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N-SUBSTITUTED D-GALACTOSYLAMINES AS PROBES FOR THE ACTIVE SITE OF β-D-GALACTOSIDASE FROM *Escherichia coli*

GUNTER LEGLER AND MONIKA HERRCHEN

Institut für Biochemie, Universität Köln, Zulpicher Str 47, D-5000 Köln 1 (Federal Republic of Germany)

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ABSTRACT

Basic *N*-substituted β-D-galactosylamines are bound to the active site of β-D-galactosidase from *E. coli* $>10^2$–10^4-fold more tightly than non-basic, neutral or positively charged β-D-galactosyl derivatives of closely related structure. Acid catalysis of O-galactoside hydrolysis and tight binding of basic inhibitors is observed only when Mg^{2+} is bound to the enzyme. Formation of an ion pair by proton transfer from the acidic group required for catalysis to the bound galactosylamine is therefore proposed as the cause of the enhanced affinity. The magnitude of the observed effects indicates strong shielding of the active site from the aqueous environment. The pH-dependence of K_i for 1-D-galactosylpiperidine and D-galactosylbenzene shows that no deprotonation from the active site occurs up to pH 10. The hydrophobic nature of the aglycon site inferred from earlier studies is confirmed by the high affinity of *N*-benzyl- (K_i 9×10^{-9}M) and *N*-heptyl-β-D-galactosylamine (K_i 0.6×10^{-9}M). A model for the active site is discussed, in which the charge of an essential carboxylate group is neutralised by an adjacent cationic group.

INTRODUCTION

Two features of the active site of β-D-galactosidase from *E. coli* are important for catalysis by this enzyme[1]. Firstly, a carboxylate group adjacent, in the ES-complex, to C-1 of the D-galactopyranosyl part of the bound substrate. This group contributes to bond breaking by electrostatically assisting the formation of a galactosyl oxo-carbonium ion that is reversibly stabilised as an α-D-galactosyl ester intermediate (Scheme 1). Hydrolysis or transgalactosylation proceeds by the stereospecific attack of water or alcohol after R-OH has diffused away. Evidence for this carboxylate group is mainly indirect, coming from kinetic[2] and preliminary labelling studies with active-site-directed inhibitors[3,4].

Secondly, an acidic group able to donate a proton to the anomeric oxygen of galactosides. This provides an additional 10^2–10^5-fold acceleration over the rate observed when the leaving group is protonated by water or not all (as in 1-β-D-galactosylpyridinium salts). The correct orientation of the acid with respect to the

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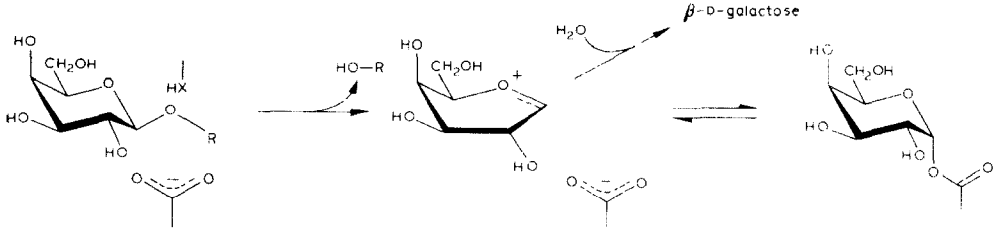

Scheme 1

galactosyl oxygen depends on the binding of Mg^{2+} or Mn^{2+} (1 g-atom/mol of subunit), which is thought to induce a conformational change that brings the acidic group into a favourable position[5].

A conceptual difficulty with this model having a permanent negative charge arises from the finding that galactosyl derivatives having a positively charged atom attached to C-1, *e.g.*, 1-β-D-galactosylpyridinium or β-D-galactosyltrimethylammonium ions, are bound no more tightly than their neutral analogues β-D-galactosylbenzene and methyl 1-thio-β-D-galactopyranoside[6]. This is in marked contrast to the β-D-glucosidase A_3 from *Aspergillus wentii*, where a carboxylate group in a similar position is assumed and where the β-D-glucosyl derivative of the pyridinium ion is bound 320-times more tightly than that of benzene[7]. Since removal of Mg^{2+} from the *E. coli* enzyme causes only a small change in the relative affinities for cationic and neutral ligands[6], the observed lack of an electrostatic binding enhancement cannot be due to neutralisation of the carboxylate charge by the metal ion which is located ~9 Å from the anomeric substituent of the galactosyl ligand[6].

The studies described herein were carried out to learn more about the charge distribution at the active site of β-D-galactosidase from *E. coli* and to obtain experimental evidence on which modifications of the present model could be based. Since glycosylamines and/or glycosylammonium ions are powerful inhibitors for β-D-glycosidases[8,9], we have studied the inhibitory properties of a number of *N*-substituted galactosylamines and compared them with their non-basic analogues.

RESULTS AND DISCUSSION

Stability and anomeric configuration of galactosylamines. — Both α- and β-D-galactosylamine and their *N*-alkyl derivatives are, in contrast to their aryl derivatives, unstable in aqueous solution and may anomerise and undergo hydrolysis[10]. Since there is a difference of ~4 pK_a units between the glycosyl derivative and the parent amine[9], the latter process can be followed by potentiometric titration at constant pH. Optical rotation, on the other hand, will be affected by anomerisation and by hydrolysis.

Titration experiments with the derivatives of benzylamine and piperidine showed that the half-life of each compound is ~20 min at pH 7 and 25°. Since stock solutions of inhibitor were made in non-aqueous solvents or used immediately after

preparation, and since measurements of substrate hydrolysis were made within 3 min after mixing, the effect of inhibitor hydrolysis on the measured rate will be 10% or less.

A more serious error will arise by anomerisation of the inhibitor, which can be very rapid[11], or by the use of inhibitor solutions where the proportion of α and β anomers is unknown. Galactosylamine, isolated as the ammonia complex of the α anomer, is rapidly converted[11] into >95% of the β anomer as pH <8.

N-Benzyl- and *N*-heptyl-D-galactosylamine each crystallised as the β anomer, as judged from the specific rotations in water and *N,N*-dimethylformamide {benzyl, $[\alpha]_{579}^{20}$ −16 (water) and −22° ($HCONMe_2$); heptyl, $[\alpha]_{579}^{20}$ −14 → +50 (water) and −10° ($HCONMe_2$)}.

1-D-Galactosylpiperidine, in contrast to the foregoing primary amine derivatives, mutarotated in *N,N*-dimethylformamide ($[\alpha]_{579}^{20}$ +3 → −26°). A similar mutarotation was observed[21] in methanol ($[\alpha]_{579}^{20}$ +14.5 → −10°). Since the change in molecular rotation (ΔM −7,100) is similar to the difference between α- and β-D-galactosylamine[11] (ΔM −6,000), we assume that the piperidine derivative crystallised as the α anomer and changes largely to the β form in *N,N*-dimethylformamide or methanol. Addition of water to an equilibrated solution in *N,N*-dimethylformamide caused the initial $[\alpha]_{579}$ value to be less negative or even positive. This was followed by a slow, positive mutarotation which we attribute to hydrolysis. With a 9:1 volume ratio for water and *N,N*-dimethylformamide, the initial specific rotation and the mutarotation corresponded to the values found in water ($[\alpha]_{579}$ +28.5 → +57.5°). It is likely that 1-D-galactosylpiperidine is present mainly as the α anomer in aqueous solution, even when originally present as the β anomer.

Inhibition of β-D-galactosidase activity. — The results of the inhibition studies are summarised in Table I. Inhibition was purely competitive in all cases. All inhibitors having a basic nitrogen are bound to the Mg^{2+}-enzyme several orders of magnitude more tightly than their weakly or non-basic analogues. This large difference is observed on comparing the basic inhibitors with their neutral counterparts (galactose and 1-thiogalactosides), a weakly basic analogue (*N-p*-tolyl-β-D-galactosylamine[9], pK_a ~1.5), or a cationic analogue (1-D-galactosylpiperidine *vs.* the pyridinium ion). The hydrophobic character of the aglycon-binding region of the active site indicated by studies with alkyl β-D-galactosides and 1-thio-β-D-galactosides[12] is confirmed by the data for the benzyl and heptyl derivatives.

No inhibition was observed with 100mM β-D-glucosylamine or 8mM nojirimycin (5-amino-5-deoxy-D-glucopyranose). These compounds inhibit β-D-glucosidases with K_i values of ~mM and ~μM, respectively[13]. Since the β-D-galactosidase studied here was not measurably affected, it is concluded that its glycon-binding region can discriminate between galactosides and glucosides better than 5 × 10^4-fold. This is in marked contrast to the 200-fold difference in affinity for *N*-bromoacetyl-D-galactosylamine and -glucosylamine reported by Viratelle *et al.*[14], who proposed a separate glucosyl sub-site in close proximity to the galactosyl site.

A comparison of the inhibition constants for the Mg^{2+}-enzyme with those for the apo-enzyme demonstrates the importance of Mg^{2+} for the strong inhibition by

TABLE I

INHIBITION OF β-D-GALACTOSIDASE BY β-D-GALACTOSYL DERIVATIVES OF DIFFERENT BASICITY AND CHARGE TYPE AT pH 7.0 AND 25° WITH 4-METHYLUMBELLIFERYL β-D-GALACTOSIDE AS SUBSTRATE

Inhibitor	K_i (μM)[a]	K_i (*without* Mg^{2+}) / K_i (*with* Mg^{2+})	*Ref.*[b]
β-D-Gal—NH_2	7	66	
β-D-Gal—NH—CO—CH_2Br	1,100	n.d.[c]	14
β-D-Gal—OH (equilib.)	21,000	n.d.	
β-D-Gal—N⟨ ⟩	9	20	
β-D-Gal—N⁺⟨ ⟩	1,120	2.9	6
β-D-Gal—⟨ ⟩	450	15	6
β-D-Gal—NH—CH_2—⟨ ⟩	0.0095	1,880	
β-D-Gal—NH—⟨ ⟩—Me	760	11	
β-D-Gal—SCH_2—⟨ ⟩	3.3	n.d.	12
β-D-Gal—NH—C_7H_{15}	0.00057	684	
β-D-Gal—SC_7H_{15}	28	n.d.	12

[a]Determined in the presence of mM Mg^{2+}. [b]This work when a reference is not given. [c]Not determined.

the basic compounds, which is in marked contrast to the weak to moderate effect of Mg^{2+}-removal on K_m values[5] or K_i values of non-basic inhibitors[6]. (The comparison of K_m and K_i is justified because the former corresponds closely to the true dissociation constant for the substrates discussed here, since degalactosylation is not rate limiting and k_{cat} is well below the dissociation rate of the ES-complex[2]). The effect of Mg^{2+} is best explained by assuming that the conformation induced by the metal ion facilitates the transfer of a proton to the nitrogen of the inhibitor, as has been postulated for the hydrolysis of galactosides[5]. If the proton is donated by a neutral acid, the resulting formation of an ion pair will provide an additional contribution to the binding energy if it takes place in a non-polar environment. The importance of a correct orientation of the basic group in the inhibitor for optimal interaction is demonstrated by the inhibition constant for 2-amino-2-deoxy-D-galactose which is 140-times the value for D-galactosylamine.

With 1-D-galactosylpiperidine, the enhancement of binding by its basic character and Mg^{2+} appears to be lowest in spite of its being the most basic inhibitor tested. We attribute this to a rapid and extensive $\beta \rightarrow \alpha$ conversion on transfer from *N,N*-dimethylformamide to buffer, as evidenced by the polarimetric data. The increase in K_i due to anomerisation is difficult to assess because only an upper limit (~10%) can be given for the proportion of the β anomer, and relative affinities for α and β anomers depend strongly on the size of the aglycon. A comparison of K_i values for α-D-galactosides with K_m for the corresponding β-anomer gives $K_i(\alpha)/K_m(\beta) = 3$ for methyl[15] and >200 for 4-nitrophenyl galactosides [no inhibition with 10mM α-D-galactoside, $K_m(\beta)$ 0.05 mM]. Thus the K_i value for 1-β-D-galactosylpiperidine may be several times smaller than the value given in Table I.

Information about the nature of functional groups at the active site which participate in the formation of the E–I complex can be obtained from the pH-dependence of the inhibition if these groups ionise in the accessible pH-range. In order to eliminate unspecific effects due to the multitude of ionisations occurring on a protein, Loeffler *et al.*[6] compared the K_i of the 1-β-D-galactosylpyridinium ion with that of the isosteric β-D-galactosylbenzene. A corresponding comparison of K_i for 1-D-galactosylpiperidine (Fig. 1) shows that binding of the basic inhibitor is influenced by changes in pH similar to that of the cationic binding. The only difference is that it behaves more like the neutral inhibitor than the latter.

Loeffler *et al.*[6] interpreted their data in terms of the perturbation of the pK_a of an acid near the active site, possibly Tyr-501, by the cationic inhibitor. Deprotonation of this acid causes a 24-fold increase in affinity for the pyridinium ion rela-

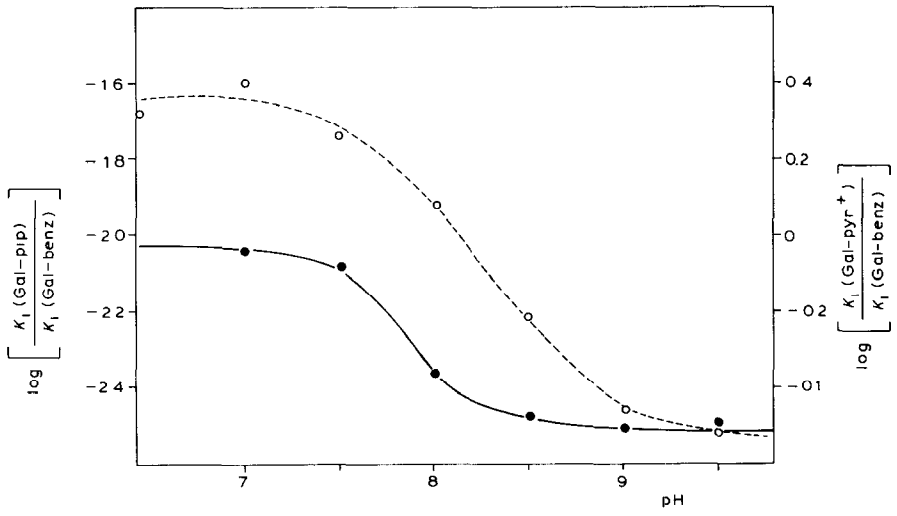

Fig. 1. pH-Dependence of the inhibition constants K_i for 1-D-galactosylpiperidine (—●—, left ordinate) and 1-β-D-galactosylpyridinium ion (---○---, right ordinate[6]) relative to K_i for β-D-galactosylbenzene.

tive to the unionised state. A similar explanation, with a smaller increase in affinity, could be advanced for the pH curve in Fig. 1 for 1-D-galactosylpiperidine, but this acid cannot be identified with HX of Scheme 1, which is responsible for catalysis of galactoside hydrolysis and protonation of basic galactosyl derivatives as proposed by Sinnott[1]. The acid HX would be present in the E–I complex as an ion pair $(Gal\text{-}NH_2R)^+X^-$, and deprotonation of the latter should be manifested in a drastic *increase* with pH of K_i (Gal-piperidine)/K_i(Gal-benzene). Since this is not observed up to pH 9.5, we conclude that pK_a is >10 for the ion pair in the E–I complex. The nature of HX is thus still an open question.

Charge distribution at the active site. — If the kinetic data of Sinnott and Souchard[2], and the formation of an alkali-sensitive covalent bond in the reaction of β-D-galactosidase with D-galactal[16,17] and with conduritol C *cis*-epoxide (ref. 4 and unpublished experiments), are accepted as sufficient evidence for a carboxylate group in close vicinity to C-1 of the bound substrate, then several explanations have to be discussed for the apparent lack of electrostatic interactions between permanently cationic galactosides and the active site, in contrast to the high affinity for basic galactosyl derivatives.

Firstly, there is no substantial electrostatic interaction with cationic galactosides because the active site is freely accessible to water, where ion-pair formation is not normally observed. This proposal was made by Sinnott and Withers[15] to account for the inability of β-D-galactosidase to hydrolyse β-D-galactosyltrimethylammonium in contrast to 1-β-D-galactosylpyridinium ion. It was argued that extended desolvation would be in conflict with the observed difference in leaving-group ability.

A problem with this kind of active site is that basic galactosyl derivatives should also bind, with affinities similar to those of their non-basic analogues (which is not observed), and that it is difficult to imagine the formation of an ion-pair/galactosyl ester intermediate[1] if water were not largely excluded.

Secondly, water is largely excluded from the active site, but the electrostatic contribution to the binding energy is compensated by the difference in hydration energy between the cationic and neutral galactosides. This requires that the difference in hydration energy is about equal for the trimethylammonium and pyridinium ions. In view of the large structural differences, this appears to be unlikely.

Thirdly, the charge effect of the carboxylate is largely neutralised by a positive charge in its vicinity. This cannot be the Mg^{2+} ion, because the effect of its removal on K_i for the 1-β-D-galactosylpyridinium ion is small and in the opposite direction[6]. Because of the small influence of Mg^{2+}, we also have to exclude the model proposed for β-D-glucosidases from almonds[9], where the positive charge neutralising the essential carboxylate was ascribed to a cationic acid that donates a proton in the ES-complex to glucosides and to basic glucosyl inhibitors. Removal of Mg^{2+} from *E. coli* β-D-galactosidase, which is essential for galactoside hydrolysis, and protonation of galactosylamines changes the position of the acid and

should cause a higher affinity for the permanently cationic inhibitors.

We propose that a positively charged group distinct from Mg^{2+} neutralises the electric field of the carboxylate at the position of the cationic centre of galactosyl-trimethylammonium and -pyridinium ions. Although this should not strongly interfere with the stabilisation of the galactosyl oxocarbonium ion as α-acylal, it could shift the proposed equilibrium (Scheme 1) towards the ion pair and thus favour hydrolysis of the galactosyl enzyme. As discussed above, the acid HX forms an ion pair with basic galactosyl derivatives. It can be estimated from a comparison with non-basic analogues that this provides an electrostatic contribution to the free energy of binding which amounts to 14–26 kJ/mol (3.5–6.4 kcal/mol). Thus, water has only limited access to the active site.

EXPERIMENTAL

The following substances were synthesised according to procedures in the literature: α-D-galactosylamine ammonia complex[11], β-D-glucosylamine[19], *N*-benzyl-D-galactosylamine[20], 1-D-galactosylpiperidine[21], *N*-*p*-tolyl-β-D-galactosylamine[22], 4-methylumbelliferyl β-D-galactopyranoside[23], and *p*-nitrophenyl β-D-galactopyranoside[24]. Melting points and specific optical rotations were in agreement with published values. *p*-Nitrophenyl α-D-galactopyranoside was obtained from Serva, and nojirimycin was prepared[25] from its hydrogensulfite adduct (Bayer).

N-Heptyl-D-galactosylamine, synthesised from heptylamine and D-galactose by the procedure given for the *p*-toluidine derivative[22], had m.p. 106–108°, $[\alpha]_{579}$ −10° (*c* 1, *N*,*N*-dimethylformamide). Its ^{1}H-n.m.r. spectrum (Varian A-90, D_2O as solvent) was in agreement with the expected structure.

Optical rotations were measured with a Perkin–Elmer 141 polarimeter at 579 nm and 20°.

β-D-Galactosidase was prepared from an *E. coli* K 12 strain, with defective *lac*-repressor (MPG 23), infected with phage λ which carried the *lac* gene ($\lambda h80c_{1857}\, t_{68} dlac\, i^{q,-1}z^+y^+x^+$)[26]. The enzyme was characterised by the following kinetic constants (U connotes μmol of substrate hydrolysed per min) with 4-nitrophenyl β-D-galactoside: K_m 0.05mM, V_{max} 29 U/mg. Wallenfels and Malhotra[27] gave K_m 0.051mM and V_{max} 19.5 U/mg for the enzyme from *E. coli* ML 309; K_m 0.03mM and V_{max} 40 U/mg were reported[28] for a constitutive strain 2 E01. With 4-methylumbelliferyl β-D-galactoside, we found K_m 0.13mM and V_{max} 280 U/mg in the presence of Mg^{2+}, and K_m 0.58mM and V_{max} 5.1 U/mg in its absence.

Enzyme activity was determined fluorimetrically[9] with 0.075–0.5mM 4-methylumbelliferyl β-D-galactoside in 50mM phosphate (pH 7.0) containing 145mM NaCl and mM $MgCl_2$ at 25°, or by following the change in A_{410} with 0.02–0.5mM 4-nitrophenyl β-D-galactoside under the same conditions[28]. For experiments in the absence of Mg^{2+}, the magnesium salt was replaced by 10mM EDTA. Rates were calculated from the initial slopes of recorder traces from the optical signals.

Stock solutions of the labile galactosylamine inhibitors were prepared in

N,N-dimethylformamide at 100-times the final concentration, and were kept for 3 h in the case of 1-D-galactosylpiperidine. Appropriate aliquots were injected into the substrate solution followed immediately by the enzyme. Readings on the recorder could be taken within 15 s after the addition of the inhibitor. The K_i values were calculated from the slopes of plots of $1/v$ *vs.* 1/[s] (normally 6 different concentrations of 4-methylumbelliferyl β-D-galactoside) in the presence [slope (I)] or absence of inhibitor [slope (O)] according to

$$K_i = \frac{I}{[\text{slope (I)}/\text{slope (O)}] - 1}$$

The standard error of the K_i values was ±15%.

Measurements with *N*-benzyl- and *N*-heptyl-D-galactosylamine at ≤30nM showed a time-dependent onset of inhibition. The lag decreased with increasing concentrations of inhibitor and was no longer detectable at concentrations above 0.1μM. This is attributed to the slow formation of the E–I complex under conditions where diffusion is partially rate-limiting.

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