Carbohydrate Research, 250 (1993) 93–100
Elsevier Science Publishers B.V., Amsterdam

N-Substituted (β-D-galactopyranosylmethyl)amines as reversible inhibitors of β-D-galactosidase †

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(Received November 23rd, 1992; accepted in revised form June 14th, 1993)

ABSTRACT

Through the use of a series of *N*-substituted (β-D-galactopyranosylmethyl)amines (βGal-CH_2NHR) and the corresponding *N*-substituted *C*-(β-D-galactopyranosyl)formamides (βGal-CO-NHR), it has been determined that the inhibitor binding constant is influenced more by the pK_a of the amine group than by the nature of the hydrophobic group. It is concluded that the compounds that are the best inhibitors are those which allow proton donation in the active site to form an ion pair (βGal-CH_2NHR + AH → βGal-$CH_2NH_2R^+ + A^-$).

INTRODUCTION

Both reversible and irreversible enzyme inhibitors with high specificity, activity, and stability can be valuable for determinations of enzyme location, function, and turnover time, and for the identification of the enzyme's natural substrate(s), the function of the product(s), the structures of active-sites, catalytic mechanisms, and substrate structure specificities. Among the known classes of effective reversible glycosidase inhibitors are glycals, lactones, thioglycosides, and glycosylamines. As each class of inhibitor reveals information about the active site and catalytic mechanism (Fig. 1), new inhibitors may be envisioned. On this basis, a new class of stable, reversible inhibitors of β-D-galactosidases was designed and made[2]. The objective was to determine the relative inhibitor activities towards β-D-galactosidase of a number of *N*-substituted (β-D-galactopyranosylmethyl)amines. It was expected that protonation of the amine at the solvent-free active site would result

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† Journal paper No. 12434 of the Purdue University Agricultural Experiment Station.

SSDI 0008-6215(93)E0199-B

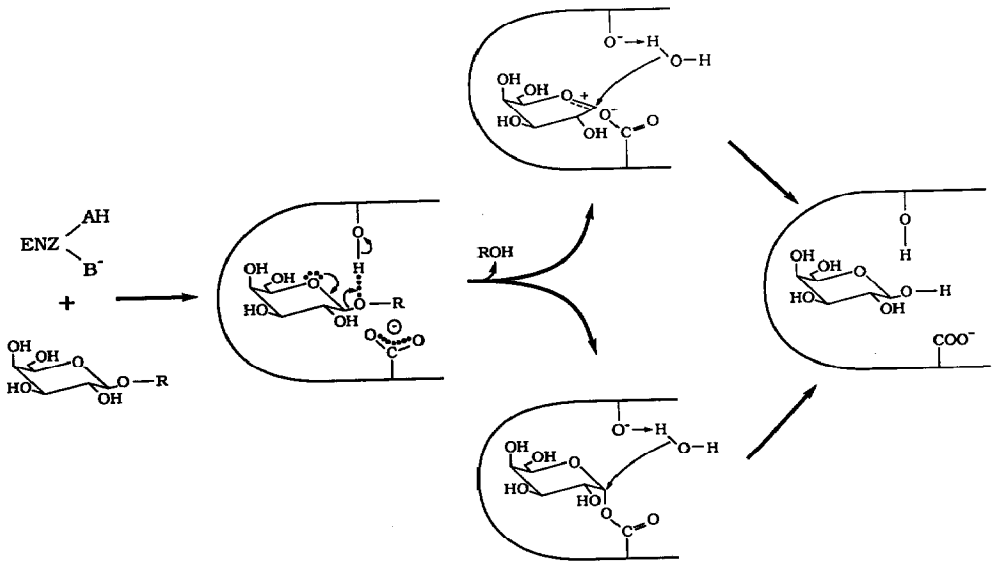

Fig. 1. Alternative proposed mechanisms of action of a retaining glycosidase, specifically *E. coli lacZ* β-D-galactosidase[1].

in tight binding, as happens with the corresponding glycosylamines, but without the subsequent hydrolysis.

RESULTS AND DISCUSSION

Using the Beer–Lambert Law, the extinction coefficient of *o*-nitrophenol was determined to be 3973 M^{-1} cm^{-1} in sodium phosphate buffer, pH 7.3.

Plots of initial velocity (v_i) vs. enzyme concentration were linear over the range of concentrations used (1.98–19.8 μg/mL).

The selection of which *N*-substituted *C*-(β-D-galactopyranosylmethyl)amines (**1**) to prepare was made to give products with a range of pK_a values in order to study the effect of pK_a on binding and inhibition. The pK_a values of the primary and secondary (glycosylmethyl)amines were consistently 1.0–1.5 units lower than those of the corresponding parent amines (Table I).

In order to vary pK_a, the *N*-substituent of the secondary (glycosylmethyl)amine must be changed; however, when this is done, the hydrophobicity : hydrophilicity ratio of the aglycon is also changed. Because the binding site of glycosidases contains a hydrophobic pocket and glycosides with hydrophobic aglycons demonstrate enhanced binding, control compounds that could be used with each potential inhibitor were needed to determine the separate contributions of the hydrophobic nature of the *N*-substituent and protonation of the amine to the strength of binding. The control compound must be basic, so as to eliminate the effect of the other variable, viz., the pK_a of the secondary (glycosylmethyl)amine,

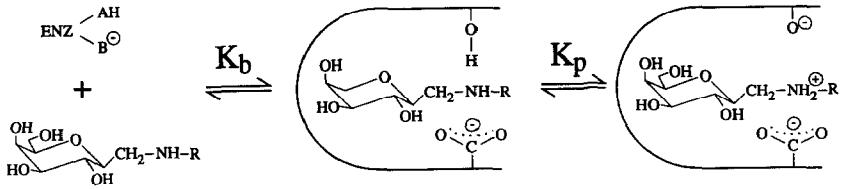

Fig. 2. Proposed mechanism of reversible binding of (β-D-glactopyranosylmethyl)amines to *E. coli lacZ* β-galactosidase: K_b, binding constant; K_p, protonation constant.

and it must have the same anomeric configuration. An oxygen or sulfur atom could be used in place of the nitrogen atom in the corresponding amine, but ethers and thioethers might also undergo protonation. Therefore, it was decided to use the corresponding amides [*N*-substituted *C*-(β-D-galactopyranosyl)formamides] (**2**) as control compounds to factor out the contribution to binding of the hydrophobic substituent on the nitrogen atom.

Each of the (β-D-galactopyranosylmethyl)amines was found to be a reversible competitive inhibitor of *E. coli lacZ* β-D-galactosidase (Table II, column IV). The binding constants of all *C*-(β-D-glactopyranosyl)formamides fell within a factor of 30 of each other and the K_m of the natural substrae, lactose (10 mM). These data indicate that the binding site accepts *C*-glycosyl compounds equally well as it does glycosides and that the carbonyl group does not interfere significantly with binding. The K_i values also increased with decreasing hydrophobicity, reaffirming that the aglycon binding site readily accepts hydrophobic groups and that hydrophobic groups actually assist binding.

The binding constants of the (β-D-galactopyranosylmethyl)amines covered a wider range. Dividing the K_i values of the β-D-(galactosylmethyl)formamides by the K_i values of the corresponding (galactosylmethyl)amines factored out the hydrophobic contribution to binding. The (β-D-galactopyranosylmethyl)amines bound up to 711-times more tightly than did their formamide counterparts (Table II, column VI).

TABLE I

Comparison of dissociation constants of protonated *N*-substituted (β-D-galactopyranosylmethyl)amines (**1**) with those of the parent amines

Parent amines		β-D-Galactopyranosylmethyl derivatives		
Compound	pK_a	Compound [a]	pK_a	ΔpK_a
N-Methylcyclohexylamine	10.6	**1a**	9.7	0.9
Methylamine	10.6	**1b**	9.1	1.5
N-Methylbenzylamine	9.6	**1c**	8.3	1.3
N-Methylmorpholine	7.4	**1d**	6.1	1.3
N-Methyl-2,2,2-trifluoroethylamine	6.0	**1f**	4.7	1.3
N-Methylaniline	4.9	**1g**	4.0	0.9

[a] See Table II for structure of R-group.

TABLE II

Reversible inhibition of *E. coli lacZ* β-D-galactosidase at pH 7.3 by *N*-substituted (β-D-galactopyranosylmethyl)amines (**1**), the corresponding formamides (**2**), and related compounds (**3**)

I Compound	II R group	III pK_a	IV K_i (μM)	V K_i^* (μM) [a]	VI $\frac{K_i(2)}{K_i(1)}$	VII $\frac{K_i(2)}{K_i^*(1)}$
1a	$-CH_2NHC_6H_{11}$	9.7	54	0.2	52	12,990
1b	$-CH_2NH_2$	9.1	505	7.8	20	1250
1c	$-CH_2NHCH_2Ph$	8.3	2.3	0.2	357	4150
1d	$-CH_2NHCH_2CH_2OMe$	8.0	19	3.1	711	4290
1e	$-CH_2N\square O$	6.1	497	467	~1	~1
1f	$-CH_2NHCH_2CF_3$	4.7	1579	1579	2.0	2
1g	$-CH_2NHPh$	4.0	171	171	11	11
2a	$-CONHC_6H_{11}$		2780			
2b	$-CONH_2$		9850			
2c	$-CONHCH_2Ph$		821			
2d	$-CONHCH_2CH_2OMe$		13299			
2e	$-CON\square O$		433			
2f	$-CONHCH_2CF_3$		3118			
2g	$-CONHPh$		1830			
2h	$-CONHPhNO_2(p)$		1637			
3a	$-CH_2OH$		10590			
3b	$-CONHNH_2$		16550			
3c	$-CH_2NHCOCH_3$		3320			
3d	$-CH_2NHCOPh$		3812			
3e	$-CO_2CH_3$		2560			
3f	$-CO_2^- Na^+$		> 50000			
3g	$-CN$		2010			
3h	$-CH_2CH{=}CH_2$		8089			
3i	$-CH_2CH_2CH_3$		4765			

[a] The K_i^* calculation is based on the concentration of the free base species. The cation is assumed to be non-inhibitory.

Amines with lower pK_a values (**1e–g**) had binding constants similar to those of the corresponding amides (**2e** and **2g**). Compounds **1c** and **1d** bound much more tightly than did their amide counterparts (**2c** and **2d**). Compounds **1a** and **1b** were already highly protonated in the pH 7.3 buffer, so little free base was present for ion pair formation. The relative effects of pK_a and hydrophobicity were examined by calculating inhibition constants from the concentration of free base present in the pH 7.3 buffer (K_i^*) under the assumption that the cation is non-inhibitory (Table II, column V). The free base form of compounds (**1a–d**) with pK_a values greater than the pH of the buffer exhibited enhanced binding when compared to the experimental binding constants determined on a mixture of protonated and unprotonated forms.

A comparison of the K_i values of the *C*-(β-D-galactopyranosyl)formamides (**2**) to the K_i values of the corresponding unprotonated amines (Table II, column VI) showed strikingly that the basic methylamines in the free-base form were better

TABLE III

Effect of pH on the inhibition of *E. coli lacZ* β-D-galactosidase by *C*-(β-D-galactopyranosylmethyl)amine (**1b**), pK_a 9.1

pH	K_i (μM)	K_i* (μM) [a]
8.0	119	8.8
7.3	505	7.8
6.0	>11000	8.7

[a] K_i* calculated from the concentration of free base species. The cation is assumed to be non-inhibitory.

inhibitors than their formamide counterparts. Unprotonated **1a** was the best inhibitor when the contribution of the hydrophobic group was factored out.

The K_i values of the neutral hydrophobic compounds **3h** and **3i** were of the same order of magnitude as those for the *C*-(glycosyl)formamides (**2**) (Table II). Compounds **3b–e** were used to determine the contribution of the carbonyl group to the binding of the formamides. Compound **3b** had a basic (protonated) group and thus did not bind well, as described above. Compound **3d** is neutral and a structural isomer of **2c**, with the carbonyl group on the opposite side of the –NH– group. Compounds **3c** and **3e** are also neutral. Compound **3a** is neutral, but contains an sp^3 rather than an sp^2 hybridized carbon atom. Their binding constants were also similar. The binding constant of **3g** suggests that a neutral sp^1 hybridized carbon atom does not affect binding at the active site. Compounds **2b**, **3a**, **3c–e** and **3g–i** bound in the 0.2–10.0 mM range, which is about the same range as the binding constants for natural substrates of *E. coli lacZ* β-D-galactosidase (0.5–10.0 mM). Apparently, the presence of a carbonyl group or other carbon atom configurations neither hindered nor enhanced binding.

The effect of inhibitor charge on the aglycon binding site was also examined by determining binding constants for compound **1b** in 0.1 M phosphate buffers, pH 6.0 and 8.0 (Table III). The K_i was found to be 119 ± 33.8 μM at pH 8.0, less than one-fourth the K_i at pH 7.3. At pH 6.0, the K_i was estimated to be greater than 11 mM, over twenty times larger than the K_i at pH 7.3. From this information, it can be affirmed that, when the amine is protonated, it is not a good competitive inhibitor of *E. coli lacZ* β-D-glactosidase, perhaps either because a proton must be donated within the binding site ($AH \rightarrow A^-$) in order for a stabilizing ion pair to be formed, or because of a barrier to the approach of a positively charged species. However, the large binding constant of **3f**, an anion, demonstrates the low affinity of this inhibitor for the *E. coli lacZ* enzyme, suggesting that there are no positively charged species at the binding site with which the anion may form an ion pair and, therefore, that the first explanation might be the correct one.

While ion pair formation is, in our opinion, the most logical explanation of the observations, there could be other processes involved. It is logical to conclude, for example, that amines with lower pK_a values (**1e** and **1f**) had binding constants similar to those of the corresponding amides (**2e** and **2f**) because they were not

basic enough to be protonated by the enzyme and thus did not form ion pairs, and that compounds **1c** and **1d** bound much more tightly than did their amide counterparts (**2c** and **2d**) because of good ion pair formation; but hydrogen bonding cannot be ruled out.

The pK_a of the proton-donating group in the active site of *E. coli lacZ* β-D-galactosidase (the hydroxyl group of a tyrosine residue) has been determined[3,4] to be 8.5.

Several conclusions and inferences can be drawn from these data: (*a*) all (glycosylmethyl)amines that are basic enough to be protonated by the proton-donating group in the environment of the catalytic site bind more tightly than do their corresponding amides; (*b*) the best competitive inhibition is given by the amine with a pK_a closest to the pK_a of the proton-donating group of the enzyme; (*c*) in general, the more hydrophobic the R group, the tighter the binding, although size and shape also play important roles; (*d*) hydrophobic groups that are six-membered rings bind well in the active site; and (*e*) binding strength is greatest when the derivative has both hydrophobic character and a pK_a value near the pK_a of the proton-donating group, with the latter effect being the predominant one.

These results suggest that, by using *N*-substituted (glycosylmethyl)amines with a range of pK_a values, the pK_a value of the proton-donating group in the active site of glycosidases (glycohydrolases) in its actual environment may be determined. Our results indicate a pK_a closer to 8.3 than to 8.0 or 9.1, agreeing with the pK_a value determined by other means[3,4].

It has been determined that *E. coli lacZ* β-D-galactosidase requires Mg^{2+} ions[5]. Basic glycosylamines bind more tightly to the enzyme than do glycosides, but only in the presence of Mg^{2+} ions[6]. Selwood and Sinnott[7] proposed an electrophilic role for Mg^{2+} and suggested that proton donation follows Mg^{2+} complex formation, at least in hydrolysis of aryl β-galactosides. For this reason, all determinations of reaction rates in this work were done in the presence of 1.0 mM Mg^{2+}.

Compound **2h** was prepared with the expectation that making the carbonyl carbon atom more electropositive might effect attack on it by the catalytic-site nucleophile (presumably a carboxylate anion) and form a relatively stable tetravalent compound. However, no evidence was obtained that this happened. Synthesis of the 2,4-dinitrophenyl amide, which would be even more likely to form a tetravalent compound, could not be accomplished.

EXPERIMENTAL

Enzymes.—*E. coli lacZ* β-D-galactosidase (β-D-galactoside-galactohydrolase; EC 3.2.1.23): Grade X; molecular weight: 540 000; $\epsilon(1\%) = 20.9$ at 280 nm; 37 mg/mL suspension) was obtained from Sigma Chemical Co., St. Louis, Missouri.

Inhibitors.—Preparation of compounds **1**, **2**, and **3** has been described[2]. Compound **1b** was prepared by an improvement of the method of Coxon and Fletcher[2,6].

Compound **3e** was prepared by the method of Fuchs and Lehmann[7]. Compound **3h** was prepared by the method of Tsvetkov et al.[8]

Substrate and other reagents.—*o*-Nitrophenyl β-D-galactopyranoside (ONP-βGal) was purchased from Sigma Chemical Co., St. Louis, Missouri and used as purchased. All buffer solutions were prepared in deionized and glass-distilled water; all pH values were determined at 25°C.

Spectrophotometry.—Spectrophotometric measurements were made on a Beckman Acta MVI or a Varian DMS 80 recording spectrophotometer. Cuvettes were maintained at a temperature of 25.0 ± 0.1°C.

The extinction coefficient of *o*-nitrophenol (ONP) was determined by serially diluting a stock solution (0.72 mM) with 0.1 M sodium phosphate buffer, pH 7.3. Absorbances of the dilutions were determined spectrophotometrically at 410 nm and 25°C.

Initial velocity vs. enzyme concentration (V_i vs. [E]).—A suspension (25.0 μL) of *E. coli lacZ* β-D-galactosidase was added to 5.00 mL of 0.1 M sodium phosphate buffer, pH 7.3. The concentration of this stock solution was determined spectrophotometrically at 280 nm and 25°C. Five concentrations of the enzyme were made by adding appropriate volume of the enzyme stock solution and buffer; final assay volume 2.50 mL. The assay solution in 0.1 M sodium phosphate buffer, pH 7.3, was 0.80 mM ($\sim 10 \times K_m$) in ONP-βGal and 1.0 mM in $MgCl_2$. An aliquot of the enzyme stock solution was added to the assay solution to begin the reaction, which was followed spectrophotometrically by measuring the change in A_{410} at 25°C.

To determine the linearity of v_i vs. [E] plots, initial velocities were determined by drawing a tangent to the slope of the curve made by the recorder. Velocities were expressed as the change in absorbance per minute. The initial velocities were plotted as a function of enzyme concentration with 0.0 min^{-1} (velocity) and [E] = 0.00 M represented as a point. All concentrations of enzymes used subsequently gave velocities that fell within the linear range.

pK_a Determinations.—To determine pK_a values, solutions of (glycosylmethyl)amines (**1a–d**, **1f** and **1g**) were titrated with 100-μL aliquots of 31.80 mM HCl. The solutions were stirred magnetically and kept at a constant 25°C under N_2. Gran plots[9] were used to calculate equivalence points. The pK_a of **1e** was determined directly as the pH of a solution of one-half neutralized substrate.

Reversible inhibition activity assays.—Production of ONP from ONP-βGal (K_m 86.5 ± 2.8 μM; [S] = $\sim 0.5, 1, 2, 4 \times K_m$) in the presence of various concentrations of inhibitor and 1.0 mM $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.3, was monitored spectrophotometrically at 410 nm and 25°C. The reaction was initiated by addition of enzyme ($\sim$ 1.2 μg); total reaction volume 1.0 mL.

Determination of Michaelis–Menton and inhibition constants.—At least four substrate concentrations were utilized to determine K_m. Four concentrations of inhibitor were used with each substrate concentration when determining the

binding constant, K_i, of the inhibitor. The resulting 4×4 matrix was performed in triplicate.

Stock assay solutions were added to cuvettes which were allowed to equilibrate at 25°C in the cell holder for at least 5 min before the enzyme was added. The assay solution was stirred with a battery-operated micro-mixer as the aliquot of the stock enzyme solution was added. The recorder was started immediately, and rate data were obtained from the change in A_{410} vs. time. Initial velocities were determined by drawing a tangent to the slope of the curve. Velocities were expressed as the change in A_{410}/min. Kinetic parameters were calculated by linear least-squares treatment of the resulting data. Control velocities were determined for every evaluation of K_i and K_m at a constant substrate concentration (0.2 mM) in order to normalize the determinations.

The K_i of **1b** was also determined in pH 6.0 and 8.0 sodium phosphate buffers, 0.1 M.

ACKNOWLEDGMENTS

The authors acknowledge the help of J.A. Bohn and V.N. Kalabokis who, along with M.P. Yadav, prepared the *C*-glycosyl compounds[2].

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