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Exposure to galactose oxidase of GM1 ganglioside molecular species embedded into phospholipid vesicles

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Abstract

The exposure of GM1 molecular species present in the native ganglioside, carrying C18:1 or C20:1 long-chain bases (LCB), to *Dactylium dendroides* galactose oxidase was studied. When native GM1 (49.3% C18:1 and 50.7% C20:1 LCB, respectively), was inserted in dipalmitoylphosphatidylcholine vesicles and partially oxidized (10%), the proportion of C18:1 and C20:1 species in the oxidized GM1 was 59.6% and 40.4%, respectively, suggesting a preferential action of the enzyme on the shorter species. The V_{max} of the enzyme was higher on C18:1 GM1 than on C20:1 GM1. The molecular species were affected without any preference after partial (10%) oxidation of GM1 incorporated in egg phosphatidylcholine vesicles or in micellar form. These data indicate that the exposure of the terminal galactose moiety of GM1 ganglioside to galactose oxidase is affected by the ganglioside ceramide composition as well as the phospholipid environment, that presumably determine the distribution (molecular dispersion, segregation) of the ganglioside within the membrane.

Key words: Galactose oxidase; Ganglioside; Artificial membrane

1. Introduction

Glycosphingolipids are inserted with their ceramide portion into the outer leaflet of the plasma membrane, their oligosaccharide moiety protruding toward the external environment. The degree of exposure of the oligosaccharide moiety affects its availability to interact with external ligands, like enzymes and antibodies, and contributes to modulate the function of glycosphingolipids (crypticity and antigenicity of glycosphingolipids) [3–5]. Little is known about the role played by the hydrophobic moiety of glycosphingolipids on the exposure of the saccharide portion [3,6,7]. However, the changes of the physico-chemical and biochemical properties of the membrane caused by insertion of glycosphingolipids [8–10] as well as the intrinsic attitude of glycosphingolipids to undergo lateral phase separation [11], support the notion that the ceramide portion of these molecules do affect the exposure and the recognition availability of the oligosaccharide portions [6,12,13]. Galactose oxidase (GAO; EC 1.1.3.9) from *Dactylium dendroides*, a model of soluble protein interacting with the cell surface, has been used to examine the exposure of glycosphingolipid oligosaccharide portions in both natural [3,14,15] and artificial membranes [3,16]. In the present study we studied the influence of the long chain base (LCB) components of the ceramide portion of ganglioside GM1 on the exposure to GAO of the terminal galactose residue of the same ganglioside. The study model was constituted by 'native' GM1 ganglioside from bovine brain or GM1 molecular species containing a single LCB, C18:1 or C20:1, inserted into the outer layer of dipalmitoylphosphatidylcholine (DPPC) or egg phosphatidylcholine (EPC) vesicles, an artificial system mimicking the ganglioside distribution in the cell membrane [17].

2. Materials and methods

2.1. Materials

DPPC and EPC were from Fluka AG (Buchs, Switzerland); GAO (1 unit/2.3 μg, as protein) and *N*-acetylneuraminic acid (NeuAc) from Sigma Chemical Co. (St. Louis, MO, USA); Sodium boro-[^{3}H]hydride (6.5 Ci/mmol) from Amersham (Little Chalfont, Buchs, UK).

2.2. Preparation and radiolabeling of gangliosides

Native GM1 ganglioside, homogeneous in the saccharide portion but not in the lipid moiety, was extracted and purified from beef brain as described [18]. Preparation of GM1, tritium labeled at the 3-position of the LCB, and fractionation of native (radiolabeled or not) GM1 ganglioside into molecular species with homogeneous LCB (C18:1 and C20:1) in the erythro configuration was performed as described [19,20]. Identification, structural analysis, and purity assays of ganglioside were performed as described [20]. The final purity of all gangliosides was over 99%. The LCB composition of native GM1 was assessed by HPLC [21]. Ganglioside-bound sialic acid (NeuAc) was determined as described [22] using NeuAc as standard.

2.3. Preparation of LUVs containing GM1

DPPC and EPC, dried and lyophilized from a chloroform/methanol (2:1, v/v) solution, were resuspended in 50 mM Tris–HCl, pH 7.0, and extruded 10 times through 10 μm pore filters (Nucleopore, Plessanton, CA, USA), using a N_2 pressure-operated extruder (Lipoprep, Ottawa, Canada). The insertion of native GM1, C18:1 GM1 or C20:1 GM1 asymmetrically embedded into the outer leaflet of LUVs was done as

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Abbreviations: GAO, Galactose oxidase (EC 1.1.3.9); DPPC, dipalmitoylphosphatidylcholine; EPC, egg phosphatidylcholine; LUVs, large unilamellar vesicles; LCB, long-chain base; HPLC, high performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; GM1-ox, oxidized GM1; NeuAc, *N*-acetylneuraminic acid. Ganglioside nomenclature follows Svennerholm [1] and the IUPAC-IUB recommendations [2].

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described [17,23]. Vesicles containing 10% (molar) GM1 were prepared and used within 1 day from the preparation.

2.4. Preparation of micellar ganglioside dispersions

A known amount of ganglioside, dissolved in chloroform/methanol (2:1, v/v) was dried by a gentle flow of N_2, resuspended in 50 mM Tris–HCl buffer, pH 7.0, and vortexed. The mixture was allowed to stand overnight at room temperature.

2.5. LCB composition after treatment of GM1 with GAO

The incubation mixtures contained 0.8 mM native GM1, in micellar form or inserted into DPPC (or EPC), LUVs, and known amounts of GAO, in a final volume of 50 μl of 50 mM Tris–HCl, pH 7.0. 1 U, 0.2 U and 50 U of GAO were added to GM1/DPPC vesicles, GM1/EPC vesicles or micellar GM1, respectively. The oxidation, started by the addition of the enzyme, was performed under shaking at 37°C for different periods of time to obtain a different extent of oxidation (from 10% to 99%). At the end, samples were submitted to ganglioside extraction [18]; ganglioside extracts were evaporated, lyophilized and dissolved in 50 μl of *n*-propanol/water (8:2, v/v). [^{3}H]$NaBH_4$ (400 μCi) were then added to reduce oxidized GM1 (GM1-ox) to [^{3}H]GM1 [24,25]. To ensure complete reduction, one additional ml of Tris–HCl buffer and 100 μmol of $NaBH_4$ were added and allowed to react for 5 min. The samples were dialyzed and lyophilized. C18:1 and C20:1 [^{3}H]GM1 were separated by reverse-phase HPTLC in the solvent system methanol/acetonitrile/water (18:6:1, v/v/v) and quantitated by a radiochemical method [26,27]. Three replicate determinations were performed in 5 independent experiments.

2.6. Kinetics of GAO activity on LUV dispersion of C18:1 and C20:1 GM1

Incubation mixtures containing, in a final volume of 30 μl of 50 mM Tris-HCl buffer, pH 7.0, 0.05–1 mM C18:1 GM1 or C20:1 GM1 (carrying 250,000 dpm corresponding to 0.15 nmol of the corresponding radiolabeled molecular species) embedded into DPPC or EPC LUVs; 1 U (GM1/DPPC LUVs) or 0.2 U of GAO (GM1/EPC LUVs), were incubated for given periods of time in order to obtain about 10% oxidation. In the control incubation mixtures (blanks), the enzyme was omitted. At the end of incubation the samples were submitted to lipid extraction and GAO activity was measured by radiochemical quantification [27] of formed GM1-ox, separated from residual GM1 by HPTLC in the solvent system chloroform/methanol/water (110:40:6, v/v/v) followed by chloroform/methanol/$CaCl_2$ 0.2% (50:42:11, v/v/v). Five replicates were performed for each experiment. Unless otherwise stated, S.D. values were < 7% of the mean values. K_m and V_{max} values were determined from the double-reciprocal plot according to Lineweaver-Burk [28], by linear regression. The correlation constants (c) were $0.999 > c > 0.994$.

2.7. Statistical analysis

The differences were evaluated for statistical significance using Student's *t*-test.

3. Results

3.1. Treatment of native GM1 with GAO

Galactose oxidase was allowed to act on native GM1 embedded in LUVs. The results are reported in Fig. 1. The proportion of C18:1 and C20:1 species present in the starting GM1 at 0 time of incubation was 49.3% and 50.7%, respectively. When the extent of GM1 oxidation was about 10%, the proportion of C18:1 and C20:1 species present in GM1-ox, detected through its reduction to [^{3}H]GM1, was 59.6% and 40.4%, respectively, using DPPC LUVs, and 48.3% and 51.7% using EPC LUVs, respectively. The compositional difference between treated and untreated DPPC LUVs was statistically highly significant ($P < 0.01$).

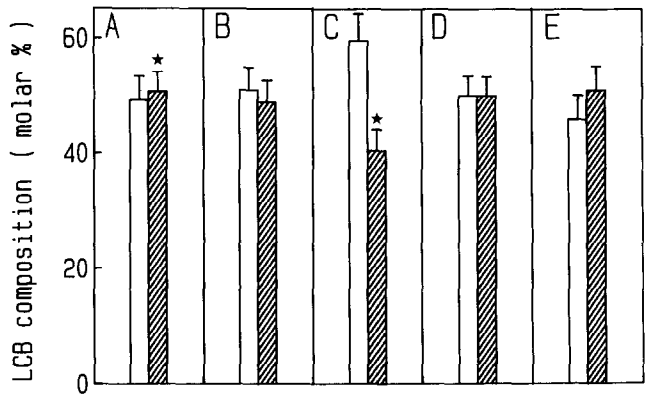

Fig. 1. Molecular species composition of oxidized GM1 formed by partial (10%) or exhaustive (99%) GAO oxidation of native GM1 embedded in LUVs. (A) Native GM1 ganglioside (0 time incubation with GAO). (B) Native GM1 inserted in DPPC LUVs after exhaustive oxidation. (C) Native GM1 inserted in DPPC LUVs after partial oxidation. (D) Native GM1 inserted in EPC LUVs after exhaustive oxidation. (E) Native GM1 inserted in EPC LUVs after partial oxidation. Bars indicate the S.D. values. Open bars = C18:1 GM1; hatched bars = C20:1 GM1. Statistical analysis by Student's *t*-test: data in panel A vs. data in panel C: $P < 0.01$ ($n = 5$).

A partial (10%) oxidation of native GM1 in micellar form produced C18:1 and C20:1 GM1-ox in the proportion of about 51% and 49%, matching the composition of starting native GM1 [21]. After exhaustive (99%) enzymatic oxidation of native GM1 embedded in both DPPC and EPC LUVs no differences in LCB proportion were detected between treated and untreated vesicles (Fig. 1).

3.2. Kinetics of GAO upon C18:1 GM1 or C20:1 GM1 embedded into LUVs

The *V/S* plots referring to GAO acting on GM1 molecular species in DPPC and EPC LUVs are reported in Fig. 2A and B. The corresponding values of V_{max} and K_m are given in Table 1. In the case of GM1/DPPC LUVs the activity was higher on the C18:1 than the C20:1 species (69.9 and 52.9 nmol GM1ox·min^{-1}·mg of protein, respectively). Similar results were obtained using an equimolar mixture of C18:1 GM1 and C20:1 GM1 (total GM1, 10% molar) inserted in DPPC vesicles. In this case radiolabeled C18:1 GM1 was mixed with cold C20:1 GM1 or viceversa (Table 1). For GM1 in EPC vesicles the kinetic parameters were alike for the two molecular species. K_m and V_{max} values for the GM1/EPC system were significantly different from those obtained for GM1/DPPC.

4. Discussion

The evidence emerging from the present study is the ability of GAO to preferentially affect, at the initial stages of oxidation, GM1 ganglioside molecules carrying

C18:1 rather than C20:1 LCBs when inserted in DPPC vesicles, but not when inserted in EPC or in micellar form. These results show that the capability to affect GM1 in dependence of its LCB composition is not an intrinsic property of the enzyme but depends on the particular features of the membrane-mimicking system containing the gangliosidic substrate.

The susceptibility of GM1 molecular species to GAO action might be related to differences in the orientation of the saccharide chains, depending on the ceramide moiety composition and controlling accessibility to the enzyme. These possibilities seem to be ruled out by previous data [29,30] indicating that the lipid chain length does not significantly affect the conformation of saccharide moiety. Moreover, it has been reported that in the case of galactosylceramide embedded in PC vesicles [10], GAO preferentially affects the molecular species carrying longer lipid chains, their saccharide moiety being more exposed above the apolar–polar interface of the bilayer. This is an entirely different situation than ours. Since it has been shown that GAO is very sensitive to the degree of segregation of GM1 [16], we believe that the preferential action on C18:1 GM1 rather than C20:1 GM1 inserted in DPPC vesicles reflects a difference in the extent of lateral phase separation of the two ganglioside species. This possibility is suggested by calorimetric investigations [11,31] that showed a higher tendency to undergo lateral phase separation by C20:1 than C18:1 species in DPPC vesicles. Presumably, the triggering factor resides in the difference in length and unsaturation between the ganglioside and phospholipid moieties. Of course, in case of a different extent of segregation GAO would preferentially act on the GM1 molecular species that is more molecularly dispersed, or less packed in the segregated form.

In conclusion, the ceramide composition of glycosphingolipids together with the chemical characteristics of the surrounding lipid environment appear to control the exposure of the sphingolipid saccharide portions to external ligands. As a consequence, some biological functions taking place at the plasma membrane level, may result to be modulated by the surface features of the oligosaccharide moieties protruding from the membrane surface.

Table 1
Kinetic parameters of GAO upon C18:1 GM1 and C20:1 GM1 inserted in DPPC or EPC LUVs

	V_{max}[a]	K_m (μM)
(a) DPPC LUVs		
+ C18:1 GM1 (10% mol)	69.9	760
+ C20:1 GM1 (10% mol)	52.9	770
+ C18:1 GM1 (5%) + C20:1 GM1 (5%)		
(C18:1 GM1)	70.0	740
(C20:1 GM1)	53.1	730
(b) EPC		
+ C18:1 GM1 (10% mol)	210	150
+ C20:1 GM1 (10% mol)	201	160

[a] V_{max} expressed as nmoles of oxidated substrate · min^{-1} · mg $protein^{-1}$.

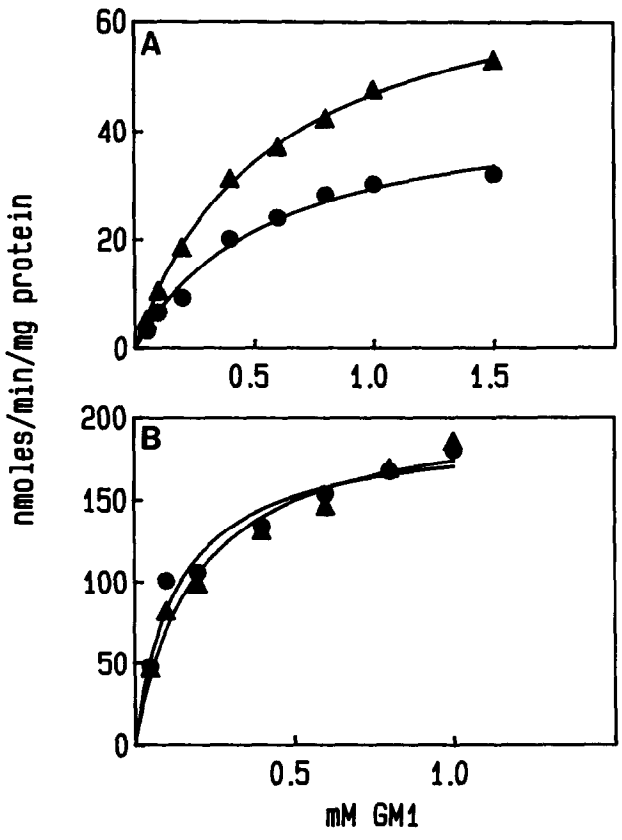

Fig. 2. Kinetics of GAO action on GM1 molecular species inserted into DPPC LUVs (A) or EPC LUVs (B). ▲ = C18:1 GM1; ● = C20:1 GM1.

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