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The Effect of Glycophorin A on Oxidation of Globoside by Galactose Oxidase

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The interaction of galactose oxidase with native and desialylated glycophorin A was studied by oxidizing human erythrocytes and globoside/phospholipid vesicles with the enzyme. Oxidation of the glycolipid was improved in the presence of vesicle-incorporated glycophorin A. Although galactose oxidase is a very basic protein, it was not adsorbed on native human erythrocytes. Instead, neuraminidase-treated cells bound a substantial amount of galactose oxidase, but the enzyme seemed to be released into the buffer when desialylated glycoproteins had been oxidized.

Glycosphingolipids are thought to play a role in cell-cell interaction, microbial adhesion, cell differentiation, and malignant transformation [1-5]. A large proportion of cell-surface glycolipids is, however, inaccessible to external ligands [6]. Several investigators have found that neuraminidase treatment of cells increases the exposure of small neutral glycolipids to specific antibodies and to galactose oxidase [7-9]. These findings suggest that sialoglycoconjugates and neutral glycolipids have some interactions on the cell surface. On the other hand, sialic acids could affect galactose oxidase or anti-glycolipid antibodies.

Galactose oxidase has an isoelectric point above pH 10 [10], and therefore it might bind non-specifically to sialic acid residues. There is also some evidence that sialoglycoconjugates could inhibit galactose oxidase activity [11]. We have investigated these factors in the present study.

Materials and Methods

Enzymes

Galactose oxidase (*Dactylium dendroides*, type V), horseradish peroxidase (type II), and bovine liver catalase were obtained from Sigma Chemical Co. (St. Louis, MO). *Vibrio cholerae* neuraminidase (1 U/1.1 ml) was from Boehringer (Mannheim, W. Germany).

Abbreviation: PBS, 0.01 M sodium phosphate - 0.15 M NaCl, pH 7.4

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Isotopes

Tritiated sodium borohydride (7 Ci/mmol) was purchased from Amersham International (Amersham, England) and handled as described [12]. Carrier-free ^{125}I (3.7 GBq/ml) was from Amersham.

Cells

Freshly drawn human red cells were used in all experiments. The cells were washed four times with PBS (0.01 M sodium phosphate - 0.15 M NaCl, pH 7.4) before use.

Assay of Enzyme Activity from Cell Suspensions

Human red cells were suspended in an equal volume of PBS and pre-treated with 50 mU neuraminidase/ml packed cells for 30 min at 37°C. Control cells were incubated with the buffer only. Thereafter the cells were oxidized with five units galactose oxidase/ml cells at 37°C. During incubation, small samples were taken from the cell suspension and centrifuged at 4°C. Enzyme activity was assayed from the supernatant as recommended by Sigma Chemical Co.

Isolation of Globoside

Globoside was isolated from human erythrocyte membranes by the procedure of Saito and Hakomori [13] and purified by Bio-Sil A chromatography.

Labeling of Globoside Standard

Tritium-labeled globoside standard was prepared as previously described [14].

Isolation of Glycophorin A

Glycophorin A was isolated from human erythrocyte membranes by the method of Hamaguchi and Cleve [15].

Purification of Phosphatidylcholine

Egg lecithin was obtained from Merck A.G. (Darmstadt, FRG) and further purified by aluminium oxide chromatography as described [14].

Preparation of Unilamellar Lipid Vesicles

Small unilamellar lipid vesicles were prepared as described [14]. Glycophorin A-containing vesicles were prepared by suspending 25 mg phosphatidylcholine, 400 nmol globoside and 1.0 mg glycophorin A in 1.5 ml PBS. The mixture was sonicated for 1.5 h in a bath sonicator at +6°C (Bransonic 32, GWB) and centrifuged (Beckman LB-70 ultracentrifuge) at 150,000 x *g* for 35 min. The supernatant was removed and used for further studies. The amount of glycolipid was estimated by using tritium-labeled globoside as a tracer. Vesicles containing only globoside and phosphatidylcholine were prepared in the same way.

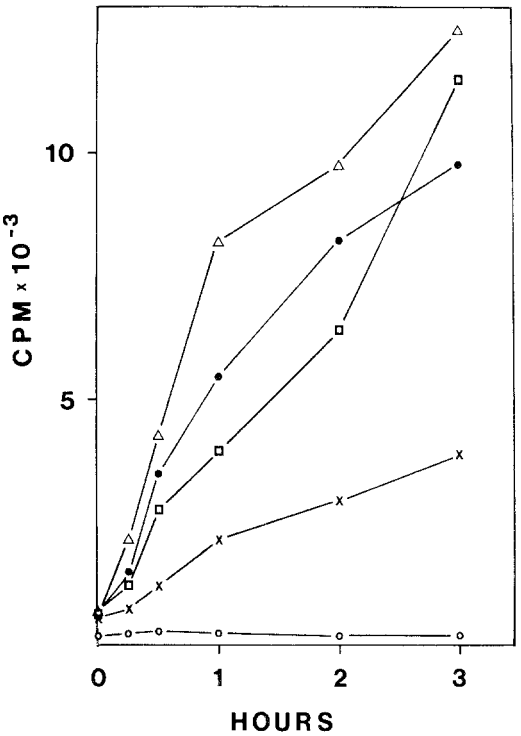

Figure 1. The effect of glycophorin A on oxidation of globoside/phosphatidylcholine vesicles by galactose oxidase. Glycophorin A incorporated in the same vesicles as globoside (△); neuraminidase-treated vesicles containing glycophorin A and globoside (●); globoside and glycophorin A in different vesicles (□): vesicles containing globoside and phospholipid (x); vesicles containing glycophorin A and phospholipid (○). - Radioactivity incorporated into the glycolipid was measured.

Sialic Acid Determination

The sialic acid content of glycophorin A vesicles was determined by the method of Miettinen and Takki-Luukkainen [16]. About 20% of glycophorin A (and almost twice as much globoside) was found in the small vesicle fraction, when vesicles containing only glycophorin A or vesicles containing both globoside and glycophorin A were studied.

Labeling of Vesicles

Vesicles containing 10 nmol globoside and/or 14 μg glycophorin A were incubated at 37°C with 2.5 units galactose oxidase and 2.5 units catalase in PBS. For desialylation of glycophorin A vesicles, five milliunits of neuraminidase were used. The total volume of the incubation mixture was 500 μl. Samples of 50 μl were taken into ice-cold tetrahydrofuran

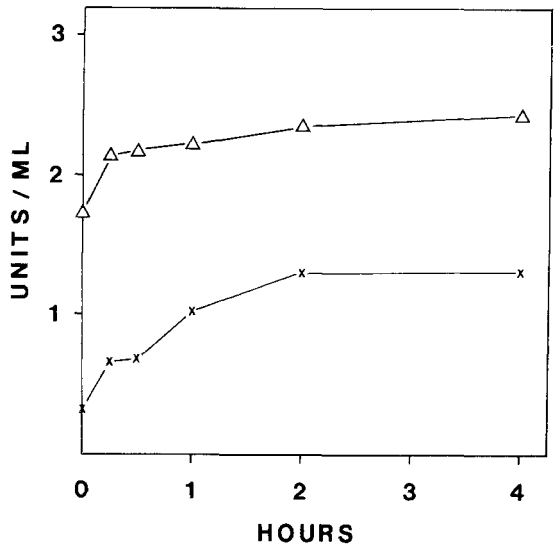

Figure 2. Activity of galactose oxidase in the supernatant from a red cell suspension. Activity from a suspension containing native red cells (△); activity from a suspension containing neuraminidase-treated cells (x). Galactose oxidase (2.5 units) was originally added per ml of the incubation mixture.

and immediately frozen at -20°C. When all samples had been collected, they were warmed to room temperature and reduced with 100 μCi NaB^3H_4 overnight. The samples were washed with solvent partition as described [17]. The final purification of all samples was done by thin layer chromatography.

Radio-iodination of Galactose Oxidase

The chloramine T method [18] was used for radio-iodination of galactose oxidase.

Results

Fig. 1 shows typical results from an experiment, in which phospholipid vesicles containing globoside and/or glycophorin A were incubated with galactose oxidase. The enzyme oxidizes terminal galactose and *N*-acetylgalactosamine residues in oligosaccharide chains, and the aldehyde groups thus formed can be reduced with NaB^3H_4. In our experiments, radioactivity incorporated into the glycolipid was measured. When glycophorin A was present in the incubation mixtures, globoside was oxidized several times faster than in those suspensions containing only glycolipid/phospholipid vesicles. Glycophorin/phospholipid

Table 1. Binding of ^{125}I-galactose oxidase to native and neuraminidase treated red cells. Human erythrocytes were incubated at 37°C with 10 units of galactose oxidase/ml cells. ^{125}I-Labeled galactose oxidase (2.3 x 10^6 cpm/ml cells) was added as a tracer. After incubation, the cells were isolated by centrifugation and washed twice with phosphate-buffered saline.

	% ^{125}I-Galactose oxidase bound			
	Incubation time (min)			
	0	30	60	90
Neuraminidase-treated cells	28.5	21.1	18.2	16.9
Native cells	3.2	3.2	3.8	3.8

vesicles were used as controls, and when the oxidized and reduced control samples were analyzed by thin layer chromatography, no radioactivity was found in the area corresponding to globoside standards.

Relatively high amounts of galactose oxidase were needed to oxidize susceptible galactose residues in erythrocytes. Therefore we could easily measure the enzyme activity remaining in the incubation buffer, when the red cells were first removed by centrifugation. Most of the enzyme activity was found in the supernatant when native red cells were treated with galactose oxidase (Fig. 2). In contrast, desialylated erythrocytes bound more than 80% of the enzyme activity at the very beginning of the incubation. Experiments with ^{125}I-labeled galactose oxidase gave similar results (Table 1). Moreover, some of the enzyme seemed to bind irreversibly to desialylated erythrocytes, probably by formation of Schiff bases between amino groups of the protein and galactose aldehydes. This might be the reason why globoside in desialylated glycophorin A vesicles was not oxidized as efficiently as it was oxidized in suspensions containing native glycophorin A (Fig. 1).

According to Kosman *et al.* [10], a substantial proportion of galactose oxidase might be lost by binding to vessel walls if dilute solutions (less than 0.2 mg enzyme/ml) are used in assays. We did not observe this phenomenon in our studies. However, production of hydrogen peroxide in the reaction may decrease the enzyme activity.

Discussion

Our results show that although galactose oxidase is a basic protein, it does not bind to native red cells having a large amount of sialic acid. Instead, the enzyme binds to desialylated erythrocytes and is released into the buffer as incubation proceeds. Bretting and Jacobs found that galactose oxidase oxidized β(1-6)-linked galactose residues at a rate eight times

higher than D-galactose was oxidized. β(1-3)-Linked galactose residues were half as good substrates as D-galactose, and lactose (Galβ1-4Glc) was completely inactive [19]. However, lactosylceramide can be oxidized by galactose oxidase in 50% tetrahydrofuran [14, 20]. These results indicate that terminal galactose residues on the red cell surface are poor substrates for galactose oxidase, and the enzyme substrate complex has a relatively long half-life. Similar oligosaccharides could be useful in purification of the enzyme; agarose and melibiose-polyacrylamide gels have previously been used as affinity matrixes for galactose oxidase [21-23].

The experiments with glycophorin/glycolipid vesicles indicate that galactose oxidase is not inhibited by sialoglycoproteins. In fact, oxidation of globoside is improved in the presence of glycophorin A, whether or not the protein is in the same vesicles as the glycolipid. This suggests that glycophorin A may have a stabilizing effect on the active form of the enzyme. Neuraminidase treatment of the vesicles had little effect on the oxidation rate. However, we cannot exclude that glycolipids and glycophorin A might have some interaction. Endo *et al.* found that the haptenic activity of Forssman glycolipid was affected by glycophorin, and the interaction of lectins with glycophorin was also affected by glycolipids. They incorporated the protein and glycolipids in the same phospholipid vesicles [24].

The concentration of glycophorin A in human erythrocytes is higher than in the vesicles we have used [25]. Therefore, the lateral distribution of red cell glycolipids should be more restricted. Human En(a-) cells lack glycophorin A, and the amount of sialic acid is decreased. As compared to normal erythrocytes, small glycolipids were present in En(a-) cells in similar amounts but were much more easily labeled [26]. This could correspond to a situation where normal red cells are treated with neuraminidase; glycophorin A molecules partially lose their negative charge and might cluster on the red cell membrane.

In conclusion, galactose oxidase is not inhibited by sialoglycoproteins or adsorbed on a layer of sialic acids on the cell surface. Neuraminidase treatment of red cells could lead to clustering of glycophorin A molecules, which might expose small glycolipids to galactose oxidase.

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