FABRY'S DISEASE AS AN α-GALACTOSIDOSIS: EVIDENCE FOR AN α-CONFIGURATION IN TRIHEXOSYL CERAMIDE.[1]

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SUMMARY. Pure α-galactosidases, devoid of β-galactosidase activity, were purified from coffee beans, ficin (a crude extract from figs), rat and rabbit small intestine. With the exception of the coffee bean enzyme, all α-galactosidase preparations released galactose from ^{3}H- or ^{14}C-labeled trihexosyl ceramide obtained from patients with Fabry's disease. Galactose liberation was specifically inhibited by α-galactosides, such as melibiose and stachyose, while lactose had no effect. Our results corroborate the α-galactosidase deficiency reported in Fabry's disease and establish that the terminal galactosyl residue of the trihexosyl ceramide stored in this condition has an α-configuration.

Fabry's disease is a sex-linked inborn error of sphingolipid metabolism (1). Trihexosyl ceramides (CTH) are stored in most tissues. A digalactosyl ceramide accumulates mainly in the kidney. The carbohydrate sequence and the site of the linkage were readily established (2,3) as galactopyranosyl-(1→4)-galactopyranosyl-(1→4)-glucopyranosyl-(1→1)-ceramide, but the nature of the glycosidic bonds remained unsettled. Brady et al. (4) identified and purified an intestinal enzyme capable of cleaving the terminal galactose from CTH. This enzyme preparation did not release galactose from natural or artificial β-galactosides, but glycosides containing α-galactosidic linkages were not tested. It was thus implied that "ceramide trihexosidase" was a special type of galactosidase exclusively involved in CTH degradation. Gatt (5), however, showed that erythrocyte

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and kidney CTH could be rapidly hydrolyzed by a rat brain galactosidase proven to possess β-galactosidase activity towards synthetic substrates but in which α-galactosidase activity was not reported. Kidney CTH was described as containing three β-glycosidic bonds but no evidence for that structure was presented. Rat brain is now known to possess an α-galactosidase active towards natural and synthetic substrates (6).

The antigenic activity of the blood group B substance involves a terminal α-glycosidic bond. Although blood group B activity had been observed in lipid extracts (7), the characterization of such lipids has only been achieved recently (8). CTH with a terminal α-glycosidic bond has now been recognized in mouse sarcoma (8) human erythrocytes and hamster kidney fibroblasts (10). On the basis of a study by nuclear magnetic resonance, Sweeley et al. (11) claimed that the terminal galactosyl residue in CTH from Fabry's disease could be a β-configuration. This is difficult to reconcile with the α-galactosidase deficiency discovered by Kint (12). This deficiency has been confirmed by Romeo and Migeon (13) who, moreover, were able to demonstrate two fibroblast phenotypes (with and without α-galactosidase activity) in female carriers of the Fabry's gene. In order to find conclusive evidence for the anomeric configuration of CTH in Fabry's disease we studied the degradation of CTH by purified α-galactosidase preparations devoid of β-galactosidase activity.

MATERIALS AND METHODS. CTH was obtained from a lymph node of a patient with Fabry's disease (14). Galactopyranosyl-(1→4)-glucopyranosyl-(1→1)-ceramide (CDH) was a gift from Dr. Jerzy Hildebrand. The glycolipids were tritiated (15) and purified by column and thin layer chromatography. ^{14}C-labeled CTH was also prepared from skin fibroblasts taken from a patient with Fabry's disease and cultured in the presence of 1-^{14}C-galactose. "Ceramide trihexosidase" was prepared from rabbit and rat small intestine (4). α-galactosidases were purified from both coffee beans (16) and ficin (10). α- and β-D-galactopyranosyl derivatives of p-nitrophenol and 4-

methylumbelliferone were used as substrates to follow enzyme purification. The degradation of labeled glycolipids by the purified enzymes was studied in 0.01M sodium acetate buffer for 18 h at 37°C (17). There was no release of galactose from β-galactosides or ^{3}H-CDH under these conditions.

RESULTS. The purified α-galactosidase from coffee beans, rat intestine and ficin had similar specific activities towards synthetic substrates, (table 1) but they were devoid of β-galactosidase activity. The α-galactosidase from rat intestine had a pH optimum of 5.0 and the release of ^{3}H-galactose from CTH was depressed by 20mM of melibiose but not by lactose at the same concentration. Coffee bean α-galactosidase however was inactive towards either the ^{3}H- or ^{14}C-labeled CTH. α-galactosidase could be readily purified from ficin in good yield. The enzyme from this source was then

Table 1. Specific activity of purified α-galactosidase.

Origin	α-D-galactosyl-4-Methylumbelliferone	^{3}H-trihexosyl ceramide
Rat intestine	9.0	0.107
Coffee bean	8.0	0
Ficin	7.1	0.065

All the values were corrected for boiled enzyme controls. Results are expressed as μmoles/mg of protein/hr.

used for further studies. This enzyme was able to hydrolyze the terminal galactose of ^{3}H-CTH with a sharp pH optimum at 4.5; the activity between pH 4.9 and 7.9 was negligible (fig. 1A). The amount of labeled galactose liberated was proportional to the amount of enzyme added up to 4.8 mg of protein. The apparent Michaelis constant (Km) was calculated from the Lineweaver-Burk plot (fig. 1B). Several inhibitors were found (table 2) and the data suggested a competitive inhibition with stachyose and melibiose.

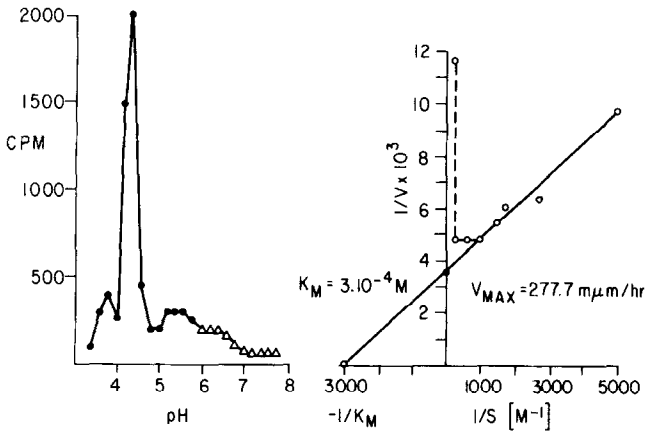

Fig. 1A. (left) Effect of pH on the activity of α-galactosidase from ficin. Experimental conditions described in Table 2. In order of increasing pH, the buffers were: sodium acetate (●-●) and sodium phosphate (Δ-Δ). Total CPM per assay, 10,000.

Fig. 1B. (right) p-nitrophenol α-galactopyranoside + ficin α-galactosidase. Sodium acetate buffer pH 4.5, 0.05M, 15 minutes at 37°C.

Table 2. Effect of various substances on the activity of α-galactosidase from Ficin.

Additions	Concentration	Activity (%)
None	--	100
Melibiose	10mM	55
Melibiose	20mM	50
Melibiose	30mM	48
Stachyose	20mM	65
Lactose	20mM	100
D-galactose	20mM	40
D-galactose	40mM	25
Sodium cholate	2mg/ml	75

Activity was measured as cpm of galactose liberated by α-galactosidase (Ficin) incubated for 18 hr with ^{3}H-trihexosyl ceramide. Each assay, run in duplicate and with a blank, contained 1 ml of 0.01M sodium acetate buffer pH4.5, 1g% of Albumin, 2.4mg of Enzyme, and water to a total volume of 1.5ml.

DISCUSSION. Despite its activity on a wide range of α-galactosides, including blood group B substance (16), the α-galactosidase from coffee beans was not able to hydrolyze labeled CTH from patients with Fabry's disease. Ficin α-galactosidase which had been shown to degrade erythro-

cyte CTH (10) also acted on the lymph node and fibroblast CTH. Substrate analogues such as galactose, melibiose and stachyose inhibited the reaction while an equivalent amount of a β-galactopyranosyl substrate, lactose, had no influence.

The "ceramide trihexosidase" from rat intestine had been conclusively shown to be devoid of activity against β-galactosides, including O-β-D-galactopyranosyl-(1→4)-O-β-D-galactopyranosyl-(1→4)-D-glucose (4), an oligosaccharide identical to the carbohydrate moiety of the CTH structure recently defended (11). Our results confirmed the lack of activity of "ceramide trihexosidase" on synthetic β-galactosides, and indicated that such purified enzyme was characterized by a highly specific α-galactosidase activity, that, to our knowledge, had never been shown before. The definite inhibition of ficin α-galactosidase by sodium cholate is contrary to the results of Brady et al. who showed a stimulation of the enzyme at the same detergent concentration (4). However the apparent activating effect may simply reflect the optimum amount of detergent required to adequately solubilize the CTH substrate. The detergent-free conditions of our assay (17) avoided this problem and increased its sensitivity.

The relative lack of specificity regarding the aglycone group is a well-established characteristic of acid hydrolases (18). However, the aglycone group has a marked effect on the rate of reaction and the Km value. α-galactosidases from different sources exhibit both similarities and differences regarding their substrate specificity (19). The α-galactosidase from Mortierella vinacea acts on α-(1→6)- and α-(1→4)- linked D-galactosides (19), while coffee bean α-galactosidase has been reported to cleave α-(1→3)- and α-(1→6)-linked D-galactosides (16). The site of the glycosidic linkage therefore seems to have a role in determining the substrate specificity. The coffee bean α-galactosidase has not been shown to degrade α-(1→4) linked D-galactosides and this may account for its inability to degrade CTH.

Our data give its full meaning to the reported deficiency of α-galactosidase in Fabry's disease (12,13). The possible relationship of the CTH stored in Fabry's disease with erythrocyte degradation (1) is further strengthened by the fact that erythrocyte CTH has the same terminal α-glycosidyl linkage (10). It is clear, however, that other tissues such as the skin fibroblasts or the kidney which are not known to have a role in erythrocyte degradation are able to synthesize their own CTH. This may account for some of the striking clinical and pathological differences observed between Fabry's and Gaucher's disease, despite the fact that the pathway of erythrocyte degradation has been implicated in both conditions (20).

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