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PROPERTIES OF IMMOBILIZED FIG α-GALACTOSIDASE AND EFFECT ON CERAMIDE-3 CONTENT OF PLASMA FROM PATIENTS WITH FABRY'S DISEASE

ANDRE W. SCHRAM, MIC N. HAMERS, ELLY OLDENBROEK-HAVERKAMP, ANNEKE STRIJLAND, ALBERTINE DE JONGE, FRANK A.J.TH.M. VAN DEN BERGH and JOSEPH M. TAGER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam (The Netherlands)

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Summary

The possibility of lowering the level of ceramide-3 (galactosyl-α(1 → 4)-galactosyl-β(1 → 4)-glucosyl-β(1 → 1)-ceramide) in the plasma of patients with Fabry's disease was investigated.

An immobilized α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) was prepared by coupling purified fig α-galactosidase to Sepharose 4B. The pH optimum for the hydrolysis of the artificial substrate *p*-nitrophenyl-α-D-galactopyranoside was shifted by approx. 0.5—1.0 pH unit to higher pH values upon coupling of the enzyme to Sepharose 4B. The immobilized enzyme was more stable than the native enzyme to incubation at 60°C.

The immobilized enzyme was able to hydrolyse ceramide-3 either at pH 4.5 or at pH 7.4 in an artificial system in which sodium taurocholate was used to solubilize the substrate. In contrast, when the immobilized enzyme was incubated with normal plasma or plasma from a patient with Fabry's disease, in which elevated levels of ceramide-3 occur, no hydrolysis of the glycosphingolipid could be detected.

The results suggest that lowering of the level of ceramide-3 in plasma from patients with Fabry's disease by enzymic means is not feasible.

Introduction

Fabry's disease, an X-linked inborn error of metabolism, is characterized by a partial deficiency of α-galactosidase (α-D-galactoside galactohydrolase, EC

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein.

3.2.1.22) activity as measured with the artificial substrates *p*-nitrophenyl-α-galactopyranoside and 4-methylumbelliferyl-α-D-galactopyranoside [1].

Beutler and Kuhl [2] showed that there are two α-galactosidase isoenzymes (α-galactosidase A and B) in man. The A isoenzyme is absent in Fabry patients [3]. These findings have been confirmed by many other investigators studying various tissues and body fluids [4—9]. The residual activity present in Fabry tissues is identical to α-galactosidase B from normal liver [10—12].

It has been suggested that Fabry's disease may be particularly amenable to enzyme replacement therapy, since there is little or no involvement of the central nervous system and the disease has a slow progression [13]. However, several investigators have shown that cross-reactive immunological material is absent in Fabry tissues (refs. 10, 12, 14; contrast ref. 15), so that enzyme replacement therapy in this lysosomal storage disease could give rise to severe immunological problems. The deficiency of α-galactosidase A leads to accumulation of ceramide-3 (galactosyl-α(1 → 4)-galactosyl-β(1 → 4)-glucosyl-β(1 → 1)-ceramide) in the lysosomes [1,16]. The ceramide-3 accumulated in the tissues may arise either from endogenous or from exogenous sources.

Plasma glycosphingolipids may be the main source of the ceramide-3 accumulated in the walls of blood vessels, for the following reason. It has been found that the neutral glycosphingolipids like ceramide-3 and its next higher homologue ceramide-4 (*N*-acetylgalactosaminyl-β(1 → 3)-galactosyl-α-(1 → 4)-galactosyl-β(1 → 4)-glucosyl-β(1 → 1)-ceramide) are associated with the lipoprotein fractions in plasma [17—21], and Bierman and Albers [22] have shown that lipoproteins are taken up by smooth-muscle cells. Thus a mechanism may be provided for lipid accumulation in these and perhaps other tissues. Lowering the concentration in plasma of ceramide-3 and ceramide-4 by enzymic hydrolysis of the glycosphingolipids may therefore be of therapeutic interest. To investigate whether this can be achieved in plasma from patients with Fabry's disease, two requirements had to be fulfilled. Firstly, a stable enzyme which is active at neutral pH was required. Secondly, in order to avoid immunological complications, an immobilized enzyme which could be used in an extracorporeal shunt was needed. We have therefore isolated α-galactosidase from figs (see refs. 23 and 24) and immobilized it by coupling it to Sepharose-4B. We have investigated the properties of the immobilized enzyme, particularly with regard to the possibility that it might bring about the hydrolysis of ceramide-3 present in plasma from Fabry patients at pH 7.4.

Materials and Methods

α-Galactosidase was isolated from figs essentially according to Hakomori et al. [23]. 25 g Ficin (Nutritional Biochemicals Corp.) were incubated in 220 ml distilled water for 4 h at 0°C after which the suspension was centrifuged at 50 000 × *g* for 30 min. The supernatant was dialysed against 2 × 2000 ml distilled water for about 20 h. The dialysed extract was centrifuged at 50 000 × *g* for 30 min. The supernatant was treated twice with cold acetone (48 ml/100 ml extract), and incubated at 0° C for 30 min, after which the second precipitate was collected by centrifugation at 50 000 × *g* for 30 min. This precipitate was taken up in distilled water and dialysed against 50 mM phosphate buffer

(pH 6.0). The dialysed solution (volume 25 ml) was chromatographed on a Sephadex G-200 column (5 × 130 cm) and the fractions containing the α-galactosidase activity were pooled and concentrated by ultrafiltration (Amicon PM-30). Protein was measured according to Eggstein and Kreuz [25], using crystalline egg albumin as standard.

Coupling of 4.7 units enzyme to 50 ml Sepharose 4B (Pharmacia, Sweden) was carried out essentially as described by Axen et al. [26], using 150 mg CNBr/ml gel and 50 mM sodium phosphate (pH 6.5) as the coupling buffer. After coupling, the immobilized enzyme was washed with 100 ml of 500 mM NaCl to remove adsorbed material. Measurement of the washing solution indicated that all the protein originally present in the α-galactosidase-containing fraction was coupled.

Assays with the native enzyme using the artificial substrate were carried out at 37°C in a reaction mixture (final volume, 0.5 ml) containing 100 mM sodium acetate (pH 4.6) and 2 mM *p*-nitrophenyl-α-galactoside (Koch-Light, U.K.). The reaction was stopped by adding 1 ml of a 300 mM glycine-NaOH solution (pH 10.6), and the amount of *p*-nitrophenol formed was measured spectrophotometrically at 405 nm, using a micromolar extinction coefficient of 18.6 [27]. 1 unit of enzyme activity is defined as 1 μmol substrate hydrolysed/min.

Assays with the immobilized enzyme were carried out at the same pH and the same substrate concentration as the assays with the native enzyme, but the final volume was 1 ml.

The reaction was started by adding immobilized enzyme, the latter being dispensed in a Finn pipette using a tip in which the point was cut off to widen the opening. In order to ensure that a uniform volume was dispensed, the stock suspension was kept dilute and was stirred vigorously. The reaction was stopped by adding 2 ml glycine-NaOH. The mixture was centrifuged at 200 × *g* for 5 min and the absorption of the supernatant was measured at 405 nm.

Hydrolysis of ceramide-3 was measured using ceramide-3 labelled with ^3H in the terminal galactose residue (see ref. 12). The substrate was solubilized in the presence of 0.4% sodium taurocholate (Mann Research, U.S.A.). The buffer used was a 100 mM sodium acetate solution (pH 4.6), and the volume of the reaction mixture was 100 μl. The reaction was started by addition of enzyme. The reaction was stopped by adding 100 μl 0.4 mM galactose in water and 800 μl chloroform/methanol (2 : 1, v/v), resulting in a partition according to Folch et al. [28]. 200 μl of the upper phase was counted in a liquid scintillation counter (Nuclear Chicago, U.S.A.), using as scintillation fluid toluene/ethanol (3 : 1, v/v), 2,5-diphenyloxazole (2 g/l) and 1,4-bis-(4-methyl-5-phenyloxazole-2-yl)-benzene (25 mg/ml). The counting efficiency was 25—35%.

Human plasma (50 ml of normal plasma or 15 ml of plasma from a Fabry patient) was incubated with immobilized fig α-galactosidase (330 munits of *p*-nitrophenyl-α-galactosidase activity) at 37°C. The pH of the incubation mixture was 7.4—7.6 (measured both at the start and at the end of the incubation). To prevent bacterial growth 0.01% azide was added; this compound had no effect on the activity of the enzyme. After incubation the samples were centrifuged at 200 × *g* for 5 min. The supernatant was removed and lyophilized, and glycosphingolipids were processed and determined gas chromatographically according to Sweeley and Tao [29]. To assess inactivation of the immobilized

enzyme after incubation in human plasma the immobilized enzyme was centrifuged down after the incubation, washed with distilled water, and assayed with the artificial substrate.

Antisera against ceramide-3 and ceramide-4 were raised in rabbits as described in ref. 30.

Antiserum against the apoprotein of low density lipoprotein (apo-B protein) was a gift from Dr. P.H.E. Groot (Department of Biochemistry, Erasmus University, Rotterdam) and had been raised in goats.

All incubations of antisera with plasma from a Fabry patient were carried out first for 30 min at 37°C and then for 48 h at 0°C. The incubation mixture was subsequently centrifuted at 10 000 × *g* for 4 min at room temperature. The pellets were washed twice with 1 ml of cold, phosphate-buffered saline (pH 7.4) and centrifuged at 10 000 × *g* for 4 min. Pellets were dissolved in 0.5 ml of 0.1 M NaOH and the absorbance at 280 nm was measured on a Zeiss spectrophotometer.

Results and Discussion

The α-galactosidase preparation purified from figs as described in Materials and Methods contained 7 units of *p*-nitrophenyl-α-galactosidase activity and 180 mg protein. The specific activity was 39 munits/mg protein and the recovery was 44%. No loss of activity occurred during coupling, indicating that the attachment to the matrix does not dramatically change the catalytic site of the enzyme. This suggestion is confirmed by the experiment summarized in Fig. 1, showing the Lineweaver-Burk plots for the hydrolysis of *p*-nitrophenol-α-D-galactoside. Both the native enzyme and the immobilized enzyme exhibit substrate inhibition. Maximum activity was at a substrate concentration of about 2 mM (Fig. 1A), and the apparent K_m, as determined from the lower substrate concentrations, was about 70 μM (Fig. 1B).

The effect of pH on the activity towards the artificial substrate is shown in Fig. 2. A shift in pH optimum (0.5—1 pH unit) to higher pH values occurs. This shift could be due to an influence of the matrix on the environment of the catalytic site of the enzyme or to a conformation change of the enzyme protein due to attachment to the matrix. The ratio between activity at pH 7.4 and that at the optimum pH changes from 0.23 to 0.27 upon coupling.

An important parameter is the stability of the enzyme. Table I shows the heat inactivation data for the native and the immobilized enzyme. The immobilized enzyme was more stable than the native preparation; after heating for 2 h at 60°C, the immobilized enzyme still exhibited 30% of the initial activity while the activity of the native enzyme was reduced to 11%. This may be due to the fact that covalent attachment of the enzyme to Sepharose 4B leads to a more rigid conformation.

The hydrolysis at pH 4.6 of ceramide-3 by the immobilized enzyme was investigated using 0.4% taurocholate to solubilize the glycosphingolipid. The hydrolysis of the substrate was linear in time, as was the relation between rate of hydrolysis and amount of enzyme present in the assay system (not shown). A linear Lineweaver-Burk plot for the hydrolysis of ceramide-3 was obtained, indicating an apparent K_m of 1.2 mM (not shown). At pH 7.4 the activity with

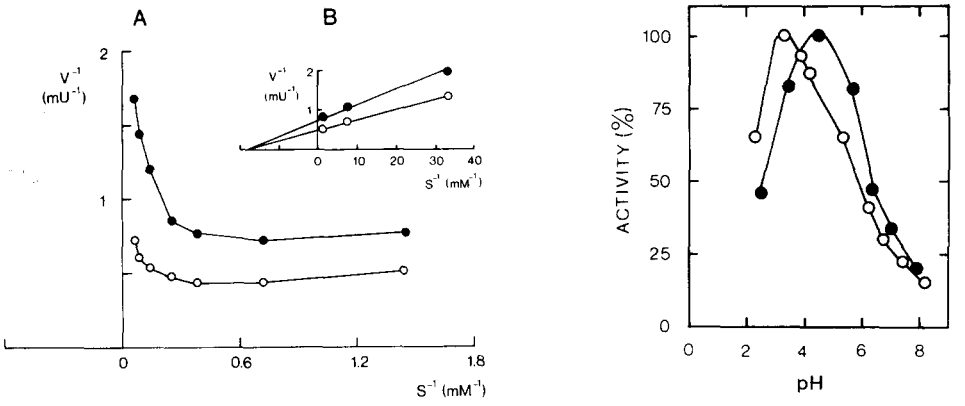

Fig. 1. Lineweaver-Burk plots for hydrolysis of *p*-nitrophenyl-α-galactoside by native (○———○) and immobilized (●———●) ficin α-galactosidase. The substrate concentration was varied between 0.7 and 18 mM (A). In B, the substrate concentration was varied between 0.03 and 0.7 mM, in order to be able to determine the K_m accurately.

Fig. 2. Effect of pH on activity of native and immobilized ficin α-galactosidase, measured with 2 mM *p*-nitrophenyl-α-galactoside. ○———○, native enzyme; ●———●, immobilized enzyme. The activities are expressed as percentage of that at the pH optimum.

ceramide-3 as substrate in the presence of 0.4% taurocholate was about 20% of the activity at pH 4.6. With *p*-nitrophenyl-α-galactoside as substrate, the activity was about 25% of that at pH 4.5.

The ability of immobilized fig α-galactosidase to hydrolyse plasma ceramide-3, which is localized in the high and low density lipoprotein fractions [17—21], was investigated as follows. Immobilized α-galactosidase (330 munits as measured with 2 mM *p*-nitrophenol-α-galactoside) was incubated with 50-ml samples of normal human plasma. Ceramide-3, the initial concentration of which was 3 μM, and other neutral glycosphingolipids were measured before incubation and after 4, 8 and 16 h. As a control, plasma was incubated with inactivated enzyme. No significant decrease in glycosphingolipid concentration could be detected after incubation either with active or with inactivated enzyme (Fig. 3). Similarly, incubation of immobilized enzyme with plasma

TABLE I

EFFECT OF HEATING AT 60°C ON ACTIVITY OF NATIVE AND IMMOBILIZED FICIN α-GALACTOSIDASE

Activity measured at 37°C with 2 mM *p*-nitrophenol-α-D-galactoside.

Time of heating (min)	Percentage of initial activity	
	Native enzyme	Immobilized enzyme
0	100	100
10	54	68
30	24	40
60	14	35
120	11	30

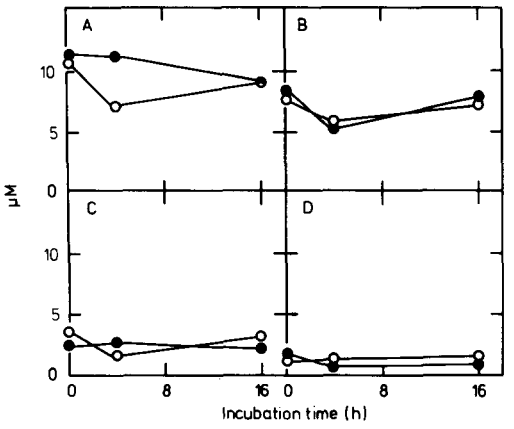

Fig. 3. Effect of incubation of normal plasma with immobilized fig α-galactosidase on glycolipid levels. Immobilized α-galactosidase (330 munits) was incubated with 50 ml normal human plasma for the times indicated and the levels of ceramide-1 (A, glucosyl-β(1 → 1)-ceramide), ceramide-2 (B, galactosyl-β(1 → 4)-glucosyl-β(1 → 1)-ceramide), ceramide-3 (C), and ceramide-4 (D) were measured as described in the text. ○———○, immobilized fig α-galactosidase; ●———●, immobilized fig α-galactosidase inactivated by heating at 100°C for 5 min.

from a Fabry patient, which contained 15 μM ceramide-3 (cf. refs. 31 and 32), did not lead to significant change in the concentration of the glycosphingolipids, even after 48 h (not shown). This failure to hydrolyse ceramide-3 could not have been due to inactivation of the enzyme during incubation in plasma. Measurement of the activity towards the artificial substrate after incubation showed that at least 60% of the initial activity was present after 48 h.

A possible explanation of the failure to hydrolyse ceramide-3 is that the concentration of the substrate is too low in relation to the amount of enzyme added.

Another explanation is that plasma ceramide-3 is so situated in the lipoprotein molecule that the oligosaccharide moiety is inaccessible to the enzyme. To clarify this point an immunological approach was developed, based on the assumption that incubation of plasma with antisera raised against a glycosphingolipid should lead to specific precipitation of lipoproteins, provided that the oligosaccharide moiety of the glycosphingolipid (which forms part of the antigenic determinant [30]) is accessible to the antibodies.

We therefore incubated different volumes (0.01—5 ml) of plasma from a Fabry patient with 200 μl anti-ceramide-3 serum, anti-ceramide-4 serum or normal rabbit serum. After centrifuging the immunecomplexes the absorbance at 280 nm of the pellet, dissolved in 0.5 ml NaOH, was determined. In all incubations, two precipitation optima were obtained, one at about 0.5 ml plasma and one at 2.5 ml plasma (not shown). The amount of precipitated material in the optimum at 2.5 ml plasma was very similar using the anti-glycosphingolipid sera and the control serum. The amount of precipitated material at the optimum found when using 0.5 ml plasma was somewhat greater with anti-glycosphingolipid sera than with control serum, suggesting that a precipitation of lipoprotein material by anti-glycosphingolipid sera might have occurred. However, this possibility is ruled out by the results of the experiment shown in Table II.

TABLE II

EFFECT OF PREINCUBATION OF 2 ml PLASMA FROM A FABRY PATIENT WITH ANTI-GLYCOSPHINGOLIPID SERA OR CONTROL SERUM ON THE AMOUNT OF PROTEIN PRECIPITATED WITH ANTI-APO-B SERUM OR CONTROL SERUM

The plasma was preincubated with 0.8 ml serum as indicated for 30 min at 37°C followed by 24 h at 0°C. After removal of the immune complexes by centrifugation at 10 000 × *g* for 4 min at room temperature, the supernatant was incubated with the sera indicated in the second column. For further experimental details, see Materials and Methods.

Preincubation with	Incubation with	Protein in precipitate after incubation (A_{280nm})
Anti-ceramide-4 serum	Anti-apo-B serum	9.2
	Normal goat serum	0.83
Anti-ceramide-3 serum	Anti-apo-B serum	9.9
	Normal goat serum	0.50
Normal rabbit serum	Anti-apo-B serum	9.8
	Normal goat serum	0.59
	Anti-ceramide-4 serum	0.47
	Anti-ceramide-3 serum	0.39

Fabry plasma was preincubated with anti-ceramide-4, anti-ceramide-3 or normal rabbit serum. No significant difference in amount of precipitated material was obtained upon subsequent incubation with anti-apo-B serum of plasma from a Fabry patient which had been preincubated either with anti-glycosphingolipid sera or with normal rabbit serum. Since low density lipoproteins (LDL) and very low density lipoproteins (VLDL) contain 35—70 and 10—15% of the plasma glycosphingolipids, respectively [17,18], it must be concluded that neither class of lipoproteins can be precipitated by anti-glycosphingolipid sera. These results support our suggestion that the oligosaccharide moiety of glycosphingolipids in plasma lipoproteins is inaccessible to antibodies or enzyme.

Conclusion

The results described above show that incubation of plasma with an immobilized α-galactosidase preparation, capable of hydrolysing ceramide-3 at pH 7.4 in the presence of taurocholate, does not lead to hydrolysis of the endogenous ceramide-3 even after 48 h.

Brady et al. [33] have observed that 40 min after injection of purified α-galactosidase into patients with Fabry's disease the ceramide-3 level in the plasma declines. They suggest [33] that this decline could be due either to hydrolysis of ceramide-3 in plasma or to hydrolysis of endocytosed plasma glycosphingolipid by added enzyme in the liver. They dismissed the first possibility, partly on the grounds of their observation that the activity of placental ceramide-trihexosidase is greatly diminished in the presence of whole blood.

However, in an assay system using taurocholate, the rate of hydrolysis of ceramide-3 is decreased by adding excess protein, which binds the detergent (ref. 34, see also ref. 35). Thus the decrease in activity observed by Brady et al.

[33] on adding whole blood was probably due to binding of detergent. Nevertheless, our results support the conclusion of Brady et al. [33] that the decline in plasma ceramide-3 levels in patients after injection of α-galactosidase could not have been due to hydrolysis in the plasma.

An alternative therapeutic approach in Fabry's disease could be to apply the method used by Thompson et al. [36] and by Moorjani et al. [37] in the treatment of hypercholesterolaemia. Removal of lipoprotein material from plasma led to a decrease in concentration of lipoprotein-bound lipid material like cholesterol and triglycerides in the plasma. It is likely that a similar decrease in plasma levels of neutral glycosphingolipids can be achieved by the same means.

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