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Binding energy and catalysis

Fluorinated and deoxygenated glycosides as mechanistic probes of *Escherichia coli (lacZ)* β-galactosidase

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Kinetic parameters for the hydrolysis of a series of deoxy and deoxyfluoro analogues of 2′,4′-dinitrophenyl β-D-galactopyranoside by *Escherichia coli* (*lacZ*) β-galactosidase have been determined and rates found to be two to nine orders of magnitude lower than that for the parent compound. These large rate reductions result primarily from the loss of transition-state binding interactions due to the replacement of sugar hydroxy groups, and such interactions are estimated to contribute at least 16.7 kJ (4 kcal)·mol^{-1} to binding at the 3, 4 and 6 positions and more than 33.5 kJ (8 kcal)·mol^{-1} at the 2 position. The existence of a linear free-energy relationship between $\log(k_{cat.}/K_m)$ for these compounds and the logarithm of the first-order rate constant for their spontaneous hydrolysis demonstrates that electronic effects are also important and provides direct evidence for oxocarbonium ion character in the enzymic transition state. A covalent intermediate which turns over only extremely slowly ($t_{\frac{1}{2}}$ = 45 h) accumulates during hydrolysis of the 2-deoxyfluorogalactoside, and kinetic parameters for its formation have been determined. This intermediate is nonetheless catalytically competent, since it re-activates much more rapidly in the presence of the transglycosylation acceptors methanol or glucose, thereby providing support for the notion of a covalent intermediate during hydrolysis of the parent substrates.

INTRODUCTION

Escherichia coli (*lacZ*) β-galactosidase (EC 3.2.1.23) catalyses the hydrolysis of galactopyranosides by cleavage of the C–O bond between the sugar and the aglycone with overall retention of anomeric configuration [1]. A double-displacement mechanism for glycosidases of this type has been proposed [2,3], involving the formation ('glycosylation' step) and breakdown ('deglycosylation' step) of a glycosyl-enzyme intermediate via oxocarbonium-ion-like transition states. Support for this transition-state structure comes from kinetic-isotope-effect measurements [4–6] and transition-state analogue studies [7], whereas support for the covalent nature of the intermediate is provided by isotope effects [3] and by a preliminary study [8] which showed that such a covalent intermediate can be trapped when β-galactosidase is allowed to react with 2-deoxy-2-fluoro-β-D-galactosyl fluoride, thereby inactivating the enzyme. The present paper, in part, provides a more detailed examination of the trapping of this intermediate, as well as providing direct evidence for the electron-deficient nature of the transition state.

A second major question addressed by the present paper is the role of non-covalent interactions between the non-reacting sugar hydroxy groups and the protein in transition-state stabilization, since the stabilization of a developing oxocarbonium ion by an enzymic nucleophile and electrophilic [3] or acid catalysis [4,9,10] is unlikely to account for the full catalytic power of the enzyme. Such non-covalent stabilization has been discussed much previously [e.g. 11–15] and, in the case of sugar binding enzymes, must involve hydrogen-bonding interactions [16–18]. Estimates of the contribution of such hydrogen-bonding interactions to the stabilization of both ground-state and transition-state complexes may be obtained by deletion of individual hydrogen bonds, either through point mutation of the protein or by ligand modification, followed by measurement of protein–ligand dissociation constants or decreases in $k_{cat.}/K_m$ values [e.g. 19–22]. The optimal replacements for a hydroxy group are hydrogen and fluorine [20], since not only are they sterically conservative replacements, but also they differ in their ability to partake in hydrogen-bonding, since the hydrogen in a deoxy sugar cannot possibly be involved in any significant hydrogen-bonding interactions, and the fluorine cannot act as a hydrogen-bond (proton) donor, but can arguably act as an acceptor (for a full discussion, see [20]). The contributions of the hydrogen bonds estimated in this way do not reflect the full strengths of the hydrogen bonds formed, since the binding process actually involves an exchange of the hydrogen bonds initially present between the ultimate partners and water with those formed in both the complex and in bulk water and, as discussed in detail elsewhere [13,19–21], the value measured is essentially equal to the *difference* between these hydrogen-bond strengths. Values for these transition-state interaction energies with each of the galactoside sugar hydroxy groups are presented here.

MATERIALS AND METHODS

Materials and general procedures

Escherichia coli (*lacZ*) β-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23), BSA, βGalONP (see the abbreviations footnote for definitions of galactopyranoside analogues) and βGalPNP were purchased from the Sigma Chemical Co. NaH_2PO_4, Na_2HPO_4 and $MgCl_2$ were of analytical grade. Methanol (BDH) was spectrophotometric grade. Details of the

Abbreviations used: βGalPNP, 4′-nitrophenyl β-D-galactopyranoside; βGalONP, 2′-nitrophenyl β-D-galactopyranoside; βGalDNP, 2′,4′-dinitrophenyl β-D-galactopyranoside; βGluDNP, 2′,4′-dinitrophenyl β-D-glucopyranoside; IPTGal, isopropyl thio-β-D-galactopyranoside; 2FβGalDNP, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside; 3FβGalDNP, 2′,4′-dinitrophenyl 3-deoxy-3-fluoro-β-D-galactopyranoside; 4FβGalDNP, 2′,4′-dinitrophenyl 4-deoxy-4-fluoro-β-D-galactopyranoside; 6FβGalDNP, 2′4′-dinitrophenyl 6-deoxy-6-fluoro-β-D-galactopyranoside; 4dβGalDNP, 2′4′-dinitrophenyl 4-deoxy-β-D-*xylo*-hexopyranoside; 6dβGalDNP, 2′,4′-dinitrophenyl 6-deoxy-β-D-galactopyranoside.

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syntheses of the deoxy and deoxyfluoro 2′,4′-dinitrophenyl glycopyranosides described herein are available from S. G. W. on request. 2′-Deoxy-2′-fluorolactose, obtained as its per-*O*-acetate, was generously given by Dr. D. H. Dolphin (Department of Chemistry, University of British Columbia) [23]. These compounds all provided satisfactory ^{1}H and (where relevant) ^{19}F-n.m.r. spectra as well as elemental analyses. All absorbance measurements were made on a Pye–Unicam PU-8800 u.v.–visible spectrophotometer equipped with a circulating water bath using 1 cm-pathlength cells. β-Galactosidase was dialysed before use against 50 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-Mg^{2+} to remove $(NH_4)_2SO_4$. The absorption coefficient (ϵ) of 2,4-dinitrophenolate at 400 nm in 50 mM-sodium phosphate buffer, pH 7.0, at 25 °C, was determined, by careful measurement of absorbances of accurately made solutions of the dried phenol, to be 11 100 $M^{-1} \cdot cm^{-1}$. 2′,4′-Dinitrophenyl glycopyranoside concentrations were determined from their absorbance at 255 nm (ϵ 10 700 $M^{-1} \cdot cm^{-1}$ for βGluDNP at 37 °C in 50 mM-sodium phosphate buffer, pH 6.8). The ϵ value for the glucoside was determined both by measuring absorbances of accurately weighed solutions and also by measuring the absorbance of such solutions at 400 nm after complete hydrolysis. Values of ϵ for the deoxy- and deoxyfluoro-glycosides were assumed to be the same as that of the unsubstituted glucoside.

Determination of K_m and $k_{cat.}$ for hydrolyses of 2′,4′-dinitrophenyl β-D-glycopyranosides by β-galactosidase

All kinetic measurements were performed at 25 °C in 50 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-Mg^{2+} (added as $MgCl_2$, $6H_2O$) and 0.1 % BSA, and the reaction was initiated by the addition of enzyme (10 μl) to thermally equilibrated cells containing substrate solutions. Enzyme concentrations and reaction times were chosen so that less than 10 % of the total substrate was hydrolysed. The enzyme sample used for each substrate was also assayed with 0.3 mM-βGalPNP ($10 \times K_m$), and all $k_{cat.}$ values are standardized with respect to a value for βGalPNP of 156 s^{-1} [4]. Values of K_m and $k_{cat.}$ were determined by measuring rates at six to eight substrate concentrations and ranging from, if possible, 7 times to 0.15 times K_m. The rate of spontaneous glycoside hydrolysis in each cell, if significant, was subtracted from the observed rate. A modified procedure was employed for the deoxy substrates because of their high rates of spontaneous hydrolysis. In these cases, thermally pre-equilibrated buffer was added to a weighed quantity of the deoxygalactoside, the stock concentration was determined by absorbance at 255 nm and appropriate volumes added to thermally pre-equilibrated cells containing buffer and BSA. Enzyme was added immediately, and the rates were determined as above. Values of K_m and $k_{cat.}$ were calculated by non-linear regression analysis. The K_m value for 4dβGalDNP was much higher than its achievable concentration; thus, in this case, an approximate value for $k_{cat.}/K_m$ was determined from the slope of the plot of v against [S]. This procedure was also applied to 6dβGalDNP as a check, and it gave a $k_{cat.}/K_m$ value comparable with that obtained by full analysis.

Methanol competition experiments

Experiments with the parent compound were performed as described previously [25] by measuring rates of the enzyme-catalysed reaction with saturating substrate in the presence or absence of methanol. Owing to the limited quantities of some of the deoxy and deoxyfluoro 2′,4′-dinitrophenyl glycopyranosides available and their relatively high K_m values, a stopped-assay procedure was employed in which 5–10 μl of enzyme was added to 50–100 μl of substrate stock in a pair of matched, thermally equilibrated, quartz cells, in the presence or absence of 1–2.5 M-methanol. After 5–15 min incubation at 25 °C, the reaction mixtures were diluted approx. 50-fold with buffer, effectively halting the reaction, and the absorbance of the cells at 400 nm was immediately determined. Incubation times and enzyme concentrations were chosen so that less than 50 % of the substrate was consumed, and all such measurements were made in triplicate.

Determination of K_i and k_i for 2FβGalDNP by time-dependent inactivation

Samples of 2FβGalDNP (final concns. 22, 33, 49, 110, 280, 500 and 1100 μM) in buffer containing 0.1 % BSA were incubated at 25 °C. Enzyme was added to each inactivation mixture and aliquots were removed at intervals, residual activity at each time interval being determined by diluting aliquots (10 μl) of the inactivation mixture into 1.6 ml of 0.3 mM-βGalPNP and measuring the rate of release of 4-nitrophenolate. The rate constant at each inactivator concentration, $k_{obs.}$, was determined by plotting the logarithm of the fraction of remaining active enzyme against time. K_i, the dissociation constant, and k_i, the rate constant for inactivation, were found by fitting the observed rate constants so determined to the expression:

$$k_{obs.} = \frac{k_i[I]}{K_i + [I]}$$

using a non-linear regression program (GraFit [26]).

Determination of K_i and k_i for 2FβGalDNP by dinitrophenolate burst

Quartz cells (1 cm) containing several concentrations of 2FβGalDNP in buffer were incubated at 25 °C. Enzyme (~ 4 mg·ml^{-1}) was added, the cells were mixed, and the increase in absorbance at 400 nm was monitored for 5–25 min, depending on the concentration of inactivator, at which point the enzyme was ~ 90 % inactivated. Longer incubation times showed only a slight linear increase in absorbance which corresponded to the spontaneous hydrolysis rate of 2FβGalDNP at that concentration. The rate of dinitrophenolate release at each inactivator concentration ($k_{obs.}$) was determined by fitting the data to a single exponential function using an Applied Photophysics kinetic workstation on an Archimedes 410/1 computer. K_i and k_i were then determined by using the GraFit program as described above.

Re-activation of covalently inactivated β-galactosidase

Inactivated enzyme was prepared by incubating β-galactosidase with 2FβGalDNP (~ 0.4 mM; approx. $\frac{1}{2}$ K_i and fifty times the estimated enzyme concentration) for several hours at 25 °C to ensure complete inactivation. Inactivated enzyme was freed of excess inactivator by centrifugation (3 × 30 min; 5500 rev./min; $r_{av.}$ 9.0 cm; 4 °C) in a Millipore Ultrafree-PF filter unit fitted with a polysulphone membrane (30 kDa molecular-mass limit) using a Sorvall RC-5B centrifuge with an SS-34 rotor. The sample was reduced to a volume of approx. 50 μl, then diluted with approx. 2 ml of buffer after each centrifugation, and finally diluted with 2.5 ml of buffer. The end-point activity was calculated from the ratio of the absorbances at 280 nm before and after centrifugation multiplied by the initial activity (prior to inactivation). Samples of the inactivated enzyme were then incubated in 50 mM-phosphate buffer, pH 7.0, containing 1 mM-Mg^{2+} at 25 °C in the presence of any desired transglycosylation acceptors. Re-activation was monitored by the removal of aliquots at appropriate time intervals and assaying for activity by addition to 2.5 ml of 0.3 mM-βGalPNP. The observed rate constant, $k_{obs.}$, for each re-activation process was determined by fitting the data to a single exponential function

using an Applied Photophysics kinetic workstation as described previously. A dissociation constant, K_d, for the binding of glucose at the transglycosylation site of the enzyme and the transglycosylation rate constant, $k_{trans.}$, were found by fitting the observed rate constants determined at various glucose concentrations to the expression:

$$k_{obs.} = \frac{k_{trans.}[glucose]}{K_d + [glucose]}$$

using the GraFit program. Control samples with non-inactivated enzyme were assayed for activity with βGalPNP over the same time course.

RESULTS

Inactivation of β-galactosidase with 2-deoxy-2-fluoroglycosides

Incubation of β-galactosidase with 2FβGalDNP resulted in time-dependent inactivation according to pseudo-first-order kinetics. Results are presented (for purposes of illustration only) in the form of a semi-logarithmic plot in Fig. 1(*a*), slopes of the lines yielding apparent rate constants for inactivation ($k_{obs.}$) at each concentration. The reciprocal replot of these slopes against inhibitor concentration is presented in Fig. 1(*b*). Values for the inactivation parameters ($k_i = 0.010\ s^{-1}$ and $K_i = 0.78$ mM) were obtained by non-linear-regression analysis of the data. The expected level of protection against inactivation was afforded by the competitive inhibitor IPTGal ($K_i = 0.085$ mM) [27], since addition of 0.5 mM-IPTGal to an inactivation mixture containing 0.32 mM-2FβGalDNP reduced the pseudo-first-order rate constant for inactivation from 0.0022 to 0.00027 s^{-1}.

Pre-steady-state formation of the 2-fluorogalactosyl-enzyme was also monitored by measuring the release of dinitrophenolate spectrophotometrically (400 nm), and a typical trace for such an experiment is shown in Fig. 2(*a*). Product release occurs according to a first-order process, followed by a low steady-state rate due to spontaneous hydrolysis of 2FβGalDNP. This experiment was performed at a total of four different concentrations of inactivator and four different traces of identical burst magnitude, but differing time course, were obtained. A reciprocal replot of $k_{obs.}$ values obtained versus inhibitor concentration is shown in Fig. 2(*b*). Values for k_i (0.011 s^{-1}) and K_i (0.62 mM), essentially identical with those determined by monitoring the inactivation process, were obtained by non-linear-regression analysis of the data. Incubation of the enzyme with 2.9 mM-2′-deoxy-2′-fluorolactose [4-*O*-(2-deoxy-2-fluoro-β-D-galactopyranosyl)-D-glucopyranose] did not result in any inactivation above that observed in a control sample over a period of 18 h.

Turnover of the 2-fluorogalactosyl-enzyme

Re-activation of the purified (inactivator-free) 2-fluorogalactosyl β-galactosidase was monitored by measuring the return of enzyme activity upon incubation in the presence and absence of methanol. In both cases the re-activation followed a single exponential time course, yielding half-lives for re-activation in the absence and presence of 1 M-methanol of 45 and 20 h. These correspond to rate constants of $4.2 \times 10^{-6}\ s^{-1}$ and $9.4 \times 10^{-6}\ s^{-1}$ respectively. Re-activation by glucose was studied more extensively, and the rate of re-activation was found to depend upon the concentration of glucose added in a saturable fashion. Semi-logarithmic plots of the return of activity versus time at different glucose concentrations are shown in Fig. 3(*a*) and clearly show that this process also follows pseudo-first-order kinetics. A reciprocal replot of the pseudo-first-order rate constants for re-activation versus time is shown in Fig. 3(*b*). Non-linear-regression analysis of this data yielded values of the maximal re-activation rate constant in the presence of glucose

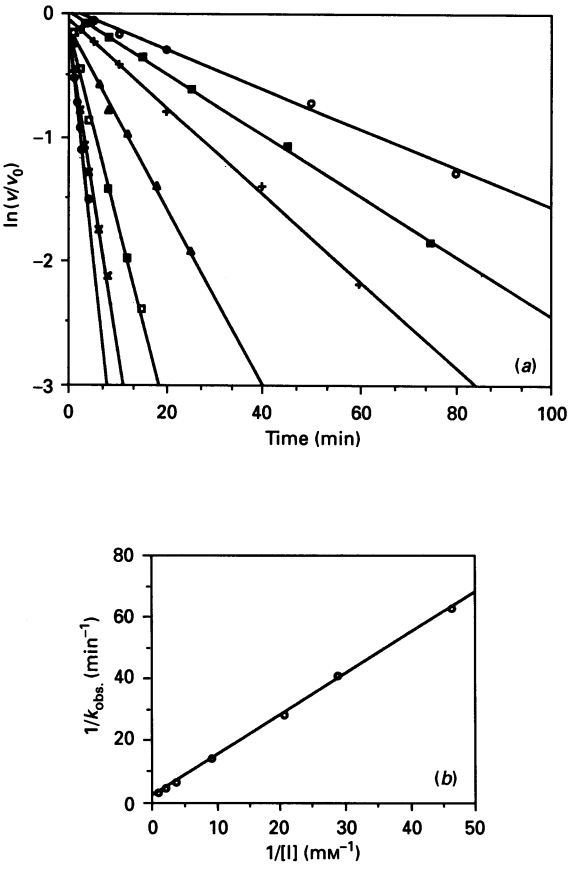


Fig. 1. Inactivation of β-galactosidase by 2FβGalDNP

(*a*) Semi-logarithmic plot of residual activity versus time at the following inactivator concentrations: ○, 0.022 mM; ■, 0.033 mM; +, 0.049 mM; ▲, 0.11 mM; □, 0.28 mM; ×, 0.50 mM; ●, 1.1 M. (*b*) Double-reciprocal plot of first-order rate constants from (*a*).

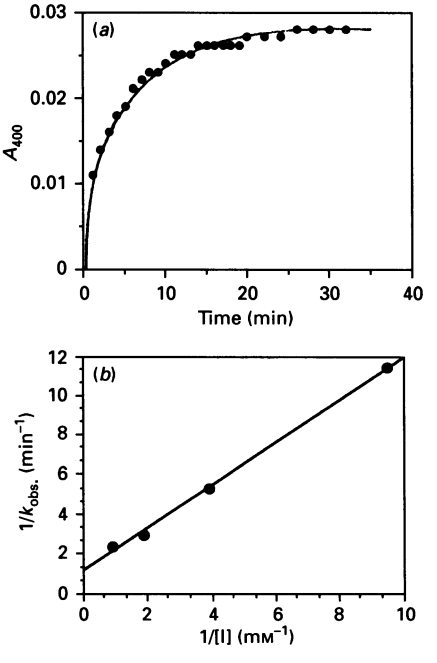


Fig. 2. Reaction of β-galactosidase with 2FβGalDNP

(*a*) Typical trace of 2,4-dinitrophenolate burst observed upon incubation of β-galactosidase with 2FβGalDNP (0.41 mM). (*b*) Double-reciprocal replot of first-order rate constants versus inactivator concentration.

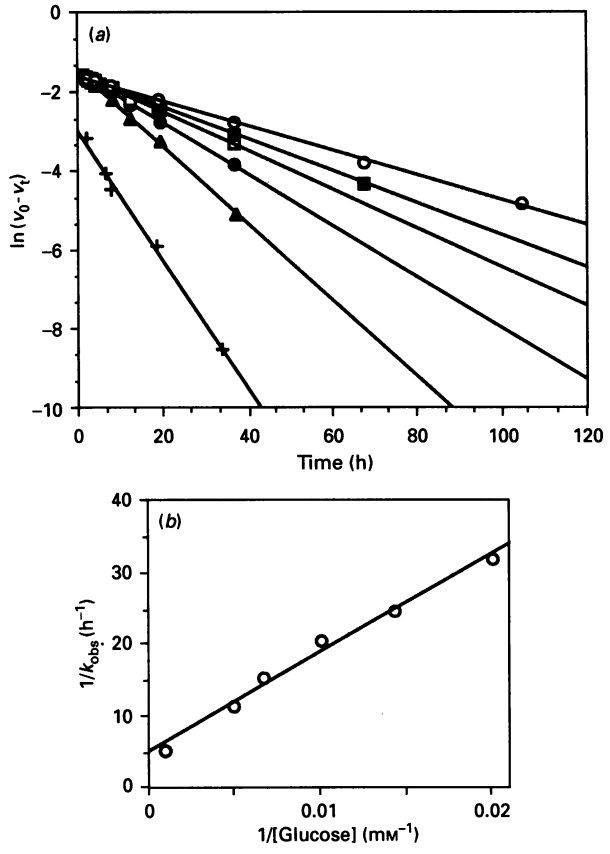


Fig. 3. Re-activation of 2-deoxy-2-fluorogalactosyl-enzyme by D-glucose

(*a*) Semi-logarithmic plot of activity versus time at the following concentrations of D-glucose: ○,† 50 mM; ■,† 70 mM; □,† 100 mM; ●,† 150 mM; ▲,† 200 mM; +,‡ 1000 mM. Conditions are as described in text (†enzyme concentration: 0.089 mg·ml^{-1}; ‡enzyme concentration: 0.070 mg·ml^{-1}). (*b*) Double-reciprocal plot of first-order rate constants from (*a*).

($k_{trans.}$) of 8.0×10^{-5} s^{-1} ($t_{\frac{1}{2}} = 2.4$ h) and a dissociation constant for binding at the transglycosylation site (K_d) of 460 mM.

Kinetic parameters of modified 2′,4′-dinitrophenyl glycopyranosides

Substrate activities of the other deoxygenated and fluorinated dinitrophenyl glycosides were determined as described in the Materials and methods section. The kinetic parameters determined ($k_{cat.}$ and K_m) as well as values of $k_{cat.}/K_m$, and the increase in activation free energy $\Delta\Delta G^0‡$ as a consequence of each modification are presented in Table 1. The inactivation and re-activation parameters for the 2-deoxy-2-fluoro compound are also presented for comparison purposes. Nucleophilic competition experiments with methanol and the modified dinitrophenyl glycosides gave the following results. Rate accelerations were seen with the 2-fluoro- (see above), 4-fluoro- (17% increase in rate of hydrolysis of 6.2 mM-glycoside in the presence of 2.5 M-methanol) and 6-fluoro- (29% increase in rate of hydrolysis of 0.70 mM-glycoside in the presence of 1 M-methanol) dinitrophenyl glycosides. No rate increase was seen for the 3-fluoro compound (8.1 mM) at methanol concentrations up to 2.5 M. Results of assays with the 4-deoxy- and 6-deoxy-glycosides were equivocal, since their high K_m values precluded saturation of the enzyme and thus expression of the full rate increase.

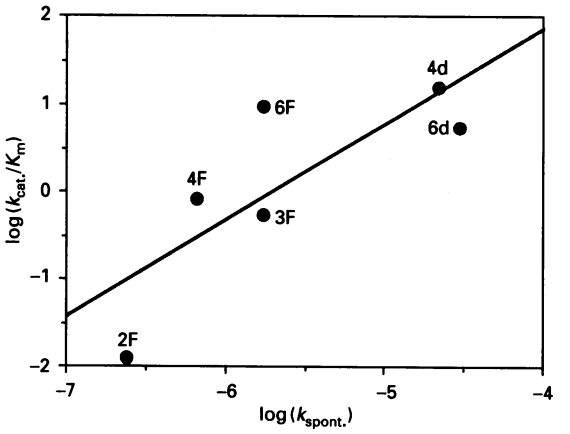


Fig. 4. Linear free-energy relationship between $k_{cat.}/K_m$ for the β-galactosidase-catalysed reaction and the spontaneous hydrolysis of a series of deoxy- and deoxyfluoro-2′,4′-dinitrophenyl glycopyranosides

Abbreviations: 2F–6F, 2-fluoro to 6-fluoro derivatives; 4d, 4-deoxy derivative; 6d, 6-deoxy derivative.

Fig. 4 shows the linear free-energy relationship ($\rho = 0.8$) obtained when the logarithms of values of $k_{cat.}/K_m$ for β-galactosidase catalysed hydrolysis of the substituted glycosides are plotted against the first-order rate constants for their spontaneous hydrolysis (0.025 M sodium phosphate buffer/

Table 1. Kinetic parameters of 2′,4′-dinitrophenyl β-D-glycopyranosides with *E. coli (lacZ)* β-galactosidase

2′,4′-Dinitrophenyl glycopyranoside*	K_m (mM)	$k_{cat.}$† (s^{-1})	$k_{cat.}/K_m$ ($s^{-1}\cdot mM^{-1}$)	$\Delta\Delta G^0‡$‖ [kJ(kcal)·mol^{-1}]
βGalDNP	0.14±0.01	1100	7900	–
6dβGalDNP	4.2±0.5‡	23	5.4	18 (4.3)
4dβGalDNP	–	–	16§	15.5 (3.7)
6FβGalDNP	0.61±0.06	5.5	9.0	16.7 (4.0)
4FβGalDNP	2.9±0.3	2.2	0.76	23 (5.5)
3FβGalDNP	1.2±0.1	0.63	0.53	23.9 (5.7)
2FβGalDNP	$K_i = 0.78 \pm 0.02$	$k_i = 0.010$, $k_{cat.}$¶ $= 4.3 \times 10^{-6}$	$k_i/K_i = 0.013$	33 (7.9)

* All measurements were made in 50 mM-sodium phosphate buffer/1 mM-Mg^{2+}, pH 7.0 at 25 °C.
† $k_{cat.}$ values are reported relative to a βGalPNP $k_{cat.}$ of 156 s^{-1} (4) and are ± approx. 10%.
‡ The maximal concentration of 6dβGalDNP in buffer is approx. 40% of the reported K_m.
§ Saturation of enzyme not possible owing to very poor binding of substrate, i.e. [S] ≪ K_m.
‖ $\Delta\Delta G^0‡ = -RT\ln\{(k_{cat.}/K_m)_2/(k_{cat.}/K_m)_1\}$ where $(k_{cat.}/K_m)_2$ is the value for the analogue and $(k_{cat.}/K_m)_1$ is the value for βGalDNP.
¶ $k_{cat.}$ = rate constant for reactivation of 2-fluorogalactosyl-enzyme.

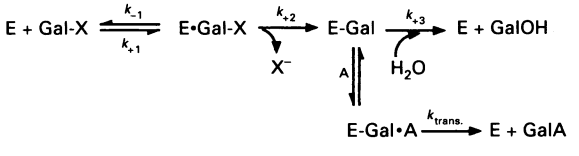
0.8 M-KCl, pH 6.5, 37 °C) (M. N. Namchuk, J. D. McCarter, R. S. Stewart and S. G. Withers, unpublished work). The point for 2FβGalDNP is the value of k_i/K_i, since this value clearly also refers to the glycosylation step.

DISCUSSION

The general mechanism proposed [2,3] for a 'retaining' glycosidase is shown in Scheme 1. When the aglycone (X) is a sufficiently activated leaving group (e.g. 2,4-dinitrophenolate), the rate of glycosylation will be increased relative to deglycosylation such that deglycosylation becomes rate-limiting, i.e. $k_{+2} \gg k_{+3}$. The rate constant for the rate-limiting step of the reaction, $k_{cat.}$, will then be equal to k_{+3}. If k_{+3} is sufficiently small compared with k_{+2}, then the glycosyl-enzyme intermediate will accumulate, and if k_{+3} approaches zero, the enzyme will be essentially inactivated. Under these latter conditions k_i, the rate constant for inactivation, will be equal to the rate of glycosylation, k_{+2}, and the dissociation constant K_i will be equal to k_{-1}/k_{+1}.

Such a situation is clearly obtained in the interaction of β-galactosidase with 2FβGalDNP, since time-dependent inactivation was observed accompanied by release of 2,4-dinitrophenolate. The finding of essentially identical kinetic parameters for the inactivation of the enzyme and for release of dinitrophenolate demonstrates that the two phenomena are associated with the same event, as required. Further, the protection against inactivation by the competitive inhibitor IPTGal indicates that this event is occurring at the active site. The identical size of the burst of dinitrophenolate released at different inactivator concentrations is also consistent with the expected stoichiometry, 1 mol of dinitrophenolate being released/mol of enzyme inactivated. These results therefore strongly support the previous proposal [8] that the closely related compound 2-deoxy-2-fluoro-β-D-galactopyranosyl fluoride, inactivates β-galactosidase by accumulation of a stable 2-fluorogalactosyl-enzyme intermediate. They are also consistent with recent work on the β-glucosidase from *Agrobacter* [28], which showed that addition of 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside to the enzyme results in the accumulation of a 2-deoxy-2-fluoro-α-D-glucopyranosyl-enzyme intermediate whose covalency and stereochemistry were directly determined by ^{19}F n.m.r. Presumably an analogous intermediate accumulates in the β-galactosidase-catalysed reaction. Significantly, incubation of the enzyme with 2′-deoxy-2′-fluorolactose [4-*O*-(2-deoxy-2-fluoro-β-D-galactopyranosyl)-D-glucopyranose] did *not* result in inactivation, demonstrating the necessity of a sufficiently rapid glycosylation step to permit accumulation of the 2-deoxy-2-fluorogalactosyl-enzyme. This is consistent with the fact that the $k_{cat.}$ value for the parent compound, lactose, the natural substrate of the enzyme, is only 40% of that of βGalPNP, a substrate for which glycosylation is rate-limiting [5,29]. Thus the β1,4-linked glucose moiety is not a sufficiently good leaving group to make the intermediate kinetically accessible.

The catalytic competence of this intermediate is demonstrated by the fact that re-activation of the 2-fluorogalactosyl-enzyme intermediate occurs through hydrolytic turnover when freed from excess inactivator. Further, this process is significantly accelerated by the addition of methanol, a reagent known to act as a more efficient nucleophile than water in reaction with the galactosyl-enzyme [25]. Indeed, the 2.25-fold rate acceleration is essentially the same as the 2.5-fold rate increase afforded by 1 M-methanol to the β-galactosidase-catalysed hydrolysis of 2′,4′-dinitrophenyl β-D-galactopyranoside, providing good evidence that the introduction of the fluorine has not perturbed the normal mechanism, other than by slowing the rates of glycosylation and deglycosylation. Re-activation of the 2-fluorogalactosyl-enzyme is also accelerated very significantly (over 18-fold above the hydrolytic rate), in a saturable fashion (K_d = 460 mM) by glucose. Such behaviour is well-precedented, since many glycosidases are known to exhibit transglycosylation activity [27,30,31], this simply being the reverse of the normal disaccharide hydrolysis reaction. A second 'aglycone' sugar-binding site was observed in these cases also and, in the case of β-galactosidase, it was concluded [27,31] that the binding of glucose to the galactosyl-enzyme is considerably tighter than to the free enzyme (K_d = 17 mM versus 630 mM), thus suggesting some change in structure of this binding site on formation of the intermediate. Turnover by transglycosylation to glucose (rate constant 380 s^{-1}) was still, however, less effective than by hydrolysis (rate constant 1200 s^{-1}) [31]. The poorer binding of glucose to the 2-fluorogalactosyl-enzyme indicates that the glucose subsite of the galactosyl-enzyme and the 2-fluorogalactosyl-enzyme are somewhat different in structure, suggesting that the 2-hydroxy group of the galactose moiety plays a role in ordering the structure of the glucose subsite, and therefore, conversely, that the binding of the glucose moiety of lactose assists in the structuring of the galactose site, certainly around the 2-hydroxy group. Such a notion of synergy between the two sites is quite appealing, since it is completely consistent with the concept of the utilization of binding energy in catalysis, and helps to explain why lactose, a substrate with an extremely chemically unreactive leaving group, is turned over at a rate comparable with that of the chemically reactive substrate βGalPNP. It is also consistent with the observation that glucose is much more effective at promoting the transfer of the 2-fluorogalactosyl moiety than the galactosyl moiety.



Scheme 1. Kinetic mechanism of β-galactosidase

Kinetic parameters of modified 2′,4′-dinitrophenyl glycopyranosides

Interpretation of the kinetic parameters given in Table 1 requires prior consideration of their true significance. For the two-step mechanism proposed, values of $k_{cat.}/K_m$ should correspond to the rate of the first irreversible step, in this case presumably the glycosylation step, k_{+2} [14]. Since this is a second-order rate constant, the values reflect the height of the activation barrier from free enzyme and substrate to the transition state for this first irreversible step. The changes in this activation barrier upon substitution ($\Delta\Delta G^{0\ddagger}$) are given in the final column. The methanol-competition experiments revealed that the actual rate-determining step for the parent compound and the 2-, 4- and 6-fluoro derivatives is the deglycosylation step, k_{+3}. Although this is probably also true for the deoxy derivatives, the high K_m values and low solubilities of the sugars preclude such investigation. It does not, however, appear to be true for the 3-fluoro derivative, since no increase in rate was seen upon addition of methanol, even at substrate concentrations of $8 \times K_m$. Whether this is the result of some artifact, or whether it indeed indicates that another step, presumably glycosylation, is rate-limiting, is not clear. In light of this uncertainty it is therefore less ambiguous to interpret relative values of $k_{cat.}/K_m$ rather than $k_{cat.}$.

Two major factors must be considered in attempting to understand the effects of substitution on the rate constants.

Firstly, intrinsic electronic effects arising from the different response of the cationic transition state to changes in electronegativity of the substituents will have a significant effect upon rates. The relatively electronegative fluorine will tend to destabilize the oxocarbonium-ion-like transition state, thus slowing the reaction, whereas the relatively electropositive hydrogen atom will tend to stabilize it, speeding up the reaction. Such effects have been seen previously [32–35] in the acid-catalysed hydrolysis of glycosides, where the reaction is known to proceed via an oxocarbonium-ion-like mechanism. Secondly, binding effects, which can be enormous [21], will be significant, since the deletion of enzyme–substrate hydrogen bonds, which serve to stabilize the transition state, will decrease rates of reaction. These two effects (electronic and binding) will, of course, be superimposed on each other in the enzymic process.

Evidence for the importance of such intrinsic electronic effects, and thereby also evidence for the oxocarbonium-ion-like structure of the enzymic transition state, is obtained through the linear free-energy relationship shown in Fig. 4. Such a correlation, albeit weak, due largely to the superimposition of binding phenomena, demonstrates that the two transition states (enzymic and spontaneous) are being affected in a similar way by substitution at each position. Since it has been demonstrated that the principal effect of substitution of a hydroxy group by a hydrogen or a fluorine atom on spontaneous hydrolysis rates is electronic in nature [34,35], then the presence of this correlation provides direct evidence for similar oxocarbonium-ion character in the enzymic transition state.

The scatter on the linear free-energy relationship is entirely expected, and is a consequence of the fact that binding effects are of considerable importance to the enzyme-catalysed process, but are not a component of the spontaneous process. The importance of such binding interactions to enzymic catalysis is perhaps best probed by examining the data for the deoxyglycopyranosides rather than the fluorinated analogues. Purely on the basis of electronic effects, the deoxy substrates would be expected to undergo enzymic hydrolysis faster than the parent galactopyranoside, whereas in fact they are hydrolysed more *slowly* by the enzyme. Thus *all* the rate reduction in these cases must be due to the loss of interactions, most likely hydrogen-bonding. Values of $\Delta\Delta G^{0}\ddagger$ will therefore represent a *minimum* estimate of the contributions of the hydrogen bonds at each hydroxy group to stabilization of the transition state. The real value may be somewhat higher, since the intrinsic electronic effect will tend to mitigate, to some extent, the deleterious binding effect.

Values of around (4 kcal)·mol^{-1} are observed for the 4- and 6-hydroxy groups. In addition, on the basis of the value of 23.8 kJ (5.7 kcal)·mol^{-1} for the 3-fluoro sugar, and knowing that the intrinsic electronic effect at the 3-position is less than that at the 4-position [34,35], it is likely that interactions contributing at least 16.7 kJ (4 kcal)·mol^{-1} are present at the 3-hydroxy group. Interestingly, in previous work [39] on β-galactosidase-catalysed hydrolysis of modified glycosides, a $k_{cat.}/K_m$ value for 2′,4′-dinitrophenyl α-L-arabinopyranoside (missing the complete C-6 hydroxymethyl group) of 52.6 mM^{-1}·s^{-1} was obtained, corresponding to a $\Delta\Delta G^{0}\ddagger$ of 13 kJ (3.1 kcal)·mol^{-1}. This $k_{cat.}/K_m$ value is significantly higher than that obtained in the present work for 6dβGalDNP (5.4 mM^{-1} s^{-1}) in which only the C-6 hydroxy group has been removed, possibly indicating that, during hydrolysis of the α-L-arabinoside, a water molecule may occupy the space vacated by the C-6 hydroxymethyl group, thereby satisfying some of the hydrogen-bonding requirements of the protein, as has been seen by X-ray crystallography in the binding of α-L-arabinopyranose to the D-galactose-binding protein [17]. This would not be possible for the 6-deoxygalactoside. Values of $k_{cat.}/K_m$ reported for 6dβGalDNP and the 6-chloro derivative are gratifyingly close to those found in the present work for 6dβGalDNP and 6FβGalDNP.

Unfortunately, owing to the anticipated extreme lability of the 2-deoxyglycoside of 2,4-dinitrophenol, no data are available on the rate of glycosylation in the absence of the 2-hydroxy group. However, very valuable insight can be obtained from previously published data on the β-galactosidase-catalysed hydration of D-galactal. This reaction has been shown to proceed, with a rate constant of $k_{+3} = 2 \times 10^{-3}$ s^{-1}, through the intermediacy of a 2-deoxygalactopyranosyl-enzyme intermediate whose hydrolysis is rate-limiting [36,37]. Indeed, similar studies [38] on the mechanistically related β-glucosidase from *Aspergillus wentii* showed that the rate-determining step for both the hydration of D-glucal and the hydrolysis of *p*-nitrophenyl β-D-glucopyranoside was deglycosylation, and identical rate constants were measured for both processes. In the case of β-galactosidase, therefore, the hydrolysis of the 2-deoxygalactopyranosyl-enzyme intermediate is some 5.5×10^{5} times slower than hydrolysis of the galactopyranosyl-enzyme intermediate, suggesting that the interactions at the 2-position contribute at least 33.5 kJ (8 kcal)·mol^{-1} to the stabilization of this transition state. The true stabilization is probably considerably larger, since the inductive effect associated with the hydrogen at the 2-position will mitigate the rate decrease (2-deoxyglycosides undergo acid-catalysed hydrolysis approx. 2000-fold faster than the parent compounds [32,33]). As stated previously, this value of > 33.5 kJ (> 8 kcal)·mol^{-1} refers to a different step (deglycosylation) than do those calculated for the other positions. However, it is probable that the two transition states are quite similar in terms of their hydrogen-bonding interactions, so a comparable value can be expected for the two steps.

Interactions at the 3-, 4-, and 6-positions therefore appear to contribute approx. 16.7 kJ (4 kcal)·mol^{-1} each, or slightly more, to the stabilization of the transition state, whereas interactions at the 2-position are considerably more important, contributing *at least* 33.5 kJ (8 kcal)·mol^{-1}. This is completely consistent with the notion that reaction proceeds through an oxocarbonium-ion-like transition state which necessarily has a distorted structure, most likely a half-chair conformation, in which C-5, O-5, C-1 and C-2 are co-planar to accommodate the double-bond character between O-5 and C-1. If the enzyme has evolved to bind such a structure selectively while still retaining affinity and specificity for a D-galactopyranoside substrate in a $^{4}C_{1}$ conformation, then the most important transition state interactions, *by far*, would be expected to be at the 2-position, since that is the hydroxy group which is undergoing the most extensive restructuring between the ground state and the transition state. Interactions at the other three positions would simply need to be sufficient, in sum, to hold the rest of the sugar ring in position, as indeed appears to be the case.

The magnitude of these interactions is of particular interest in light of recent measurements of the contributions of hydrogen bonds to the stability of protein–ligand complexes [9,20,22]. Hydrogen bonds between two neutral partners were shown to contribute up to approx. 6.3 kJ (1.5 kcal)·mol^{-1}, while those between a neutral and a charged partner contribute over 12.5 kJ (3 kcal)·mol^{-1}. On this basis the interactions at each of the 3-, 4- and 6-positions could probably be contributed by a single charged partner, or by several neutral ones. The interactions at the 2-hydroxy group likely involve several charged residues involved in co-operative hydrogen-bonding. However, interactions of this magnitude are well precedented, a recent review by Wolfenden & Kati [21] having listed a number of enzymes in which interactions of this magnitude have been detected. In each of these cases the hydroxy group in question was one which changed in position considerably between the ground and transition states. One in

particular was the β-glucosidase from *A. wentii*, for which interactions worth at least 29.3 kJ (7 kcal)·mol^{-1} at the 2-position were noted [38], though the strengths of the interactions at the other positions were not fully documented in this case.

The effect of the fluorine substituent at C-2 upon the rate constant for deglycosylation is also of considerable interest, particularly in light of the role of this substituent in effecting inactivation of the enzyme. Whereas substitution by hydrogen at this position decreases the rate constant for deglycosylation some 5.5×10^5-fold, substitution by fluorine reduces it over 400-fold more, to a value of 4.3×10^{-6} s^{-1}. This difference is likely a consequence of the different intrinsic electronic effects of the two substituents at that position. The ratio of the two rates ($k_{deoxy.}/k_{fluoro} = 465$) is considerably less than the estimated ratio of the corresponding rates for spontaneous hydrolysis of the substrates ($k_{deoxy.}/k_{fluoro} = 45000$). {This latter rate ratio is based upon the rate measured for the spontaneous hydrolysis of 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside ($k_{spont.} = 1.5 \times 10^{-7}$ s^{-1} at 37 °C in 0.025 M-sodium phosphate buffer/0.8 M KCl, pH 6.5) (M. N. Namchuk & S. G. Withers, unpublished work) and the estimated rate of spontaneous hydrolysis of 2′,4′-dinitrophenyl 2-deoxy-β-D-glucopyranoside ($k_{spont.} = 6.7 \times 10^{-3}$ s^{-1} at 37 °C) obtained by extrapolation of the excellent linear free-energy relationship ($\rho = 0.99$) discovered between rates of acid-catalysed hydrolysis of substituted glucopyranosyl phosphates (for which the hydrolysis rate constant for the 2-deoxy derivative was determined) [34,35,40], and rates of spontaneous hydrolysis of 2′,4′-dinitrophenyl β-D-glycopyranosides.} Differences may be due to different degrees of oxocarbonium-ion character for the enzyme-catalysed and non-enzymic processes. They may also be due to the fact that the fluorine substituent could be involved in significant stabilizing hydrogen-bonding interactions at the transition state, thus the full binding energy involved in transition-state stabilization would not be lost, tending to decrease the rate difference between the two. Whatever the cause, it is clear that only through substitution at the 2-position, preferably with small electronegative elements such as fluorine, can the rate constants be sufficiently manipulated to allow stabilization of the glycosyl-enzyme intermediate with a sufficient lifetime to allow its observation by such techniques as n.m.r.

REFERENCES

1. Wallenfels, K. & Weil, R. (1972) Enzymes 3rd Ed. **7**, 617–663
2. Koshland, D. E. (1953) Biol. Rev. **28**, 416–420
3. Sinnott, M. L. (1990) Chem. Rev. **90**, 1171–1202
4. Sinnott, M. L. & Souchard, I. J. L. (1973) Biochem. J. **133**, 89–98
5. Rosenberg, S. & Kirsch, J. F. (1981) Biochemistry **20**, 3189–3196
6. Sinnott, M. L. & Withers, S. G. (1974) Biochem. J. **143**, 751–762
7. Legler, G. (1990) Adv. Carb. Chem. Biochem. **48**, 319–384
8. Withers, S. G., Rupitz, K. & Street, I. P. (1988) J. Biol. Chem. **263**, 7929–7932
9. Ring, M., Bader, D. E. & Huber, R. E. (1988) Biochem. Biophys. Res. Commun. **152**, 1050–1055
10. Lehmann, J. & Schlesselmann, P. (1983) Carbohydr. Res. **113**, 93–99
11. Haldane, J. B. S. (1930) in Enzymes (Plimmer, R. H. A. & Hopkins, F., eds.), p. 182, Longmans, Green & Co., London
12. Pauling, L. (1946) Chem. Eng. News **24**, 1375–1376
13. Jencks, W. P. (1975) Adv. Enzymol. Related Areas Mol. Biol. **43**, 219–410
14. Fersht, A. R. (1985) in Enzyme Structure and Mechanism, 2nd edn., pp. 293–346, W. H. Freeman & Co., San Francisco
15. Wolfenden, R. & Frick, L. (1987) in Enzyme Mechanisms (Page, M. I. & Williams, A., eds.), pp. 97–122, The Royal Society of Chemistry, London
16. Johnson, L. N., Cheetham, J., McLaughlin, P. J., Acharya, K. R., Barford, D. & Phillips, D. C. (1988) Curr. Top. Microbiol. Immunol. **139**, 81–134
17. Quiocho, F., Wilson, D. & Vyas, N. (1989) Nature (London) **340**, 404–407
18. Cygler, M., Rose, D. R. & Bundle, D. R. (1991) Science **253**, 442–445
19. Fersht, A. R., Leatherbarrow, R. J. & Wells, T. N. C. (1986) Trends Biochem. Sci. **11**, 321–325
20. Street, I. P., Armstrong, C. & Withers, S. G. (1986) Biochemistry **25**, 6021–6027
21. Wolfenden, R. & Kati, W. M. (1991) Acc. Chem. Res. **24**, 209–215
22. Street, I. P., Rupitz, K. & Withers, S. G. (1989) Biochemistry **28**, 1581–1587
23. Shelling, J. G., Dolphin, D., Wirz, P., Cobbledick, R. E. & Einstein, F. W. B. (1984) Carbohydr. Res. **132**, 241–253
24. Wilkinson, G. N. (1961) Biochem. J. **80**, 324–332
25. Sinnott, M. L. & Viratelle, O. M. (1973) Biochem. J. **133**, 81–87
26. Leatherbarrow, R. J. (1990) GraFit Version 2.0, Erithacus Software Ltd., Staines
27. Deschavanne, P. J., Viratelle, O. M. & Yon, J. (1978) J. Biol. Chem. **253**, 833–837
28. Withers, S. G. & Street, I. P. (1988) J. Am. Chem. Soc. **110**, 8551–8553
29. Huber, R. E. & Gaunt, M. T. (1983) Arch. Biochem. Biophys. **220**, 263–271
30. Huber, R. E., Kurz, G. & Wallenfels, K. (1976) Biochemistry **15**, 1994–2001
31. Huber, R. E., Gaunt, M. T. & Hurlburt, K. L. (1984) Arch. Biochem. Biophys. **234**, 151–160
32. Capon, B. (1969) Chem. Rev. **69**, 407–498
33. BeMiller, J. N. (1967) Adv. Carb. Chem. Biochem. **22**, 25–108
34. Withers, S. G., MacLennan, D. J. & Street, I. P. (1986) Carbohydr. Res. **154**, 127–144
35. Withers, S. G., Percival, M. D. & Street, I. P. (1989) Carbohydr. Res. **187**, 43–66
36. Wentworth, D. F. & Wolfenden, R. (1974) Biochemistry **13**, 4715–4720
37. Viratelle, O. M. & Yon, J. M. (1980) Biochemistry **19**, 4143–4149
38. Roeser, K.-R. & Legler, G. (1981) Biochim. Biophys. Acta **657**, 321–333
39. Marshall, P., Reed, C. G., Sinnott, M. L. & Souchard, I. J. L. (1977) J. Chem. Soc. Perkins Trans II, 1198–1202
40. Percival, M. D. & Withers, S. G. (1988) Can. J. Chem. **66**, 1970–1972

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