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INHIBITION OF HUMAN LIVER β-GALACTOSIDASES AND β-GLUCOSIDASE BY *N*-BROMOACETYL-β-D-GALACTOSYLAMINE

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Summary

N-Bromoacetyl-β-D-galactosylamine is an irreversible inhibitor of the 'acid' and the 'neutral' β-galactosidases (β-D-galactoside galactohydrolase, EC 3.2.1.23) of human liver. The inactivation of acid β-galactosidase appears to involve a group with a pK_a = 4.5. The inhibition of neutral β-galactosidase only occurs above pH 8.0. Both enzymes are protected against inhibition by the presence of substrates, suggesting that the inhibitor reacts with the active site of the enzymes.

Other lysosomal hydrolases are not inhibited by *N*-bromoacetyl-β-D-galactosylamine, with the exception of 'neutral' β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21). The pH dependence of neutral β-glucosidase inactivation is essentially identical to that of the neutral β-galactosidase. Inhibition of β-glucosidase by this galactose derivative suggests that the same enzyme may bind glucosides and galactosides. Furthermore, both neutral β-galactosidase and β-glucosidase are inactivated at 52°C with a half-life of 7.5 min. The presence of a single enzyme with both β-glucosidase and β-galactosidase activities is also supported by mixed-substrate experiments.

Introduction

Human liver contains two distinct β-galactosidases (β-D-galactoside + H_2O ⇌ alcohol + D-galactose, β-D-galactoside galactohydrolase, EC 3.2.1.23), an 'acid' β-galactosidase with optimal activity at pH 4.0 and a 'neutral' β-galactosidase with an optimal activity between pH 6 and 7. Antiserum prepared against purified acid β-galactosidase does not cross-react with the neutral β-galactosidase [1—3]. The two enzymes are genetically unrelated, since patients with GM_1 gangliosidosis are deficient in acid β-galactosidase but do not have reduced levels of neutral β-galactosidase [2,4]. In this report we compare the mechanism of inhibition of these two enzymes by the active-site directed re-

agent *N*-bromoacetyl-β-D-galactosylamine. This inhibitor is known to alkylate a methionine residue near the active site of *Escherichia coli* β-galactosidase [5].

Human tissues also contain two distinct β-glucosidases (β-D-glucoside + H_2O = alcohol + D-glucose, β-D-glucoside glucohydrolase, EC 3.2.1.21), an acid β-glucosidase with optimal activity at pH 4.0 and a neutral β-glucosidase with a higher pH optimum [6]. The two β-glucosidases can be separated by cellulose acetate electrophoresis [7]. We have obtained results which suggest that the neutral β-galactosidase and neutral β-glucosidase activities may reside in the same enzyme. Preliminary reports of our results have appeared [1,8].

Materials and Methods

N-bromoacetyl-β-D-galactosylamine was synthesized according to a published method [9] by Pharm-Eco, Inc., Simi Valley, Calif., 93065. The melting point (191—192°C decomposition) and optical rotation of the product ($[\alpha]_{25}^{D}$ = +13.4) were in good agreement with reported values. (Found: C, 31.9; H, 4.7; N, 4.9; Br, 26.5. Calculated for $C_8H_{14}NO_6Br$: C, 32.0; H, 4.7; N, 4.7; Br, 26.6).

β-Galactosidase and β-glucosidase were assayed with 5 mM concentrations of *p*-nitrophenyl-β-D-galactoside or *p*-nitrophenyl-β-D-glucoside (Sigma) as previously described [10]. Assays were performed in Buffer A (0.1 M acetate, pH 5.0 containing 0.1 M NaCl) for the acid β-galactosidase, and in Buffer B (0.05 M phosphate, pH 6.6 containing 0.14 M NaCl) for the neutral β-galactosidase. One unit of β-galactosidase catalyzes the hydrolysis of one μmol of *p*-nitrophenyl-β-D-galactoside per h.

Separate preparations of acid and neutral β-galactosidase were employed in this study. Acid β-galactosidase was purified 300-fold from normal human liver (spec. act. 75 units/mg protein) [2,10,11]. Neutral β-galactosidase was obtained from a previously described patient with GM_1 gangliosidosis, who carried a defective structural gene for acid β-galactosidase [2]. 5 g of liver was homogenized with a Polytron Homogenizer in 9 vol. of 0.25 M sucrose buffered with 0.05 M Tris, pH 7.5. The supernate obtained by centrifugation of the homogenate at 78 000 × *g* for 75 min is referred to in the text as Extract I. The same procedure was employed to prepare an extract from normal human liver (Extract II). Interaction with *N*-bromoacetyl-β-D-galactosylamine is not affected by the degree of purification of the enzyme (Meisler, M., unpublished).

For studies of enzyme inhibition by *N*-bromoacetyl-β-D-galactosylamine, the enzyme preparations were incubated with a large excess of the inhibitor at 37°C in a total volume of 0.1 ml. The inactivation reaction was stopped by dilution with 0.9 ml of cold assay buffer. Residual enzyme activity was then assayed. Controls were preincubated under identical conditions without the inhibitor. The residual activities are expressed as % of activity in control tubes to compensate for losses of enzyme activity during incubation. Continued inhibition of residual β-galactosidase during the assay period was negligible as a result of the ten-fold dilution of inhibitor and the presence of substrate. The buffers for studies of pH dependence of inactivation were: pH 4.0—7.2, ci-

trate/phosphate buffer [12]; pH 7.5—8.5, 0.05 M Tris · HCl; pH 8.7—9.5, 0.1 M glycine/NaOH.

Fluorescence of 4-methylumbelliferone was measured with a G.K. Turner fluorimeter using filter 6-70 for the exciting radiation and filters 4B and Wratten ND 2.00 for the emitted light.

Results

The inhibition of human acid β-galactosidase by excess *N*-bromoacetyl-β-D-galactosylamine appears to proceed by a first-order reaction (Fig. 1). The rate of inactivation is dependent upon the concentration of the inhibitor. From the half-life of enzyme activity in the presence of varying concentrations of *N*-bromoacetyl-β-D-galactosylamine, we calculated the apparent rate constants of inactivation which are plotted in Fig. 2. At high concentrations of inhibitor, saturation kinetics are observed, suggesting an initial reversible binding of inhibitor to the active site prior to inactivation. From the double reciprocal plot, we determined that the K_I for the reversible phase of the inhibition is approximately 6.3 mM. This is ten-fold higher than the K_M for *p*-nitrophenyl-β-D-galactoside [10]. 5 mM *p*-nitrophenyl-β-D-galactoside is sufficient to prevent inactivation of the enzyme by 5 mM *N*-bromoacetyl-β-D-galactosylamine. The inhibition of acid β-galactosidase was irreversible in our experience; we were not able to reactivate the inhibited enzyme by dialysis for up to 48 h against buffers containing mercaptoethanol or dithiothreitol under conditions which reactivate *E. coli* β-galactosidase [5].

The effect of pH on the rate constant of inhibition of acid β-galactosidase

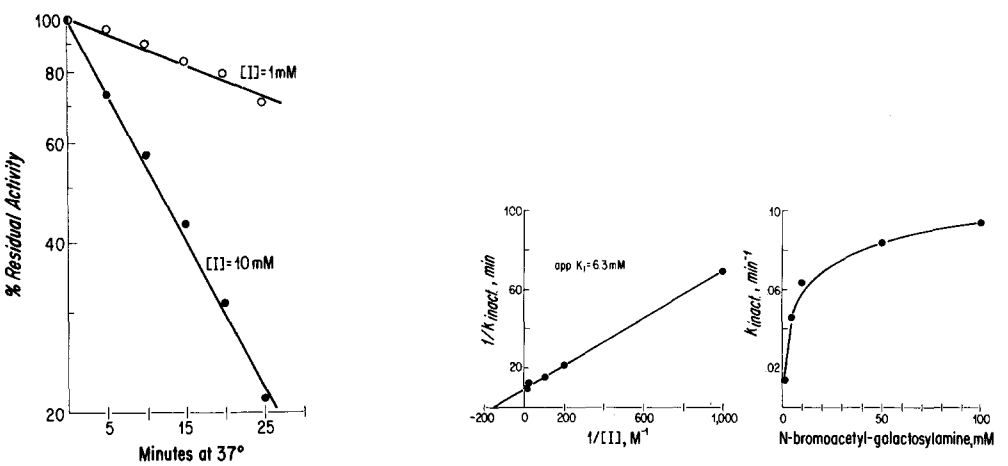

Fig. 1. Inhibition of acid β-galactosidase by *N*-bromoacetyl-β-D-galactosylamine. One-tenth unit of purified enzyme was incubated with the indicated concentration of *N*-bromoacetyl-β-D-galactosylamine in 0.1 ml of Buffer A (pH 5.0) for the indicated time. Residual enzyme activity was then determined in the same buffer as described in Methods.

Fig. 2. Effect of *N*-bromoacetyl-β-D-galactosylamine concentration on the rate of inactivation of acid β-galactosidase. The apparent rate constants of inactivation at each concentration of *N*-bromoacetyl-β-D-galactosylamine were calculated from the half-life of the enzyme determined as in Fig. 1.

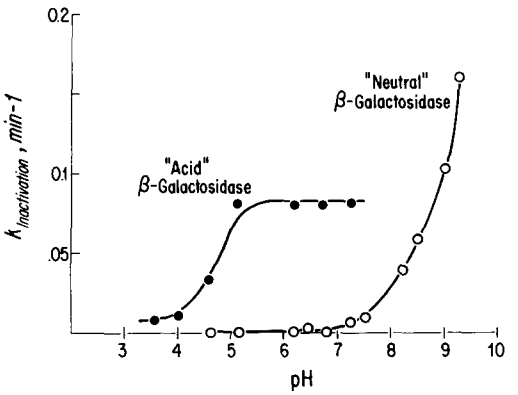

Fig. 3. pH Dependence of inactivation by *N*-bromoacetyl-β-D-galactosylamine. One-tenth unit of each enzyme was incubated in 0.1 ml of buffer (see Methods) containing 60 mM of the inhibitor. Tubes were incubated at 37°C for various time periods as in Fig. 1. Residual enzyme activity was then determined. Apparent rate constants of inactivation were calculated from the half-life of the enzyme at each pH. In this experiment neutral β-galactoside was assayed in Extract I. The same pH dependence was observed for neutral β-galactosidase in Extract II.

is presented in Fig. 3. There is a sharp increase in the rate of inactivation of acid β-galactosidase between pH 4 and 5, which appears to depend upon dissociation of a group with $pK_a = 4.5$.

The neutral β-galactosidase of human liver is also inactivated by *N*-bromoacetyl-β-D-galactosylamine, but only at pH values above 8.0 (Fig. 3). The difference in reaction with inhibitor suggests a basic difference in amino acid residues at the active sites of the two enzymes.

To determine the specificity of the inhibitor, we examined its effects on other liver glycosidases (Fig. 4A). β-D-Glucuronidase and *N*-acetyl-β-D-hexosaminidase were not inhibited by *N*-bromoacetyl-β-D-galactosylamine, but β-glucosidase activity was strongly inhibited. Neutral β-glucosidase is only inhibited in alkaline buffers above pH 8.0 (Fig. 4B).

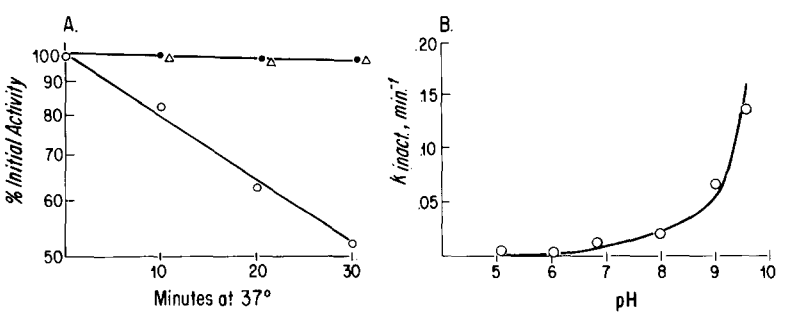

Fig. 4. (A) Specificity of glycosidase inactivation by *N*-bromoacetyl-β-D-galactosylamine. Tubes containing 50 μl of Extract II were incubated for the indicated time with 60 mM *N*-bromoacetyl-β-D-galactosylamine in 0.05 M Tris buffer, pH 8.2. Residual enzyme activities were then assayed in Buffer A. β-Glucosidase (○), β-glucuronidase (△), *N*-acetyl-β-hexosaminidase (●). (B) pH Dependence of inactivation of neutral β-glucosidase by *N*-bromoacetyl-β-D-glucosylamine. Tubes containing 50 μl of Extract I were incubated with 60 mM *N*-bromoacetyl-β-D-glucosylamine at the indicated values of pH. Residual β-glucosidase activity was then assayed in Buffer B, pH 6.6. Apparent rate constants were determined from pseudo-first-order plots as shown in Fig. 1.

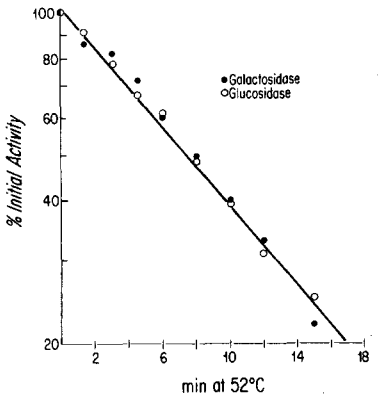

Fig. 5. Thermal inactivation of neutral β-galactosidase and neutral β-glucosidase. Tenth-ml aliquots of Extract I were incubated at 52°C in Buffer B, pH 6.6, for the indicated times. Residual β-galactosidase and β-glucosidase activities were assayed in Buffer B.

The striking similarity in pH dependence of inhibition of neutral β-glucosidase and neutral β-galactosidase suggests that the two activities might reside in the same enzyme. This hypothesis was tested with two additional experiments. The rate of inactivation at 52°C of neutral β-glucosidase and neutral β-galactosi-

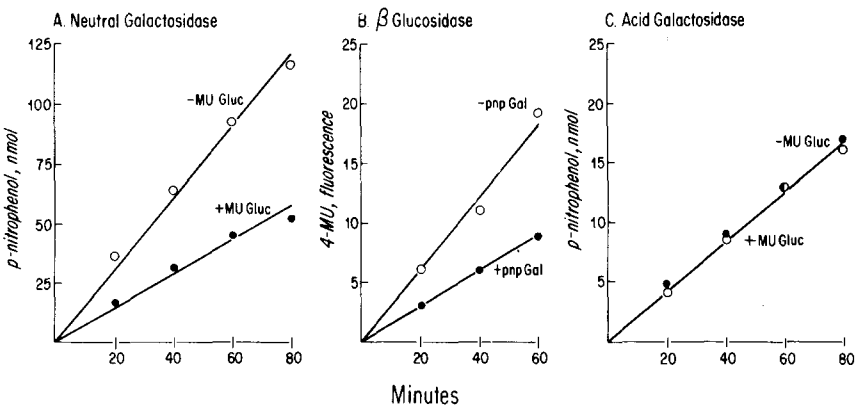

Fig. 6. Mixed-substrate experiments. (A) Neutral β-galactosidase was inhibited by 4-methylumbelliferyl-β-D-glucoside. Assays contained 50 μl of Extract I and 5 μmol of *p*-nitrophenyl-β-D-galactoside in 1 ml of Buffer B, pH 6.6 (○). 2 μmol of 4-methylumbelliferyl-β-D-glucoside were added to some tubes (●). Tubes were incubated for the indicated time at 37°C and the release of *p*-nitrophenol was determined by measurement of absorbance at 415 nm (Methods). (B) Neutral β-glucosidase is inhibited by *p*-nitrophenyl-β-D-galactoside. Assay tubes contained 25 μl of Extract I and 2 μmol of 4-methylumbelliferyl-β-D-glucoside in 1 ml of Buffer B, pH 6.6 (○). To some tubes, 2 μmol of *p*-nitrophenyl-β-D-galactoside were added (●). After incubation at 37°C for the indicated time, the fluorescence due to released 4-methylumbelliferone was measured (Methods). Prior to measurement of fluorescence, 2 μmol of *p*-nitrophenyl-β-D-galactoside were added to the tubes which did not already contain it, so that the quenching of fluorescence was comparable in all tubes. (C) Acid β-galactosidase is not inhibited by 4-methylumbelliferyl-β-D-glucoside. Tubes contained 0.1 unit of purified acid β-galactosidase and 5 μmol of *p*-nitrophenyl-β-D-galactoside in 1 ml of Buffer A (○). Some tubes also contained two μmol of 4-methylumbelliferyl-β-D-glucoside (●). After incubation of the mixtures at 37°C for the indicated time, the release of *p*-nitrophenol was determined. MU gluc, 4-methylumbelliferyl-β-D-glucoside; 4-MU, 4-methylumbelliferone; pnp Gal, *p*-nitrophenyl-β-D-galactoside.

dase is identical (Fig. 5). This similarity in stabilities is consistent with the hypothesis that a single enzyme is responsible for both activities. The results of a mixed-substrate experiment [13] are presented in Fig. 6. The presence of 4-methylumbelliferyl β-D-glucoside inhibits the hydrolysis of *p*-nitrophenyl-β-D-galactoside by a liver extract at pH 6.6 (Fig. 6A). The presence of *p*-nitrophenyl-β-D-galactoside inhibits the hydrolysis of 4-methylumbelliferyl-β-D-glucoside by the same extract (Fig. 6B). These results again suggest that the galactoside and glucoside are competing for binding at a single active site. As a control, we examined the effect of 4-methylumbelliferyl-β-D-glucoside on a β-galactosidase which does not hydrolyze glucosides [10]: purified acid β-galactosidase is not affected by the presence of the β-glucoside (Fig. 6C).

Discussion

The inhibition of mammalian β-galactosidases by *N*-bromoacetyl-β-D-galactosylamine is similar to the inhibition of the enzyme from *E. coli* [5], in that both are apparent first-order reactions which exhibit saturation kinetics. The affinity of the mammalian acid β-galactosidase for *N*-bromoacetyl-β-D-galactosylamine (K_I = 6 mM) is a little lower than that reported for the *E. coli* enzyme (K_I = 1 mM). The rate constants of inactivation are comparable in magnitude (maximum observed rate ≈ 0.2 min^{-1}). The inhibition of *E. coli* β-galactoside by *N*-bromoacetyl-β-D-galactosylamine is the result of alkylation of a methionine residue near the active site of the enzyme [5]. The protection of both human β-galactosidases by their substrates, and the saturation kinetics of the inhibition reaction, indicate that *N*-bromoacetyl-β-D-galactosylamine binds to the active sites of these enzymes.

Studies of pH dependence revealed a difference in the inhibition of the two human β-galactosidases by *N*-bromoacetyl-β-D-galactosylamine. Inhibition of acid β-galactosidase appears to require dissociation of a group with pK_a = pH 4.5. In contrast, inhibition of neutral β-galactosidase only proceeds at alkaline pH (>8.0) and closely resembles the pH dependence of inactivation of *E. coli* β-galactosidase. The characteristics of *N*-bromoacetyl-β-D-galactosylamine inhibition of several β-galactosidases are summarized in Table I. These enzymes

TABLE I

TWO CLASSES OF β-GLYCOSIDASES

Type	Example	Optimal pH for enzyme activity	pK_a of group modified by *N*-bromoacetyl-β-D-galactosylamine (pH)
I	Human acid β-galactosidase	~4	4.5*
	Mouse acid β-galactosidase	~4	4.5**
II	*E. coli* β-galactosidase	~7.5	>8.0***
	Human neutral β-galactosidase	~6—7	>8.0*
	Human neutral β-glucosidase	~6—7	>8.0*

* This paper.
** Meisler, M., unpublished.
*** Ref. 5.

appear to fall into two classes which presumably differ in the amino acid groups involved in catalysis.

N-bromoacetyl-β-D-galactosylamine inactivation of the first type (pK_a = 4.5) could result from alkylation of a carboxylate ion. Alternatively, the dissociation of a carboxyl group may produce a conformational change which permits binding of *N*-bromoacetyl-β-D-galactosylamine to these enzymes. Involvement of a carboxyl group in the hydrolysis of glycosides has been suggested previously, although the mechanism is not well understood [14]. Radiolabelled *N*-bromoacetyl-β-D-galactosylamine should be a useful new reagent for identification of the amino acid residues involved in catalysis by the two types of mammalian glycosidases.

It has been observed by others that β-glucosidase activity of human liver resembles the neutral β-galactosidase in its pH optimum, and in pattern of elution from Sephadex [15]. We have presented three lines of evidence which support the suggestion that the neutral galactosidase may also catalyze the hydrolysis of β-glucosides. (1) Neutral β-glucosidase is inhibited by the galactose derivative *N*-bromoacetyl-β-D-galactosylamine. The pH dependence of this inactivation is identical to that of the neutral β-galactosidase. (2) Neutral β-glucosidase and neutral β-galactosidase activities of human liver extracts have the same rates of thermal inactivation. (3) In mixed-substrate experiments, each activity is inhibited by the substrate of the other. Taken together, these results strongly indicate that a single enzyme in human liver can hydrolyze both β-glucosides and β-galactosides. In contrast, there is no β-glucosidase activity associated with acid β-galactosidase purified from human liver [10] or from mouse liver [16]. Unequivocal evidence that the neutral β-galactosidase does hydrolyze β-glucosides will depend upon purification of the enzyme.

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References

1 Meisler, M. (1974) Fed. Proc. 33, 1381
2 Meisler, M. and Rattazzi, M. (1974) Am. J. Hum. Genet. 26, 683—691
3 Norden, A.G.W., Tennant, L.T. and O'Brien, J.S. (1974) J. Biol. Chem. 249, 7969—7976
4 Saifer, A. (1975) in The Gangliosidoses (Volk, B.W. and Schneck, L., eds), pp. 53—116, Plenum Press, New York
5 Naider, F., Bohak, Z. and Yariv, J. (1972) Biochemistry 11, 3202—3208
6 Beutler, E. and Kuhl, W. (1970) Lancet 1, 612—613
7 Dreyfus, J.C. and Alexandre, Y. (1972) Biochem. Biophys. Res. Commun. 48, 914—920
8 Meisler, M. (1975) Am. J. Hum. Genet. 27, abstract in press
9 Thomas, E.W. (1970) J. Med. Chem. 13, 755—756
10 Meisler, M. (1972) Methods in Enzymology (Ginsberg, V., ed.), Vol. 28, pp. 820—824, Academic Press, New York
11 Tanaka, H., Meisler, M. and Suzuki, K. (1975) Biochim. Biophys. Acta, in press
12 Gomori, G. (1955) Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds), Vol. 1, p. 141, Academic Press, New York
13 Dixon, M. and Webb, E.C. (1964) Enzymes, pp. 84—87, Academic Press, New York
14 Legler, G. (1973) Mol. Cell. Biochem. 2, 31—38
15 Hultberg, B. and Ockerman, P.A. (1970) Clin. Chim. Acta 28, 169—174
16 Tomino, S. and Meisler, M. (1975) J. Biol. Chem. 250, in press