Role of the Substituent at C-5 of the Pyranose Ring in Catalysis by *E. coli*(*lacZ*)β-Galactosidase

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Removal of the C-5 hydroxymethyl group of the galactopyranosyl substrates of *E. coli* β-galactosidase has only a slight effect—usually a decrease—on the rate of the conformation-change step with aryl glycosides, on the rate of cleavage of the C–N bond of the glycosylpyridinium ion, or on the rate of hydrolysis of the glycosyl-enzyme. Values of K_m are all substantially increased. Substrate destabilisation of the particular type commonly envisaged for lysozyme catalysis therefore plays no part in catalysis by this particular glycosidase, and the C-5 hydroxymethyl group plays a role only in binding.

In the preceding paper, we present evidence that when β-galactosidase binds its substrates, the consequent destabilisation of the substrate in the enzyme–substrate (ES) complex alone accounts for a lowering of the barrier to glycone–aglycone bond fission of between about 14 and 16 kcal mol^{-1}.[1] One way in which the substrate could be destabilised is by van der Waals repulsion between the hydroxylmethyl group at C-5 of the pyranose ring and some group on the enzyme. This could force the pyranose ring into an unfavourable conformation whence departure of the aglycone would be readier. Such an effect is often considered to be responsible for a substantial portion of the catalytic power of lysozyme,[2] but convincing evidence for its

[1] C. C. Jones, M. L. Sinnott, and I. J. L. Souchard, preceding paper.

[2] *e.g.* B. M. Dunn and T. C. Bruice, *Adv. Enzymol.*, 1973, **37**, 1.

reality is hard to find. Capon and Dearie [3] made substrates containing terminal *N*-acetylxylosamine residues which, had extensive kinetic measurements been possible, would have succinctly thrown light on the problem. However, these authors were only able to show that k_{cat}/K_m values for the dinitrophenyl glycosides with terminal xylosamine residues were much lower than those for corresponding substrates with terminal glucosamine residues. If the Phillips mechanism is true, this can only be taken as evidence that the first step after binding does not involve bond breaking. Indeed n.m.r. and fluorescence measurements indicate that at least a disaccharide recognises one subsite, and, in a subsequent step, slithers down the active-site cleft to its equilibrium position.[4] Values of k_{cat}/K_m could then reflect such a process rather than bond breaking. However, if bond breaking follows immediately upon binding, for these slow-reacting substrates K_m would equal K_s, and the lower k_{cat}/K_m values observed for the derivatives with xylosamine residues than for those with terminal glucosamine residues are evidence against the Phillips mechanism. If the binding energy of the glycosides of NAG oligomers is less than the intrinsic binding[5] energy by a quantity $\Delta\Delta G^\circ$, and this is then wholly used to lower the free energy barrier to bond breaking, then k_{cat}/K_m will be completely independent of $\Delta\Delta G^\circ$. If the process is inefficient, *i.e.* if the loss of free energy of binding is not wholly used to lower the free energy barrier to reaction, then the smaller $\Delta\Delta G^\circ$, *i.e.* the less important the steric clashes on binding, the bigger will be k_{cat}/K_m, as K_m will be increased more than k_{cat}. Therefore, if a simple two-step mechanism holds, the decrease in k_{cat}/K_m observed when the C-5 hydroxymethyl group of the terminal *N*-acetylpyranosamine is removed is directly at variance with the predictions of the Phillips mechanism for catalysis.*

It therefore seemed useful to examine the applicability of the ideas of substrate destabilisation by the steric clash of the C-5 hydroxymethyl group with groups on the enzyme to β-galactosidase, an enzyme not complicated by a multiplicity of saccharide binding sites. Although it is known that β-galactosidase does indeed hydrolyse α-L-arabinopyranosides,[6a] with one exception [6b] such kinetic data as are available pertain to strongly inhibitory tris(hydroxymethyl)aminomethane buffers, and moreover there is no indication which step in the enzyme sequence is rate-determining. k_{cat} Values which really represent rate-determining conformational changes throw no light on the catalytic step and, at least for aryl galactosides slowly hydrolysed by Mg^{2+}-saturated enzyme, k_{cat} values are of this type.[7] There was at the inception of this work additionally the hope that the hydrolysis of glycosyl-β-galactosidases with differing C-5 substituents might be sufficiently unreactive for their build-up from suitable precursors to be observed by stopped-flow techniques; galactosyl-β-galactosidase is hydrolysed too fast under ordinary conditions for its build-up to be observed.

EXPERIMENTAL

(A) *Substrates.*—The 2,4-dinitrophenyl glycosides of α-L-arabinopyranose, 6-deoxy-β-D-galactopyranose, and 6-chloro-6-deoxy-β-D-galactopyranose have been described.[8] Methyl α-L-arabinopyranoside, m.p. 130—131° (lit.,[9] 131°), 2-nitrophenyl α-L-arabinopyranoside, m.p. 141—143° (lit.,[9] 139—139.5°), 4-nitrophenyl α-L-arabinopyranoside, m.p. 197—199° (decomp.) (lit.,[10] 201—202°), and phenyl α-L-arabinopyranoside, m.p. 154—155° (lit.,[11] 153—155°) were made by standard procedures.

3-Nitrophenyl and 3,5-dinitrophenyl α-L-arabinopyranosides. To a solution of 2,3,4-tri-*O*-acetyl-β-L-arabinopyranosyl bromide[12] (2.0 g) in acetone (15 ml) was added a solution of (i) 3-nitrophenol (1.35 g) or (ii) 3,5-dinitrophenol (2.0 g) in aqueous N-sodium hydroxide (10 ml). After 24 h at 22 °C, the acetone was evaporated off, the resulting gum was taken up in dichloromethane, and this solution was washed with aqueous sodium carbonate until the washings were colourless, dried ($MgSO_4$), and evaporated. The tri-*O*-acetyl derivative resisted attempts at crystallisation, and so the gum was taken up in methanolic 0.01N-sodium methoxide, and left at 0 °C for 4 h. The methoxide was then neutralised with a few drops of glacial acetic acid, and the solution was evaporated. (i) Trituration with water afforded *3-nitrophenyl α-L-arabinopyranoside* (0.48 g, 30%), $[\alpha]_D^{25}$ 32° (*c* 1 in EtOH), m.p. 154—155° (from ethanol and from acetone) (Found: C, 48.75; H, 4.8; N, 5.1. $C_{11}H_{13}NO_7$ requires C, 48.7; H, 4.8; N, 5.15%). (ii) Trituration with water and recrystallisation from acetone–ether and from methanol–ethanol–t-butyl alcohol gave *3,5-dinitrophenyl α-L-arabinopyranoside ethanolate*, m.p. 98—99° (1.0 g, 51%) (Found: C, 43.25; H, 4.65. $C_{11}H_{12}N_2O_9,C_2H_6O$ requires C, 43.1; H, 4.95%). Recrystallisation from water gave a *hydrate*, m.p. 102—103°, $[\alpha]_D^{25}$ −39° (*c* 0.8 in EtOH) (Found: C, 40.2; H, 4.4; N, 8.55. $C_{11}H_{12}N_2O_9,H_2O$ requires C, 39.5; H, 4.2; N, 8.4%).

2,5-Dinitrophenyl α-L-arabinopyranoside. This was made similarly on half the scale, but the *2,3,4-tri-O-acetyl derivative* (0.27 g, 21%), m.p. 149—150° (from methanol), crystallised out of the reaction mixture (Found: C, 46.35; H, 4.2; N, 6.3. $C_{17}H_{18}N_2O_{12}$ requires C, 46.15; H, 4.05; N, 6.35%).

* *Note added in proof*: Schindler *et al.* (M. Schindler, Y. Assaf, N. Sharon, and D. M. Chipman, *Biochemistry*, 1977, **16**, 423) have recently produced evidence that there is in fact no strain involved in the binding of a 2-acetamido-2-deoxy-D-glucopyranosyl residue in the D subsite of hens' egg-white lysozyme. This seriously weakens the case for attributing a rate enhancement of 10^4—10^8 (out of a total rate enhancement of 10^7—10^9) to steric interactions involving the C-5 hydroxymethyl group.

[3] B. Capon and W. Dearie, *J.C.S. Chem. Comm.*, 1974, 370.
[4] J. H. Baldo, S. E. Halford, S. L. Patt, and B. D. Sykes, *Biochemistry*, 1975, **14**, 1893.
[5] W. P. Jencks, *Adv. Enzymol.*, 1975, **43**, 219.
[6] (*a*) K. Wallenfels and R. Weil in ' The Enzymes,' 3rd edn., ed. P. D. Boyer, 1972, vol. 7, p. 618; (*b*) O. M. Viratelle and J. M. Yon, *European J. Biochem.*, 1973, **33**, 110.
[7] (*a*) M. L. Sinnott and I. J. L. Souchard, *Biochem. J.*, 1973, **133**, 89; (*b*) M. L. Sinnott and S. G. Withers, *ibid.*, 1974, **143**, 752.
[8] F. Ballardie, B. Capon, J. D. G. Sutherland, D. Cocker, and M. L. Sinnott, *J.C.S. Perkin I*, 1973, 2418.
[9] C. S. Hudson, *J. Amer. Chem. Soc.*, 1925, **47**, 265.
[10] J. A. Snyder and K. P. Link, *J. Amer. Chem. Soc.*, 1952, **74**, 1883.
[11] B. Helferich, S. Winkler, R. Gootz, O. Peters, and E. Gunther, *Z. physiol. Chem.*, 1932, **208**, 91.
[12] P. G. Scheurer and F. Smith, *J. Amer. Chem. Soc.*, 1954, **76**, 3224.

Deacetylation of 0.2 g (0.01N-NaOMe; 1 h) gave, on trituration with water, a crude product (120 mg). Two-fold recrystallisation from acetone–ether afforded *2,5-dinitrophenyl α-L-arabinopyranoside acetone solvate*, m.p. 150—155° (decomp.), $[\alpha]_D^{25}$ −33° (*c* 0.15 in H_2O) (Found: C, 44.4; H, 5.2; N, 8.05. $C_{11}H_{12}N_2O_9,C_3H_6O$ requires C, 44.9; H, 4.8; N, 7.5%).

(2,3,4-Tri-O-acetyl-α-L-arabinopyranosyl)pyridinium bromide, m.p. 145—150° (decomp.), $[\alpha]_D^{22}$ +1.7°, was made in 54% yield by literature procedures [13] (Found: C, 45.9; H, 4.95; N, 3.4. $C_{16}H_{20}BrNO_7$ requires C, 45.5; H, 4.8; N, 3.35%). Deacetylation with aqueous 4% hydrobromic acid yielded *α-L-arabinopyranosylpyridinium bromide*, m.p. 140.5—141.5°, $[\alpha]_D^{22}$ +70° (Found: C, 40.9; H, 4.9; N, 4.85. $C_{11}H_{14}BrNO_4$ requires C, 41.2; H, 4.45; N, 4.8%).

Kinetic Measurements.—All kinetic measurements were made at 25.0 °C in the buffer solution obtained when 0.10M-solutions of sodium dihydrogen phosphate and disodium hydrogen phosphate were mixed at 22 °C until a pH of 7.00 was registered on an EIL pH meter, standardised with B.D.H. standard buffer solution. Magnesium chloride (1mM) was added subsequently. Wild-type β galactosidase from *E. coli* as a suspension in 2M ammonium sulphate was purchased from Boehringer Ltd. (lots no. 7500408 and 7423216) or from Worthington Inc. (lot no. N56359). For measurements on alkyl and aryl glycosides, the enzyme was diluted appropriately, but for the exceedingly unreactive arabinosylpyridinium ion the enzyme was dialysed against buffer before use. The hydrolyses of aryl arabinosides were followed spectrophotometrically, at the same wavelengths (same extinction coefficients) as for the aryl galactosides.[7a] That of the pyridinium salt was also followed spectrophotometrically, at 260 ($\Delta\varepsilon$ −306$_4$), 276 (−289), or 282 nm (−87). The high concentrations of pyridinium salt used in the attempt to observe enzyme saturation and the necessity of working with a decrease of absorbance necessitated the use of 1 mm path length cells fitted with a 0.9 mm thick quartz spacer. It proved impracticable to observe enzyme saturation with this salt using its own hydrolysis as a measure of the proportion of the enzyme present as the ES complex. The K_m of the salt was therefore estimated by using it as an inhibitor (I) of the enzymic hydrolysis of 0.5mM- (*ca.* 0.1K_m) 4-nitrophenyl α-L-arabinopyranoside, data being processed by a (nine point) v_0/v against [I] plot whose slope was estimated by a least squares procedure. This procedure was also adopted for measuring the K_m value of methyl α-L-arabinopyranoside. Hydrolysis of methyl α-L-arabinopyranoside was followed by use of a Perkin-Elmer 141 polarimeter fitted with a 1 dm jacketted cell through which water, maintained at 25.0 ± 0.1 °C by a Tecam Tempuni was circulated. Each velocity measurement showed linear behaviour for several hours; mutarotation of arabinose is both comparatively fast and buffer-catalysed, and therefore probably does not interfere. All k_{cat} values are standardised with respect to a k_{cat} value for 2-nitrophenyl β-D-galactopyranoside of 1 000 s^{-1}.

RESULTS AND DISCUSSION

Evidence has been advanced [7] to show that the hydrolysis of aryl galactosides by β-galactosidase proceeds through at least three steps after the formation of the Michaelis (ES) complex. First there is a rearrangement of the ES complex without any covalency change involving the substrate. Then the aglycone is liberated and a glycosyl-enzyme formed. In a third step the glycosyl-enzyme is hydrolysed (the complicated nature of this glycosyl-enzyme is not germane to present discussions). Only exceptionally can the second step be observed with aryl glycosides, so it will not be discussed at this point. However with galactosylpyridinium salts the conformation-change step does not occur and bond-breaking takes place immediately after binding, k_{cat} values being rate constants for bond-breaking. Each of these steps will be considered in turn.

(A) *Conformational step; aryl glycosides.* For the arabinosides as for the galactosides, the k_{cat} values (Table) depend on aglycone nature and not simply on

β-Galactosidase-catalysed hydrolyses of C-5-modified substrates (*T* 25 °C, pH 7.0, $[Mg^{2+}] = 10^{-3}$M, $[Na^+] = 0.14$M)

	α-L-Arabinopyranosides		β-D-Galactopyranosides [a]	
Aglycone	k_{cat}/s^{-1}	K_m/mmol l^{-1}	k_{cat}/s^{-1}	K_m/mmol l^{-1}
Phenol	13	2.3 ± 0.2	70	0.087
2-Nitrophenol	39	1.62 ± 0.15	1 000	0.10
3-Nitrophenol	201	1.91 ± 0.15	893	0.22
4-Nitrophenol	209	4.9 ± 0.5	156	0.028
2,4-Dinitrophenol	207	2.5 ± 0.6	1 290	0.16
	(k_{cat}/K_m = 52.6)			
2,5-Dinitrophenol	122	0.48 ± 0.04	1 084	0.43
3,5-Dinitrophenol	275	2.50 ± 0.15	1 144	0.48
Methanol	1.3 [b]	65 [c]	76 [d]	8 [d]
Pyridine	0.23	84 [c]	0.6	1.25 ± 0.25
	($k_{cat}/K_m = 2.76 \times 10^{-3}$)			

2,4-Dinitrophenyl 6-deoxy-β-D-galactopyranoside has a k_{cat}/K_m value of 2 and its 6-chloro-derivative one of 7.

[a] Taken from ref. 7, except where stated. [b] From polarimetric measurements. [c] From K_m measurements against 4-nitrophenyl α-L-arabinopyranoside. [d] Taken from J.-P. Tenu, O. M. Viratelle, J. Garnier, and J. Yon, *European J. Biochem.*, 1971, **20**, 363.

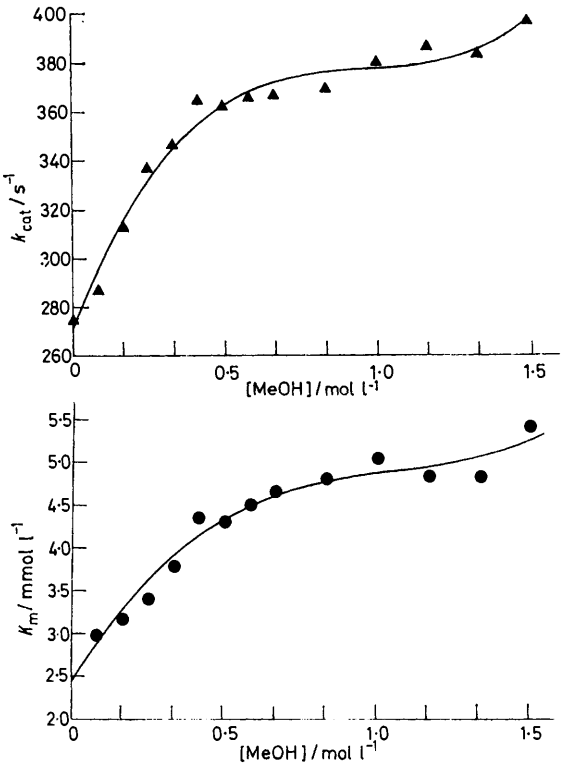
aglycone acidity. The k_{cat} values for arabinosides are usually smaller than those for the galactosides of the same aglycone,[7] but no prediction can be made as to the reactivities of the arabinosides from a knowledge of k_{cat} values of the galactosides. Thus, the 2-nitrophenyl arabinoside is hydrolysed 25 times slower than the galactoside, the 3-nitrophenylarabinoside 4.5 times slower, and the 4-nitrophenyl arabinoside 34% faster. Any residual notion that bond-breaking in the hydrolysis of aryl glycosides is indeed rate-limiting, and that the erratic behaviour of k_{cat} with aglycone structure represents unfavourable interactions of the aglycone with protein and/or catalysing groups, cannot accommodate these data. Values of K_m appear to be between 10 and 100 times higher for the arabinosides.

Bond-breaking step; hydrolysis of α-L-arabinopyranosylpyridinium bromide. The k_{cat}/K_m value for the arabinosylpyridinium salt is *ca.* 10^2 times smaller than for the galactosyl derivative, but this is almost entirely accounted for by the poor binding of this compound. The k_{cat} value—which we assume by analogy with the galactosyl compounds to represent bond breaking—

[13] E. Fischer and K. Raske, *Ber.*, 1910, **43**, 1750.

is only slightly less than for the galactosyl compound. The catalytic efficiency of the enzyme against the arabinosyl salt is not quite as close to that against the galactosyl salt as crude k_{cat} values would indicate, as the arabinopyranosyl cation is more stable than the galactopyranosyl cation: the spontaneous hydrolysis rate of the α-L-arabinopyranosylpyridinium ion is *ca.* 3 times faster at 25 °C than its galactosyl analogue.[1] Nonetheless, any major contribution from the type of strain induction proposed to play a part in lysozyme catalysis does not operate with β-galactosidase.

A speculative rationale for the origin of this difference can be put forward on the basis of the fact that lysozyme



Effects of [MeOH] on k_{cat} and K_m for 3,5-dinitrophenyl α-L-arabinopyranoside

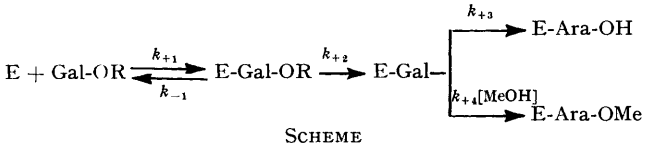
is a small extracellular enzyme with a polymeric substrate, but β-galactosidase is a large intracellular enzyme with, when *E. coli* is growing on lactose as sole carbon source, an important function in the viability of the bacterium. It is to the advantage of an organism secreting lysozyme that as much damage to invading gram-positive bacteria is done with as little expenditure of protein as possible. For this reason a very small protein is produced which can introduce strain in the pyranose ring at which cleavage of the cell-wall polysaccharide takes place by using the binding energy of adjacent saccharide residues; the contribution of binding in the D subsite to overall binding energy is negligible. Such a mechanism is only possible for a glycosidase acting upon a polysaccharide substrate; for disaccharide hydrolysis a much larger enzyme, capable of destabilising the substrate by interaction with only one pyranose ring, must be constructed. It is perhaps significant that the other β-galactosidase of *E. coli*, the evolved *ebg* enzyme, also has sub-unit molecular weight close to that of the *lacZ*-gene enzyme.[14] The reason why, nonetheless, the enzyme does not appear to use the C-5 hydroxymethyl group to destabilise the pyranose ring relative to the cation may be that some ability to hydrolyse α-L-arabinopyranosides is advantageous to the micro-organism, which can certainly possess inducible enzymes for the catabolism of L-arabinose.

Deglycosylation step. The technique of nucleophilic competition with methanol was applied to the fastest reacting α-L-arabinopyranoside discovered, the 3,5-dinitrophenyl.[15] The Figure shows the effects of methanol concentration on k_{cat} and K_m for this compound. Such an increase in k_{cat}—and large increase in K_m—is diagnostic of deglycosylation being at least partly rate limiting. Similar curves are observed for fast-reacting aryl galactopyranosides, but not for slow-reacting ones, nor are they observed for fast-reacting aryl galactosides when productively non-nucleophilic organic solvents, such as dioxan, are added. The dearabinosylation rate (k_{+3}) cannot be calculated simply from $k_{cat} = k_{+2}k_{+3}/(k_{+2} + k_{+3})$, assuming the plateau region at high methanol concentrations to represent the k_{+2} step, since even with substrates for which deglycosylation is completely rate-limiting some curving-over of plots of k_{cat} against [MeOH] is observed, due to saturation of binding sites for glycosyl acceptors—such as methanol—and/or non-specific solvent effects.[15] We therefore looked at the conformity of the initial gradient of the plot of k_{cat} against [MeOH] to that predicted from the methanol inhibition of methyl arabinoside hydrolysis. We had

$$k_{cat} = a + b[MeOH] + c[MeOH]^2 + d[MeOH]^3 \quad (i)$$

$$a/b = k_{+3}/k_{+4}[k_{+2}/(k_{+2} + k_{+3})] \quad (ii)$$

shown elsewhere that for a simple two-step mechanism if the curve of k_{cat} against [MeOH] was fitted to a polynomial (i) then equation (ii) applied, where constants of individual steps are given by the Scheme. Even



SCHEME

though acceptor sites were being saturated and non-specific solvent effects operating, k_{+3}/k_{+4} could be estimated independently from the inhibition of the enzymic hydrolysis of methyl galactoside by methanol under conditions of enzyme saturation with substrate; k_{+3}/k_{+4} was given by the ratio of gradient to intercept of a plot of $1/v$ against [MeOH]. In the case of methyl α-L-arabinopyranoside, the concentration necessary for saturation with substrate was 0.59M. At this high concentration the value of k_{+4}/k_{+3} obtained (1.3 ± 0.3

[14] B. G. Hall, *J. Mol. Biol.*, 1976, **107**, 71.

[15] M. L. Sinnott and O. M. Viratelle, *Biochem. J.*, 1973, **133**, 81.

l mol^{-1}) may be different from that obtaining in more dilute solutions: nonetheless, comparison of this value with that of a/b (1.17 l mol^{-1}) indicates that the de-arabinosylation rate is *ca.* 300 s^{-1}, whereas simply regarding the curvature of the k_{cat} *vs.* [MeOH] plot as due to change in rate-limiting step gives a value of 800 s^{-1}. The degalactosylation rate is 1 300 s^{-1}, so the rate of hydrolysis of the glycosyl-enzyme is decreased by removal of the C-5 hydroxymethyl group by about the same small factor as C–N bond fission. This hydroxymethyl group clearly then plays little part in the substrate destabilisation responsible for catalysis.

Other C-5 *substituents*. The 2,4-dinitrophenyl β-glycosides of 6-chloro-6-deoxygalactose and of fucose were insufficiently soluble in water for any saturation of the enzyme to be observed, but the k_{cat}/K_m values (Table) indicate that they are relatively poor substrates for the enzyme. In the light of the foregoing data on arabinosides, it is more probable that this reflects poor binding than poor catalysis once bound.

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