization does not reveal other critical SH groups. The different results obtained must be interpreted with regard to several thiol groups, essential for enzyme activity. No protection by the substrate UDP-glucose against mercurial-type reagents was obtained indicating that these thiol groups were not located in the nucleotide sugar binding domain. A more thorough investigation of the thiol inactivation mechanism was undertaken with NEM (*N*-ethylmaleimide), an irreversible reagent. The time dependent inactivation followed first order kinetics and provided evidence for the binding of 1 mol NEM per mol of enzyme. UDP-Glucose protected partially against NEM inactivation, indicating that the thiol groups may be situated in or near the substrate binding domain. Inactivation experiments with disulfide reagents showed that increased hydrophobicity led to more internal essential SH groups which are not obviously protected by the substrate UDP-glucose, thus not implicated in the substrate binding domain, but rather related to conformational changes of the enzyme during the catalytic process.

Abbreviations: Chaps: 3-[(3-cholamidopropyl)dimethylammonio] 1-propanesulfonate. Mops: 4-morpholinepropanesulfonic acid. PC: phosphatidylcholine. NEM: *N*-ethylmaleimide. CPDS: carboxypyridine disulfide (dithio-6,6'-dinicotinic acid). DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid). DTP: dithiodipyridine. *p*-HMB: para-hydroxymercuribenzoate. DTT: dithiothreitol. BAL: British anti-Lewisite (dimercaptopropanol). Zw 3-14: Zwittergent 3-14.

A UDP-glucose:ceramide glucosyltransferase associated with membranes of the Golgi apparatus has been shown to be cytoplasmically oriented [1, 2]. This enzyme is the initial step in the assembly of the oligosaccharide moiety of some glycolipids. Since the latter steps in the biosynthesis of gangliosides take place in the lumen of the Golgi apparatus [3], a mechanism of translocation of the nascent oligosaccharide-lipid chain must be considered before this chain may be utilized as a precursor. A similar model has been proposed for oligosaccharide-lipid synthesis in the endoplasmic reticulum [4].

Investigations into the sensitivity of the membrane-bound activity to various penetrating and non-penetrating sulfhydryl reagents [5-7] may be of interest to confirm the location of the UDP-glucose:ceramide glucosyltransferase on the cytoplasmic face of the Golgi vesicles.

On the other hand, a study of the action of sulfhydryl reagents may also provide some insight into the mechanism of the reaction cycle of the enzyme [8, 9]. As it is difficult to obtain this enzyme in a purified state, investigations into the reactivity of SH groups with various sulfhydryl reagents were then conducted on a solubilized preparation permitting reliable kinetic studies. Protection experiments by substrates and analogs were also performed which revealed the presence of a sulfhydryl group essential for catalysis.

Materials and Methods

Materials

Porcine submaxillary ganglia were obtained from a local slaughterhouse. All the reagents were of analytical grade. UDP-[U-^{14}C]Glucose (11.5 GBq / mmol) was purchased from Amersham International. Chaps, NEM, mersalyl and DTT were obtained from Sigma, St. Louis, MO, USA; Mops and DTP from Fluka, Buchs, Switzerland; Zwittergent 3-14 from Calbiochem, La Jolla, CA, USA; ceramides from Serdary Research Laboratories, Canada; DTNB from Boehringer, Mannheim, W. Germany; CPDS from Aldrich, Milwaukee, USA and BAL from Serva, Heidelberg, W. Germany.

Isolation of Golgi Vesicles

Golgi vesicles were isolated from porcine submaxillary ganglia according to the procedure of Leelavathi *et al.* [10] as already described [1]. Under these conditions, vesicle integrity was preserved and the vesicles were "right-side-out" as determined by measuring the latency of galactosyltransferase when exogenous glycoproteins were used as substrates [2].

Solubilization of UDP-Glucose:ceramide Glucosyltransferase

The Golgi fraction was suspended in 20 mM Mops buffer (pH 6.5) (8 mg/ml protein concentration). Solubilization was achieved by mixing in 20 mM Mops buffer (pH 6.5), 6.2 mg/ml Chaps, 0,1 mg/ml Zwittergent 3-14 and 1 mg/ml Golgi fraction. After 10 min at 0°C, glycerol was added to make a final concentration of 20% (by vol). After a further 5 min at 0°C, the suspension was centrifuged for one hour at 100,000 x *g*. Under these conditions, about 60-75% of enzyme activity was recovered in the supernatant as well as 80% of the protein.

Enzyme Assay

UDP-Glucose:ceramide glucosyltransferase assay on exogenous ceramides was adapted from Neskovic *et al* [11] according to [2] except that dimercaptopropanol (BAL) was omitted. Before incubation, Chaps (500 µg) and ceramide (75 µg) dissolved in chloroform/methanol, 1/1 by vol, were added into the test tubes. After solvent evaporation under a gentle stream of nitrogen, 70 µl of the enzyme fraction and 10 µl of 10 mM NADH were added to the dry lipid film and vortexed vigorously twice for 30 s. The reaction was then initiated by the addition of 20 µl of 260 µM UDP-[U-^{14}C]glucose (70 mCi / mmol). After 10 min at 37°C, the reaction was stopped by the addition of 2 ml chloroform/methanol, 2:1 by vol, and left for one hour at room temperature. The lipid extract was then partitioned according to the method of Folch *et al* [12]. Radiolabeled glucosylceramide was separated on silica-gel 60 thin-layer plates (Merck) in the solvent system chloroform/methanol/water, 60/35/8 by vol, scraped off the plates and counted by liquid scintillation.

Chemical Modification of Thiol Groups by Sulfhydryl Reagents

Sulfhydryl reagents were assayed on membrane-bound and on solubilized enzyme. For the membrane-bound enzyme, freshly prepared Golgi pellets were suspended in 70 mM Mops buffer (pH 6.5) containing 7 mM $MnCl_2$, 3.5 mM $MgCl_2$ and 0.35 M sucrose. The Golgi fraction or the solubilized enzyme (100 µl; about 500 µg protein/ml) was first incubated for 10 min at 0°C with 10 µl of the various sulfhydryl reagents to give the desired concentration. All solutions were freshly prepared.

For experiments with NEM, incubations were stopped by adding 2 mM DTT. For the others, which are reversible reagents, incubation mixtures were diluted with 10 volumes of 70 mM Mops buffer (pH 6.5), 5 mg/ml Chaps, 0.08 mg/ml Zw 3-14, 19% glycerol, 7 mM $MnCl_2$, 3.5 mM $MgCl_2$, then enzyme activity was assayed as described above using 70 µl of the medium. Each assay was done in duplicate.

Results

Inactivation of the Membrane-bound Ceramide Glucosyltransferase by Various Sulfhydryl Reagents

We have compared the membrane-bound ceramide glucosyltransferase sensitivity to different concentrations of sulfhydryl reagents. Fig. 1 shows that mercurial-type reagents like mersalyl or *p*-HMB completely inhibited the enzyme at a concentration of 200 µM. Alkylating thiol reagents like NEM reacted more slowly: a concentration of 1 mM gave 80% of enzyme inactivation. Among disulfide reagents, CPDS induced no inactivation whereas 1 mM DTNB inactivated the enzyme activity by about 50%. These results provide evidence of the involvement of thiol groups in the activity of the ceramide glucosyltransferase. Inactivation by mercurial type non-penetrating reagents which are unable to penetrate through the membranes [6, 7] confirms the location of the enzyme on the cytoplasmic face of the Golgi vesicles. For disulfide reagents, as indicated, CPDS, which is unable to penetrate through the membranes [5], does not inactivate the enzyme. This shows that the external SH

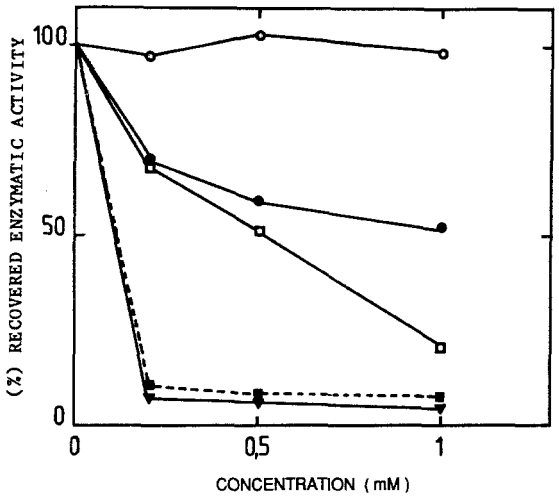

Figure 1. Effect of sulfhydryl reagents on membrane-bound ceramide glucosyltransferase.
Freshly prepared Golgi fractions were incubated for 10 min at 0°C with increasing concentrations of the various sulfhydryl reagents: ■, Mersalyl; ▼, *p*-HMB; □, NEM; ○, CPDS; ●, DTNB. Aliquots for enzyme assays were taken as described in the Materials and Methods section. Activity is expressed as % of the control without any treatment. Mean of two separate experiments. The average value for membrane-bound ceramide glucosyltransferase activity was 230 ± 40 pmol/min/mg proteins.

groups which reacted with the mercurial type reagents are not sensitive to dithiol reagents. The inhibition obtained with DTNB, another dithiol compound which is able to penetrate through the membranes [5], clearly shows the involvement of more internal SH groups for enzyme activity. NEM, a penetrant sulfhydryl reagent [6, 7] with another type of reactivity, is able to inactivate enzyme activity.

Inactivation of the Solubilized Ceramide Glucosyltransferase by Various Sulfhydryl Reagents

Investigations on the solubilized ceramide glucosyltransferase sensitivity to different concentrations of sulfhydryl reagents were also undertaken. Essentially the same pattern of inactivation as for membrane-bound enzyme was obtained. Fig. 2 shows that mercurial-type reagents like mersalyl or *p*-HMB induced almost complete inactivation (95%) at a concentration of 50 μM. Disulfide reagents like CPDS, DTNB or DTP gave increasing inactivation. CPDS inactivated about 5% of the enzyme activity at all the concentrations tested. DTNB gave 55% inactivation at a concentration of 0.5 mM. DTP was the stronger inhibitor, as only 15% of enzyme activity remained at 0.1 mM concentration. All these compounds are known for their increasing hydrophobicity [8].

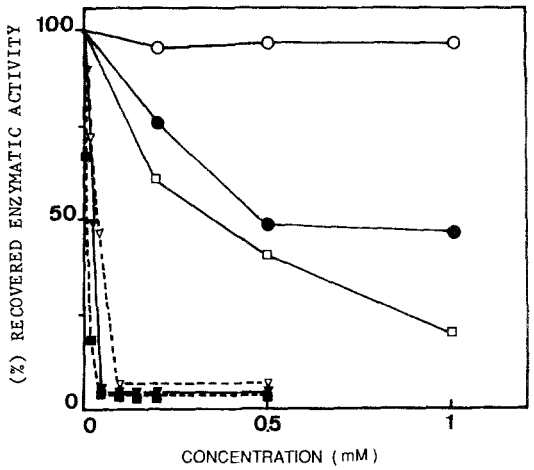

Figure 2. Effect of sulfhydryl reagents on solubilized ceramide glucosyltransferase.
Solubilized enzyme fractions were incubated for 10 min at 0°C with increasing concentrations of the various sulfhydryl reagents: ■, Mersalyl; ▼, *p*-HMB; □, NEM; ○, CPDS; ●, DTNB; ▽, DTP. Aliquots for enzyme assays were taken as described in the Materials and Methods section. Activity is expressed as % of the control without any treatment. Mean of two separate experiments. The average value for solubilized ceramide glucosyltransferase activity was 210 ± 30 pmol/min/mg proteins.

The alkylating thiol reagent, NEM, inactivated enzyme activity more slowly .At a concentration of 1 mM about 20% of the ceramide glucosyltransferase activity still remained. These results clearly indicate the involvement of several distinct thiol groups in the glucosyltransferase activity and that thiol groups do not show the same reactivity to these reagents. For all reagents tested, except NEM, the addition of an excess of DTT or BAL gave over 90% recovery of initial activity at any point in the course of inactivation, showing the reversibility of these reactions.

Protective Effect of Substrates and Analogs against Inactivation of Solubilized Ceramide Glucosyltransferase by Sulfhydryl Reagents

Substrates and analogs were assayed for their ability to protect enzyme activity from inactivation by the various types of thiol reagents. For mercurial type reagents, as shown in Table 1, protection by substrates and analogs was never obtained whether inactivation by mersalyl was complete (100% at 50 μM) or partial (41% at 15 μM).

Protective experiments with substrates and analogs were assayed by NEM inactivation. Fig. 3 shows that protection was in decreasing order from UDP-glucose, UDP, UDP-galactose (against NEM, 1 mM for 30 min at 0°C, which gives 83% inactivation; panel A), then UTP

Table 1. Protective effect of substrates and analogs on solubilized ceramide glucosyltransferase activity against mersalyl inactivation. Prior to incubation with mersalyl at the indicated concentration (10 min at 0°C) solubilized enzyme fractions were incubated for 10 min at 0°C with substrates or analogs at the chosen concentrations. Aliquots were then assayed for ceramide glucosyltransferase activity. Results are given as mean of two separate experiments.

Protector	% of control enzymatic activity
Experiment I	
No protector, (mersalyl 15 μM)	59
UDP-glucose, 10 μM and 60 μM	62
Experiment II	
No protector, (mersalyl 50 μM)	0
UDP-glucose, 10 μM and 60 μM	0
UDP-galactose, 10 μM and 60 μM	0
Glucose, 10 μM and 60 μM	0
Uridine, 10 μM and 60 μM	0
UMP, 10 μM and 60 μM	0
UDP, 10 μM and 60 μM	0
UTP, 10 μM and 60 μM	0

(against NEM, 1 mM for 10 min at 0°C which gives 50% inactivation; panel B). UMP, uridine and glucose do not protect ceramide glucosyltransferase at all against NEM inactivation. The same order of magnitude was obtained when Mn^{2+} and Mg^{2+} were added during the protection incubation. NADH, a competitive inhibitor of UDP-glucose used to circumvent hydrolysis by pyrophosphatases in ceramide glucosyltransferase assay, was unable to protect from inactivation by NEM. Finally, protection by the ceramide substrate was not obtained.

Against disulfide reagents, DTNB and DTP, used in order to give about 50% inactivation (DTNB 500 μM and DTP 50 μM for 10 min at 0°C), UDP-glucose gave only minimal protection (about 10%).

Kinetics of Inactivation of the Solubilized Ceramide Glucosyltransferase by NEM

To gain a more thorough understanding of the thiol inactivation mechanism, we studied in more detail the reaction mechanism with NEM, an irreversible reagent. Kinetic experiments could not be undertaken with the other sulfhydryl reagents using our conditions, as these reagents are reversible inhibitors.

The time dependent inactivation of the solubilized ceramide glucosyltransferase with increasing concentration of NEM is shown in Fig. 4. The plot of the log of relative enzyme activity against time was linear up to about 80% inhibition. The apparent constant of

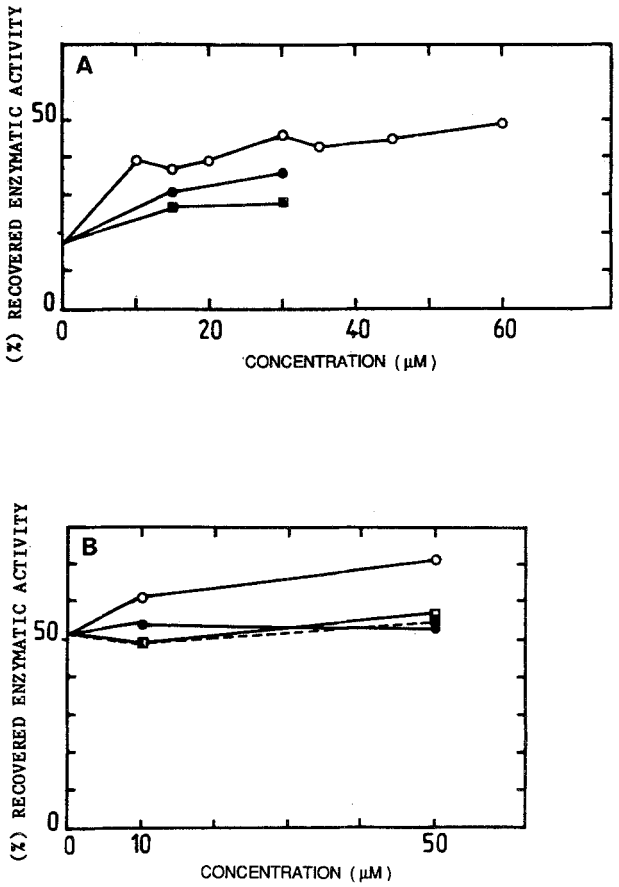

Figure 3. Protective effect of substrates and analogs on solubilized ceramide glucosyltransferase activity against NEM inactivation.
Prior to incubation with NEM, solubilized enzyme fractions were incubated for 10 min at 0°C with substrates and analogs at the indicated concentrations.
Panel A. Protection from inactivation by NEM, 1 mM for 30 min at 0°C: ○, UDP-glucose; ●, UDP; ■, UDP-galactose.
Panel B. Protection from inactivation by NEM,1 mM for 10 min at 0°C: ○, UTP; □, UMP; ■, uridine; ●, glucose.
Activity is expressed as % of the control without any treatment.

inactivation, k_{app}, was determined from the slope $\log(v/v_0)$ vs. t. Rate constants of inactivation were directly proportional to NEM concentrations over a range of 0 - 1 mM. The slope of the plot $-\log(1/t_{1/2})$ vs. log NEM was 1.07, indicating that essentially 1 mol of NEM was bound per mol of catalytic site to produce inactivation [13, 14].

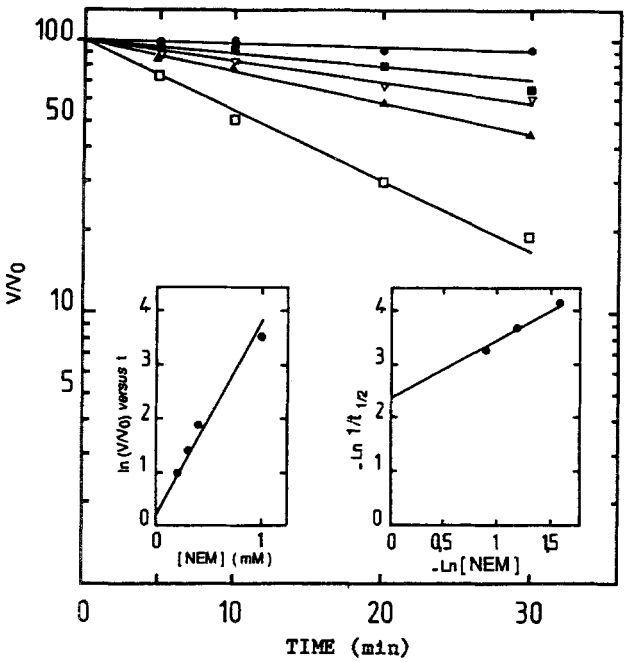

Figure 4. Rate of inactivation of solubilized ceramide glucosyltransferase as a function of NEM concentration. The solubilized enzyme fraction was incubated at 0°C with 0.1 mM (●), 0.2 mM (■), 0.3 mM (▽), 0.4 mM (▲), 1 mM (□) of NEM for increasing times. Aliquots were then assayed for ceramide glucosyltransferase activity. Activity is expressed as % of the control without any treatment.
Inset: Double reciprocal plot of the apparent pseudo-first order rate constants of inactivation vs. NEM concentrations and determination of the number,n, of molecules of NEM binding to one molecule of ceramide glucosyltransferase.

Kinetic studies were carried out on solubilized native and partially inactivated ceramide glucosyltransferase treated with 1 mM NEM for 10 minutes at 0°C. They indicated that the loss of activity was due to a reduction in V_{max} (380 pmol/min/mg protein compared to 140 pmol/min/mg protein), whereas apparent K_M values of native and partially inactivated enzyme for both substrates, UDP-glucose and ceramide, remained unchanged (13 µM and 260 µM, respectively).

Discussion

The action of various sulfhydryl reagents on UDP-glucose:ceramide glucosyltransferase from Golgi membranes was investigated both on the membrane-bound and on the solubilized form.

Using the membrane-bound enzyme our findings indicate that in sealed and "right-side-out" vesicles the ceramide glucosyltransferase is strongly inhibited by non-penetrating sulfhydryl reagents like mersalyl and *p*-HMB, providing evidence for the involvement of thiol groups in the enzyme activity and confirming its location on the cytoplasmic face of Golgi vesicles. Other types of sulfhydryl reagents have access to other thiol groups of the enzyme, according to their penetrability through the membrane.

A more thorough examination of the thiol inactivation mechanism of the enzyme was undertaken on the solubilized form. No significant differences in the sensitivity to the various sulfhydryl reagents were noticed when solubilized enzyme was used. It is obvious that solubilization of the ceramide glucosyltransferase in a micellar form maintains the enzyme roughly in the same conformation and does not reveal other critical sulfhydryl groups. This is at variance with the findings of Coves *et al.* [15] who studied a diacylglycerolgalactosyltransferase from spinach chloroplast envelope solubilized by the detergent, Chaps.

We were unable to purify this integral membranous enzyme like other membrane-bound enzymes [16-18] because of its instability after detergent solubilization. The presence of critical thiol groups has been suggested to explain such failure [15]. However, the introduction of DTT throughout the solubilization and attempts at purification did not overcome these problems.

The results obtained here on the solubilized enzyme must be interpreted considering that there are several thiol groups essential for ceramide glucosyltransferase activity. Polarity, reactivity, steric hindrance of the sulfhydryl reagents, as well as accessibility and reactivity of the SH groups of the enzyme should be taken into account.

One group is concerned with inhibition by mercurial type reagents. Such SH groups are not protected from inactivation by the substrate UDP-glucose, and therefore are probably not located in the nucleotide sugar-binding domain. It is conceivable that modification of such residues interferes with conformational changes of the transferase that are of importance in the catalytic pathway. These sites must be (relatively) outside, whereas other types of thiol groups may be located more inside the micelle and are sensitive to NEM and disulfides.

An alkylating type of reagent like NEM was able to inactivate most of the enzyme activity. The inactivation by NEM approximates first order kinetics and we found that at least one mol NEM binds per mol of ceramide glucosyltransferase according to the kinetic method of Levy *et al.* [14]. Modification of the V_{max} without modification of the K_M for the substrates showed the involvement of this reagent in the catalytic site. Protection experiments indicated that the substrate UDP-glucose is able to protect, at least partially, against inactivation by NEM, showing that this thiol group may be situated in the substrate binding domain. The decreasing order of protection by analogs of nucleotide sugar implies that the entire molecule is necessary to protect even partially against inactivation by NEM. The hydrophobic substrate ceramide does not offer any protection. The metal ion binding domain is not implicated, as Mg^{2+} and Mn^{2+} do not afford any protection.

The different disulfide reagents tested, CPDS, DTNB and DTP, while having the same type of reactivity (dithiol bond), were assayed for their increasing hydrophobicity [5, 8]. As

expected, increasing hydrophobicity gave access to more internal SH groups, some of them being largely inhibited, which involves them in enzymatic activity. However, a very slight protection by UDP-glucose was noticed, indicating that modification of thiol residues may be related to conformational changes of the enzyme during the catalytic process, rather than a direct implication in the nucleotide sugar binding site.

Purification of the enzyme activity will be necessary in order to investigate more thoroughly the reactivity of the SH groups of ceramide glucosyltransferase.

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