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THE IDENTITY OF α-GALACTOSIDASE B FROM HUMAN LIVER

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Summary

1. The identity of α-galactosidase B (α-D-galactoside galactohydrolase, EC 3.2.1.22), the minor α-galactosidase isoenzyme present in normal human tissues and the component responsible for the residual α-galactosidase activity in patients with Fabry's disease, was investigated. For this investigation, α-galactosidase B was purified from normal human liver.

2. Purified α-galactosidase B contains α-glucosidase, α-xylosidase, *N*-acetyl-α-galactosaminidase and α-galactosidase activity, as measured with *p*-nitrophenyl glycosides as substrates.

3. Incubation of purified α-galactosidase B with an antiserum against the same preparation resulted in a reduction of both α-galactosidase and *N*-acetyl-α-galactosaminidase activities. The extent of the reduction was dependent on the amount of antiserum added, an identical titration curve being obtained with both activities. On the other hand, the α-glucosidase activity was not affected by incubation with the antiserum and α-xylosidase was inactivated by incubation either with antiserum or with normal serum.

4. Incubation of purified α-galactosidase B at 50°C for 4 h led to about 34% inactivation of both α-galactosidase and *N*-acetyl-α-galactosaminidase.

5. The K_m of purified α-galactosidase B for *p*-nitrophenyl-α-galactoside (about 20 mM) is higher than that for *p*-nitrophenyl-*N*-acetyl-α-galactosaminide (about 1 mM). The maximal velocity with the latter substrate is about 2.3-fold higher than that with the former.

6. When purified α-galactosidase B is incubated with *p*-nitrophenyl-α-galactoside and *p*-nitrophenyl-*N*-acetyl-α-galactosaminide together, the rate of *p*-nitrophenol formation is considerably less than the sum of the rates observed when the substrates are added singly.

7. It is concluded that the α-galactosidase and *N*-acetyl-α-galactosaminidase activities in purified α-galactosidase B are due to the same protein containing a single catalytic site.

8. It is suggested that the so-called α-galactosidase B from human tissues is in reality an *N*-acetyl-α-galactosaminidase.

Introduction

Human ceramidetrihexosidase is an α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) responsible for the hydrolysis of the terminal α-galactosidic linkage of neutral glycosphingolipids like galactosyl (α 1 → 4) galactosyl (β 1 → 4) glucosylceramide (ceramide-3, also known as ceramidetrihexoside) [1]. Beutler and Kuhl [2] showed that there are two α-galactosidase isoenzymes in human tissues, one (ceramidetrihexosidase) being referred to as α-galactosidase A, and the other as α-galactosidase B. α-Galactosidase A, which is responsible for 80—90% of the total α-galactosidase activity with artificial substrates in normal human material, is deficient in patients with Fabry's disease [1].

The isoenzymes differ in their kinetic properties. α-Galactosidase B has a higher K_m for the artificial substrates *p*-nitrophenyl-α-galactoside and 4-methylumbelliferyl-α-galactoside than α-galactosidase A [2—7], and is stimulated by myoinositol whereas the A isoenzyme is inhibited [2,6,7]. α-Galactosidase B has only a low activity with ceramide-3 as substrate [4].

Immunological studies with antisera against the purified isoenzymes have indicated that there is no structural relationship between α-galactosidase A and B [2,9,10]. The possibility that α-galactosidase B might have another substrate specificity was first suggested by the finding of Tallman et al. [11] that one of the α-galactosidase-containing fractions isolated from human placenta contained α-hexosaminidase activity. Tallman et al. [11] proposed that the natural substrate for α-galactosidase B might be a glycolipid or glycoprotein containing a terminal, α-linked hexosamine group.

In this paper enzymological and immunological evidence is provided for the identification of α-galactosidase B as *N*-acetyl-α-galactosaminidase, an enzyme that has previously been investigated in human liver and other tissues by Callahan et al. [12] and in pig and beef liver and various tissues of the rat by Weissmann and Hinrichsen [13].

Materials and Methods

α-Galactosidase B was purified from normal human liver using concanavalin A-Sepharose 4B (Pharmacia) and carboxymethyl-cellulose chromatography (Whatman CM 11), as described in ref. 10.

To determine glycosidase activities in the preparation, the reaction mixture (final volume 0.5 ml) contained purified α-galactosidase B (0.2—6.0 munits, as determined with *p*-nitrophenyl-α-galactoside as substrate), 0.5% bovine serum albumin and the buffer and substrates shown in Table I, with the following exception. In the experiment of Fig. 1 and Table III, the buffer was 200 mM acetate (pH 4.6) and the *p*-nitrophenyl-*N*-acetyl-α-galactosaminide concentration was varied as indicated. All substrates were obtained from Koch-Light. After incubation at 37°C for 10—60 min, the reaction was stopped by adding

1 ml 0.3 M glycine/NaOH (pH 10.6). The liberated *p*-nitrophenol was estimated spectrophotometrically at 405 nm, using a molar extinction coefficient of $18.5 \cdot 10^6$ cm^2/mol [9]. In control incubations, either enzyme or substrate was omitted. One unit of enzyme activity is defined as 1 μmol *p*-nitrophenol liberated/min at 37°C.

Anti-α-galactosidase B anitibodies were prepared by injecting purified α-galactosidase B into rabbits as described in ref. 10. All incubations of purified α-galactosidase B with the antiserum or control serum were carried out first for 30 min at 37°C and subsequently for 2 h at 0°C as described in ref. 10. The incubation mixture was subsequently centrifuged at 10 000 × *g* for 4 min at room temperature. The resultant supernatant was assayed for glycosidase activities as described above.

To protect the enzyme against denaturation in dilute protein-containing solutions (see ref. 10), all serum dilutions were made in phosphate-buffered saline containing bovine serum albumin (Sigma Fraction V; final concentration, 0.1%).

Results

To investigate if glycosidase activities other than α-galactosidase are present in purified α-galactosidase B, the preparation was incubated with several *p*-nitrophenyl glycosides in which an α-anomeric linkage is present. The results are summarized in Table I. Besides α-galactosidase activity, the preparation also had α-glucosidase, α-xylosidase and *N*-acetyl-α-galactosaminidase activities.

These activities could be due in part to impurity of the α-galactosidase B preparation. Therefore, to investigate if there is a common identity between α-galactosidase B and one or more of the other glycosidase activities present, a sample of the preparation was incubated with different dilutions of antiserum against the α-galactosidase B preparation. After centrifugation, the supernatants were tested for activity with different *p*-nitrophenyl glycosides.

The α-glucosidase activity was not affected by the treatment with antiserum (not shown), either because of the absence of precipitating antibodies against

TABLE I

GLYCOSIDASE ACTIVITIES IN PURIFIED α-GALACTOSIDASE B

The activities were measured using the *p*-nitrophenyl derivatives of the glycosides indicated, as described in Materials and Methods. The amount of enzyme used in this experiment corresponded to 6 munits measured with *p*-nitrophenyl-α-galactoside as substrate.

Glycoside (mM)	Buffer (mM)	pH	p-Nitrophenyl formation (nmol/min)
α-Glucoside (17)	Sodium acetate (250)	4.0	29.1
α-Xyloside (12)	Sodium citrate (175)	4.5	16.5
N-Acetyl-α-galactosaminide (1.75)	Sodium citrate (175)	4.5	11.8
α-Galactoside (12)	Sodium acetate (200)	4.6	6.0
α-Mannoside (7)	Sodium citrate (25)	5.0	0.3
α-Fucoside (7)	Sodium acetate (35)	5.5	0
N-Acetyl-α-glucosaminide (14)	Sodium citrate (175)	4.5	0

this enzyme or because of the presence of very weak antibodies. The α-xylosidase activity totally disappeared after incubation not only with antiserum, but also with control serum (not shown), suggesting that denaturation of this activity had occurred during the incubation procedure.

On the other hand, the α-galactosidase and *N*-acetyl-α-galactosaminidase activities were both strongly reduced by incubation with antiserum, the magnitude of the reduction being dependent on the relative amount of antigen and antibodies present in the system (Fig. 1). Similar titres were obtained in both cases. Although the presence of antibodies against two separate enzymes cannot be entirely ruled out, it is very unlikely that incubation of the α-galactosidase B preparation with a mixture of antibodies against two different enzyme proteins would result in an identical precipitation curve with the same titre for both enzymes.

To investigate further the possibility that α-galactosidase B and *N*-acetyl-α-galactosaminidase have a common identity, the effect of preincubation at 50°C and pH 4.0 on the activities towards the artificial substrates was investigated. The results (Table II) show a similar degree of heat inactivation of both activities; about 34% of the initial activity was lost after 4 h at 50°C (cf. ref. 12).

Lineweaver-Burk plots of the activity of the α-galactosidase B preparation with *p*-nitrophenyl-α-galactoside and *p*-nitrophenyl-*N*-acetyl-α-galactosaminide as substrates are shown in Fig. 2. The K_m for *p*-nitrophenyl-α-galactoside was about 20 mM, while that for *p*-nitrophenyl-*N*-acetyl-α-galactosaminide was about 1 mM (cf. ref. 12). The maximum velocity using the latter substrate was about 2.3-fold higher than that with *p*-nitrophenyl-α-galactoside.

Myoinositol, which slightly stimulates α-galactosidase B when *p*-nitrophenyl-α-galactoside is used as substrate [2,6,7], was found to have a similar slight stimulatory effect on the *N*-acetyl-α-galactosaminidase activity (not shown).

These results all suggest that a single enzyme protein is responsible for both

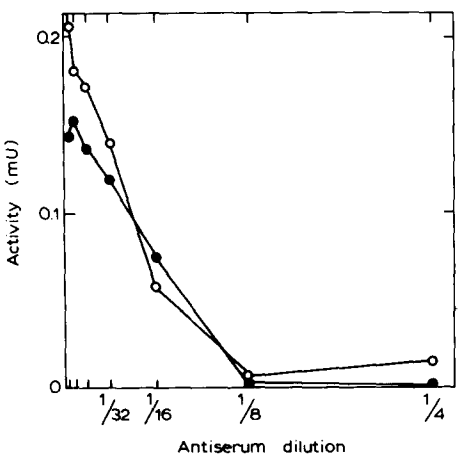

Fig. 1. Effect of an antiserum against purified α-galactosidase B on the activity of an α-galactosidase preparation with *p*-nitrophenyl-α-galactoside and *p*-nitrophenyl-*N*-acetyl-α-galactosaminide as substrates. ○———○, *p*-nitrophenyl-α-galactoside (12 mM); ●———●, *p*-nitrophenyl-*N*-acetyl-α-galactosaminide (0.525 mM). In control incubations with phosphate-buffered saline instead of antiserum, the activity with *p*-nitrophenyl-α-galactoside was 0.2 munit and with *p*-nitrophenyl-*N*-acetyl-α-galactosaminide, 0.14 munit.

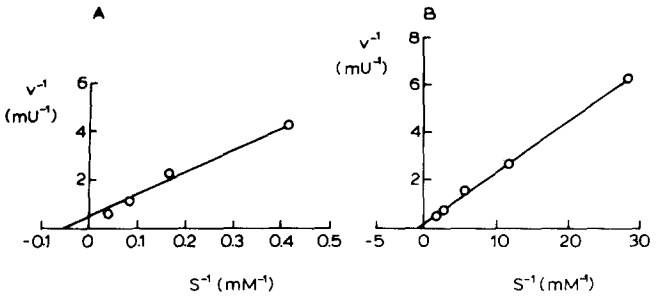

Fig. 2. Lineweaver-Burk plots for the activity of a purified α-galactosidase B preparation with *p*-nitrophenyl-α-galactoside (A) and *p*-nitrophenyl-*N*-acetyl-α-galactosaminide (B) as substrate. With *p*-nitrophenyl-α-galactoside as substrate, K_m = 19.6 mM and V = 57 munits/ml. With *p*-nitrophenyl-*N*-acetyl-α-galactosaminide as substrate, K_m = 1.07 mM and V = 130 munits/ml. The K_m was determined by the method of least squares.

activities. To investigate whether a single catalytic site is involved, an α-galactosidase B preparation was incubated with *p*-nitrophenyl-α-galactoside and *p*-nitrophenyl-*N*-acetyl-α-galactosaminide, either singly or together. Table III shows that the observed *p*-nitrophenyl formation when the two substrates were incubated together was less than that to be expected if an independent, concomitant hydrolysis of the two substrates had occurred. A possible explanation of this result is that there is competition between the two substrates for two catalytic sites. If this were the case, the following relationship would hold:

$$v = \frac{S_x \cdot V_x}{K_{m_x}(1 + S_y/K_{i_y}) + S_x} + \frac{S_y \cdot V_y}{K_{m_y}(1 + S_x/K_{i_x}) + S_y} \quad (1)$$

in which v, V, K_m, K_i and S have their usual meanings, and the subscripts x and y refer to the substrates *p*-nitrophenyl-α-galactoside and *p*-nitrophenyl-*N*-acetyl-α-galactosaminide, respectively.

However, if there is one catalytic site,

$$K_{i_y} = K_{m_y} \quad (2)$$

TABLE II

EFFECT OF HEATING AT 50°C ON ACTIVITY OF α-GALACTOSIDASE B WITH *p*-NITROPHENYL-α-D-GALACTOSIDE AND *p*-NITROPHENYL-*N*-ACETYL-α-GALACTOSAMINIDE AS SUBSTRATES

For conditions, see text. The concentration of *p*-nitrophenyl-*N*-acetyl-α-galactosaminide was 0.525 mM.

Expt.	Time of heating (min)	Percentage of initial activity with	
		p-Nitrophenyl-α-galactoside	p-Nitrophenyl-N-acetyl-α-galactosaminide
1	0	100	100
	20	90	89
	60	85	85
	120	73	75
2	250	65	67

TABLE III

α-GALACTOSIDASE AND *N*-ACETYL-α-GALACTOSAMINIDASE ACTIVITY OF AN α-GALACTOSIDASE B PREPARATION

The α-galactosidase B preparation was incubated with 12 mM *p*-nitrophenyl-α-galactoside or 0.175 mM *p*-nitrophenyl-*N*-acetyl-α-galactosaminide or both substrates together as described in Materials and Methods.

Substrate	Rate of p-nitrophenol formation (nmol/min)	
	Observed	Calculated *
p-Nitrophenyl-α-galactoside (x)	1.14	—
p-Nitrophenyl-*N*-acetyl-α-galactosaminide (y)	0.90	—
$x + y$	1.61	1.58

* The calculated rate of *p*-nitrophenol formation in the presence of both substrates together was obtained by assuming that (a) there is competition between the two substrates for the same site, (b) the K_i of *p*-nitrophenyl-α-galactoside in inhibiting *p*-nitrophenyl-*N*-acetyl-α-galactosaminidase = K_m for *p*-nitrophenyl-α-galactoside when this substrate is incubated alone (= 19.6 mM), (c) the K_i for *p*-nitrophenyl-*N*-acetyl-α-galactosaminide in inhibiting *p*-nitrophenyl-α-galactosidase = K_m for *p*-nitrophenyl-*N*-acetyl-α-galactosaminide when this substrate is incubated alone (= 1.07 mM).

and

$$K_{i_x} = K_{m_x}. \tag{3}$$

Substituting Eqns. 2 and 3 in Eqn. 1:

$$v = \frac{S_x \cdot V_x}{K_{m_x}(1 + S_y/K_{m_y}) + S_x} + \frac{S_y \cdot V_y}{K_{m_y}(1 + S_x/K_{m_x}) + S_y} \tag{4}$$

Using Eqn. 4, it can be calculated that the total *p*-nitrophenol formation should be 1.58 nmol/min. This value is in very good agreement with the observed one of 1.61 nmol/min (Table III). In another experiment, in which a different concentration of *p*-nitrophenyl-*N*-acetyl-α-galactosaminide was used (0.525 mM), the calculated and observed values were 1.89 and 1.94 nmol/min, respectively.

Discussion and Conclusions

The immunological data, the heat inactivation studies and the kinetic analysis suggest that a single enzyme protein is responsible for the α-galactosidase and *N*-acetyl-α-galactosaminidase activities in a purified α-galactosidase B preparation and that a single catalytic site is involved. The instability of the preparation in dilute solutions (see Fig. 1 in ref. 10) is very similar to that described for *N*-acetyl-α-galactosaminidase purified from beef liver (see Fig. 4 of ref. 13).

Furthermore, the modified form of α-galactosidase B formed during storage of the preparation [10] (the A-like α-galactosidase of Romeo et al. [14]) also has *N*-acetyl-α-galactosaminidase activity which reacts with anti-α-galactosidase B (Schram, A.W., Brouwer-Kelder, E.M., Hamers, M.N. and Tager, J.M., in preparation).

Since the maximal activity with *p*-nitrophenyl-*N*-acetyl-α-galactosaminide is greater than that with *p*-nitrophenyl-α-galactoside and since the affinity for the former substrate is greater than that for the latter, we suggest that α-galactosi-

dase B is in reality a *N*-acetyl-α-galactosaminidase. In this respect it should be borne in mind that the preparation has only a low activity with ceramide-3, the major natural substrate containing an α-galactosidic linkage [4].

Several glycoproteins, including blood group A substance, contain *N*-acetylgalactosamine linked by an α-glycosidic bond (see ref. 15). Thus the natural substrates for *N*-acetyl-α-galactosaminidase can be expected to be intermediates in the catabolism of these glycoproteins. Indeed, Mahadevan and Tappel [16] have shown that lysosomal *N*-acetyl-α-galactosaminidase releases *N*-acetylgalactosamine from sialic acid-free ovine summaxillary gland glycoprotein.

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