The catalytic consequences of experimental evolution

Transition-state structure during catalysis by the evolved β -galactosidases of *Escherichia coli* (*ebg* enzymes) changed by a single mutational event

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1. The first chemical step in the hydrolysis of galactosylpyridinium ions by the evolvent *ebg*^a enzyme is less sensitive to leaving-group acidity than in the case of the wild-type *ebg*^o enzyme, implying less glycone–aglycone-bond fission at the transition state. 2. The first chemical step in the hydrolysis of aryl galactosides by *ebg*^b enzyme is probably less sensitive to leaving-group acidity than in the case of *ebg*^o enzyme, possibly as a consequence of resulting in more effective proton donation to the leaving aglycone. 3. α -Deuterium kinetic isotope effects of 1.1₀ and β -deuterium kinetic isotope effects of 1.0₀ were measured for the hydrolysis of galactosyl-enzyme intermediates derived from *ebg*^a and *ebg*^b enzymes: these effects are not compatible with reaction of the sugar ring through a ⁴C₁-like conformation, or with an ionic glycosyl-enzyme intermediate. 4. The variation with pH of steady-state kinetic parameters for hydrolysis of *p*-nitrophenyl galactoside by *ebg*^a and *ebg*^b enzymes and of 3-methylphenyl β -galactoside, 3,4-dinitrophenyl β -galactoside and β -galactosyl-3-bromopyridinium ion by *ebg*^b enzyme was measured. The steep, non-classical, fall in activity against *p*-nitrophenyl galactoside at low pH observed with *ebg*^o and *ebg*^a enzymes is not observed with *ebg*^b enzymes.

INTRODUCTION

Escherichia coli cells cannot normally grow on lactose when the *lacZ* gene, which codes for β -galactosidase, is deleted. However, it was found that cells with *lacZ* deletions selected for growth on lactose had produced a new β -galactosidase, for which the name *ebg* enzyme, an acronym for evolved beta galactosidase, was coined (Campbell *et al.*, 1973; Arraj & Campbell, 1975; Hall & Hartl, 1975). The *ebg* gene maps at 66 min on the *E. coli* chromosome, whereas *lac* maps at 8 min (Bachmann *et al.*, 1976). The *ebg* system consists of a repressor encoded by *ebgR* and at least two structural genes, *ebgA* and *ebgB* (Hall & Hartl, 1975; Hall & Zuzel, 1980). Hall (1976) isolated *ebg* enzyme protein and deduced it to be a hexamer of subunit *M_r* 120000. The *ebgA* gene indeed encodes a peptide of *M_r* 119172 (Stokes *et al.*, 1985). Recently, however, a third structural gene, *ebgC*, has been detected, whose start overlaps the stop codon of *ebgA* and whose expression is necessary for a lactose-positive phenotype (P. W. Betts & B. G. Hall, unpublished work). Gel electrophoresis, under denaturing conditions, of *ebg* enzymes isolated in this laboratory always showed a fragment of *M_r* approx. 15000 (S. Osborne & P. J. Smith, unpublished work) even when isolated in the presence of phenylmethanesulphonyl fluoride (L. Hosie & M. L. Sinnott, unpublished work). This fragment had an amino acid composition very roughly corresponding to that calculated for the 19000-*M_r* peptide encoded by the *ebgC* gene (P. J. Smith, unpublished work; P. W. Betts & B. G. Hall, unpub-

lished work). The *ebg* enzymes are therefore not simple homohexamers.

lacZ-deleted mutants cannot grow on lactose because the enzymic product of the wild-type β -galactosidase gene, *ebg*^o, is too catalytically feeble (Hall & Hartl, 1974) and the wild-type repressor is too insensitive to β -galactosides (Hall, 1978). The spontaneous appearance of β -galactosidase activity is associated with simultaneous mutations in the *ebgR* gene and the structural genes coding for the β -galactosidase, and the system has been used extensively as a model for the study of acquisitive evolution (reviewed by Hall, 1982). Hall (1976) demonstrated incisively that in this system the fitness of the organism was directly related to the catalytic competence of a single enzyme, when he showed that a linear relationship existed between *k_{cat}*/*K_m* for hydrolysis of lactose by isolated enzyme and growth rate on lactose of the bacterial strain from which the enzyme was isolated. Measurement of the Michaelis–Menten parameters for ten substrates hydrolysed by each of 17 isolated *ebg* enzymes enabled the mutant enzymes to be placed into five classes (Hall, 1981). Single mutational events selected by growth on lactose gave rise to a class I mutant, of which a typical representative is *ebg*^a, whereas single mutational events selected by growth on lactulose gave rise to a class II mutant, of which a typical representative is *ebg*^b. Class IV mutants are the consequence of a mutational event of each type: a typical representative is *ebg*^{ab}. (The wild-type and a triple mutant accounted for the remaining two classes.)

Like the *lacZ* enzyme, *ebg*^o enzyme gives β -galactopyranose as first product (Burton & Sinnott, 1983). A double-displacement mechanism is therefore likely:

For such a mechanism, $k_{\text{cat.}} = k_{+2}k_{+3}/(k_{+2} + k_{+3})$ and $K_m = (k_{-1} + k_{+2})k_{+3}/k_{+1}(k_{+2} + k_{+3})$, and $k_{\text{cat.}}/K_m$ does not contain k_{+3} .

No substrates were found for ebg^o enzyme for which $k_{+2} \gg k_{+3}$ (Burton & Sinnott, 1983), but it was possible to show that for a number of aryl galactosides hydrolysed by ebg^a and ebg^b enzymes this was so, i.e. hydrolysis of the galactosyl-enzyme was rate-determining (Li *et al.*, 1983). From values of k_{+3} for these two evolvents and values of $k_{\text{cat.}}$ for the best substrates for ebg^o enzymes, we concluded that k_{+3} , a parameter that did not affect fitness, had been slowed down by experimental evolution by at least a factor of 10-fold. Other changes in the free energy profile for the hydrolysis of lactose by ebg^o , ebg^a and ebg^b enzymes that likewise did not affect $k_{\text{cat.}}/K_m$ showed no simple pattern of behaviour (Hall *et al.*, 1983).

Affinity labelling of the ebg enzymes with the suicide substrate 1- β -D-galactopyranosylmethyl-3-*p*-nitrophenyl-triazene led to the identification of a glutamate residue that was probably involved in catalysis (Fowler & Smith, 1983). It was in a peptide homologous with a peptide alkylated by conduritol C epoxide in the *lacZ* enzyme (Herrchen & Legler, 1984). The galactosyl-enzyme intermediate is thus the α -galactosyl ester of a glutamate residue, identifiable from the DNA sequence of the *ebgA* gene as Glu-408 of the *ebgA*-gene product (Stokes *et al.*, 1985).

Burton & Sinnott (1983) studied the variation of $k_{\text{cat.}}$ and $k_{\text{cat.}}/K_m$ for the hydrolysis of aryl galactosides (I) and galactosylpyridinium ions (II) with the acidity of the leaving group. The fact that defensible correlations of $\log k_{\text{cat.}}$ or $\log(k_{\text{cat.}}/K_m)$ with pK_a were obtained for both sets of substrates, coupled with the existence of deuterium kinetic isotope effects of about 10% on $k_{\text{cat.}}$ for a representative of both classes of substrate, indicated that k_{+2} governed both $k_{\text{cat.}}$ and $k_{\text{cat.}}/K_m$ for the substrates studied. It was therefore possible to use the values of structure-reactivity parameters and isotope effects to deduce the structure of the first chemical transition state for this enzyme. We now report analogous studies on ebg^a and ebg^b enzymes, undertaken to detect any change in transition-state structure as a consequence of experimental evolution.

The results are discussed in terms of the gradients of $\log(\text{rate parameter})$ -versus- pK_a plots, i.e. β_{lg} values. By analogy with the convention for reporting kinetic isotope effects on enzyme reactions (Cleland, 1982), we describe these parameters as $\beta_{\text{lg}}(V)$ or $\beta_{\text{lg}}(V/K)$ according to whether the correlation is with $k_{\text{cat.}}$ or $k_{\text{cat.}}/K_m$. β_{lg} values measure the net change in charge at the leaving group from ground state [free substrate in the case of $\beta_{\text{lg}}(V/K)$ or the enzyme-substrate complex in the case of $\beta_{\text{lg}}(V)$] to transition state. In the case of glycosylpyridinium ions (II) this is affected only by the degree of C-N cleavage, whereas for aryl glycosides it is increased by any proton donation to the leaving group as well as made more negative by increasing C-O cleavage (see, e.g., Sinnott, 1984).

We have also probed the structure of the transition state for the second chemical step, degalactosylation, of ebg^a and ebg^b enzymes by measurement of α - and β -deuterium kinetic isotope effects on $k_{\text{cat.}}$ for a substrate for which $k_{+2} \gg k_{+3}$. α -Deuterium kinetic isotope effects measure the degree of rehybridization at the reaction centre, and hence the degree of oxocarbenium ion character at the transition state, although the precise value is critically dependent on the nature of any preassociated nucleophile (Craze *et al.*, 1978; Knier & Jencks, 1980). β -Deuterium kinetic isotope effects have their origin in a hyperconjugative weakening of a β -C-H bond vicinal to an electron-deficient centre. They are geometry-dependent, being at a maximum when the C-H bond and the electron-deficient p orbital are parallel and zero (or even slightly inverse) when they are orthogonal (Cleland, 1982).

The pH variation of $k_{\text{cat.}}$ and $k_{\text{cat.}}/K_m$ for hydrolysis of various types of substrate by ebg^a and ebg^b enzymes is also reported.

METHODS AND MATERIALS

Enzymes, unlabelled substrates, and procedures for determining Michaelis-Menten parameters have been described previously (Burton & Sinnott, 1983; Li *et al.*, 1983). 3,4-Dinitrophenyl β -D-[1- ^2H]- and β -D-[2- ^2H]-galactopyranosides were made from the appropriately labelled 2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl bromide ('acetobromogalactose') as described by Sinnott *et al.* (1978). The preparation of acetobromo[1- ^2H]-galactose is given by Sinnott & Souchard (1973); that of acetobromo[2- ^2H]-galactose was the exact analogue of the preparation of acetobromo[2- ^2H]-glucose given by Bennet & Sinnott (1986). The resonances of residual protium in the deuterated acetobromogalactose precursors could not be detected at 60 MHz. The melting points (with decomposition) of the thrice-recrystallized 3,4-dinitrophenyl galactosides used for kinetic isotope measurements were (unlabelled) 189–193 °C, (1- ^2H -labelled) 188–190 °C and (2- ^2H -labelled) 186–189 °C, and were unchanged on further recrystallization (from ethanol).

Kinetic isotope effects on $V_{\text{max.}}$ were measured at 10 K_m (1.2 mM) for ebg^b enzyme, but the limited solubility of 3,4-dinitrophenyl galactoside made it impossible to use a substrate concentration of more than 7 K_m (7 mM) for ebg^a enzyme. Reported values are calculated from zero-order rates of increase of absorbance at 400 nm; quoted errors are standard deviations on at least ten pairs of runs.

Kinetic data at the pH optimum of 7.5 refer to 0.125 M-potassium phosphate buffer containing 5 mM-MgCl₂ and 25 μM -2,2'-bipyridyl at 25 °C. Variation of kinetic parameters with pH at 25 °C was studied with a swamping electrolyte of 0.15 M-KCl, to which 5 mM-MgCl₂ and 25 μM -2,2'-bipyridyl had been added. The buffering agents were 31 mM-potassium phosphate below pH 7.7, 31 mM-imidazole/imidazolium chloride at pH 8, and glycine/HCl above pH 8.5.

$k_{\text{cat.}}$ values are expressed relative to values of 14.8 s⁻¹ and 11.6 s⁻¹ for the hydrolysis of 3,4-dinitrophenyl galactoside by ebg^a and ebg^b enzymes respectively. There is pre-steady-state (Li *et al.*, 1983) and affinity-labelling (Fowler & Smith, 1983) evidence that these values are not seriously incorrect.

pH-dependences of k_{cat} or k_{cat}/K_m were analysed by using the programs BELL and HABELL (Cleland, 1979), translated from FORTRAN into BASIC and run on a BBC Master microcomputer.

RESULTS AND DISCUSSION

Structure-reactivity parameters

Table 1 gives Michaelis-Menten parameters for a series of aryl galactosides and galactosylpyridinium ions hydrolysed by ebg^a and ebg^b enzymes. The fact that, for these evolvants, k_{+3} is slowed down as compared with the wild-type enzyme, ebg^0 , means that k_{cat} values for aryl galactosides of most phenols represent k_{+3} . Indeed, pre-steady-state experiments showed that even for 2-naphthyl galactoside hydrolysed by ebg^b enzyme $k_{+2} > k_{+3}$ (Li *et al.*, 1983).

For this reason the values of $\beta_{\text{lg}}(V)$ for aryl galactosides hydrolysed by ebg^a and ebg^b are close to zero and the correlation coefficients are low (approx. -0.6) (Table 2). These $\beta_{\text{lg}}(V)$ values therefore do not give mechanistic information. Correlations of $\log k_{\text{cat}}$ or $\log(k_{\text{cat}}/K_m)$ with leaving-group $\text{p}K_a$ for pyridinium salts gave correlation coefficients better than -0.97 , and correlations of $\log(k_{\text{cat}}/K_m)$ for aryl galactoside hydrolysis by the evolvants gave coefficients better than -0.95 . We can therefore conclude that glycone-aglycone fission determines k_{cat} or k_{cat}/K_m in these cases, from the very fact that good linear free-energy relationships are observed. However, since k_{cat} values for the better pyridinium salt substrates hydrolysed by ebg^b enzyme are close to k_{+3} , the $\beta_{\text{lg}}(V)$ values for pyridinium salts hydrolysed by this evolvant will be somewhat closer to zero than the intrinsic β_{lg} value.

Table 1. Hydrolysis of β -D-galactopyranosyl derivatives by ebg^a and ebg^b enzymes at 25 °C in 0.125 M-potassium phosphate buffer, pH 7.5, containing 5 mM-MgCl₂ and 25 μ M-2,2'-bipyridyl

For sources of $\text{p}K$ data see Burton & Sinnott (1983).

Leaving group	$\text{p}K$	ebg^a enzyme		ebg^b enzyme	
		k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)
3,4-Dinitrophenol	5.42	14.8	1.03	11.6	0.891
3,5-Dinitrophenol	6.69	15.1	1.26	12.1	0.167
4-Nitrophenol	7.15	15.8	2.99	10.5	0.268
3-Nitrophenol	8.39	14.3	7.10	11.1	0.332
2-Nitrophenol	7.17	13.5	3.08		
4-Cyanophenol	7.95	9.7	6.62	8.4	0.45
4-Bromophenol	9.34	7.5	17.15	8.8	1.02
2-Naphthol	9.51	3.9	10.08	10.3	0.73
Phenol	9.99	10.3	16.6	9.3	1.71
3-Methylphenol	10.09			5.7	1.71
3-Chloropyridine	2.81	2.3	0.36	5.3	0.38
3-Bromopyridine	2.85	2.2	0.41	5.6	0.33
4-Bromoisquinoline	3.31	0.78	0.35	8.3	0.48
Pyridine	5.22	0.096	1.6	0.21	3.1
Isoquinoline	5.40	0.048	1.4	0.072	0.79
3-Methylpyridine	5.70	0.040	1.1	0.040	1.5
3,5-Dimethylpyridine	6.14			0.045	1.9

Table 2. Dependence of steady-state kinetic parameters on leaving-group acidity for hydrolysis of β -D-galactopyranosyl derivatives by ebg enzymes

Data for ebg^0 enzyme are from Burton & Sinnott (1983). Errors are standard deviations.

	ebg^0 enzyme	ebg^a enzyme	ebg^b enzyme
Aryl galactosides			
$\beta_{\text{lg}}(V)$ ($n = 9$, $\Delta\text{p}K = 4.7$)	-0.37 ± 0.12		$-0.04 \pm 0.02^*$
$\beta_{\text{lg}}(V)$ ($n = 8$, $\Delta\text{p}K = 4.6$)	-0.34 ± 0.10	$-0.37 \pm 0.04^*$	$-0.02 \pm 0.01^*$
$\beta_{\text{lg}}(V/K)$ ($n = 9$, $\Delta\text{p}K = 4.7$)	-0.39 ± 0.06		-0.31 ± 0.04
$\beta_{\text{lg}}(V/K)$ ($n = 8$, $\Delta\text{p}K = 4.6$)	-0.51 ± 0.08	-0.37 ± 0.04	-0.28 ± 0.04
Galactosylpyridinium ions			
$\beta_{\text{lg}}(V)$ ($n = 7$, $\Delta\text{p}K = 3.3$)	-0.80 ± 0.10		-0.72 ± 0.07
$\beta_{\text{lg}}(V)$ ($n = 6$, $\Delta\text{p}K = 3.1$)	-0.89 ± 0.08	-0.60 ± 0.03	-0.75 ± 0.09
$\beta_{\text{lg}}(V/K)$ ($n = 7$, $\Delta\text{p}K = 3.3$)	-0.89 ± 0.08		-0.95 ± 0.07
$\beta_{\text{lg}}(V/K)$ ($n = 6$, $\Delta\text{p}K = 3.1$)	-0.96 ± 0.08	-0.81 ± 0.03	-0.99 ± 0.08

* k_{+3} is rate-determining for most aryl galactosides

With the exceptions outlined above, the β_{lg} values in Table 2 are intrinsic to the first chemical step in the action of *ebg* enzymes and two evolvents. Unfortunately, as with most attempted structure-reactivity correlations with enzymic reactions, chemical noise, arising from adventitious interactions of the substrate substituents with the enzyme, results in large error bars on derived structure-reactivity parameters. Changes in transition-state structure can nonetheless be perceived.

The most definite of these is lowering of the β_{lg} value for pyridinium salt hydrolysis in the *ebg*^a evolvent. This is reflected in both V_{max} and V_{max}/K_m correlations and can be interpreted as a change to an earlier transition state involving less C-N fission. According to the Hammond postulate, such a change should be associated with lowering of the energy of the glycosyl-enzyme intermediate with respect to the E· β GalX Michaelis complex in the case of the $\beta_{\text{lg}}(V)$ parameter, or with respect to unbound substrate in the case of the $\beta_{\text{lg}}(V/K)$ parameter. In fact the energies of the Michaelis complexes of galactosylpyridinium ions with *ebg*^a enzyme are decreased, since K_m values are lower (Burton & Sinnott, 1983), whereas the energy of α -galactopyranosyl-*ebg*^a enzyme intermediate is increased (Hall *et al.*, 1983). Such 'anti-Hammond' behaviour is possible only if the reaction co-ordinate involves more than the extension of a single bond (cf. Jencks & Jencks, 1977).

The $\beta_{\text{lg}}(V/K)$ value for aryl galactoside hydrolysis by *ebg*^a enzyme is probably closer to zero than that for *ebg*^a enzyme or *ebg*^b enzyme, but because of the errors on β_{lg} values the conclusion is not completely firm. Since β_{lg} values for the pyridinium salts are the same for *ebg*^b enzyme and for *ebg*^b enzyme, this change is unlikely to be associated with a lesser extent of glycone-aglycone fission and is more likely to arise from a greater degree of proton transfer from *ebg*^b enzyme. Sequence data on the wild-type and mutant *ebgA* and *ebgC* genes (P. W. Betts & B. G. Hall, unpublished work) indicate that the *ebg*^b enzyme-*ebg*^b enzyme change involves only Trp⁹⁸⁸-Cys on the *ebgA* peptide: other class II mutants have this change also. The chemical nature of this mutation makes it likely that any increased proton donation to the leaving group arises from changes in geometry, rather than an effect on the pK of the acid catalytic group: major changes (Asp⁹⁹-Lys and Glu¹⁵⁶-Lys) in subtilisin produce a shift of the (k_{cat}/K_m)-pH profile of only 0.6 pH unit, and that only at low ionic strength (Russell & Fersht, 1987).

Secondary deuterium kinetic isotope effects on the degalactosylation of *ebg*^a and *ebg*^b enzymes

Since k_{+3} determines k_{cat} for hydrolysis of 3,4-dinitrophenyl galactoside by both *ebg*^a and *ebg*^b enzymes (Li *et al.*, 1983), isotope effects on V_{max} for this substrate represent degalactosylation. The α -deuterium effects are direct ($1.09 \pm 0.04_0$ and $1.10 \pm 0.01_0$ for *ebg*^a and *ebg*^b enzymes respectively), but there are no β -deuterium kinetic isotope effects ($0.98_8 \pm 0.02_5$ and $1.00_5 \pm 0.01_8$ respectively being the measured values).

Although some caution has to be exercised in interpretation of the magnitude of α -deuterium kinetic isotope effects, conclusions drawn from their direction are reasonably unambiguous. Since the observed effects are direct, hydrolysis of the galactosyl-enzyme intermediates are therefore associated with a decrease in the co-ordination number of the anomeric centre. The galactosyl-enzymes therefore cannot be ionic; if they

existed as some sort of stabilized ion-pair, inverse deuterium kinetic isotope effects would be observed. The *ebg* enzymes thus join an increasing number of retaining glycosidases for which there is direct experimental evidence that the glycosyl-enzyme intermediate is covalent; these include the *lacZ* β -galactosidase (Sinnott & Souchard, 1973) and the β -glucosidases of *Stachybotrys atra* (Van Doorslaer *et al.*, 1984) and *Alkaligenes faecalis* (Withers *et al.*, 1987).

The magnitude of the deuterium kinetic isotope effect implies a degree of oxocarbenium ion character in the transition state: on a very simple model an α -deuterium kinetic isotope effect of 1.10 at 25 °C suggests that the reaction centre is just over half rehybridized from sp^3 to sp^2 (Bennet & Sinnott, 1986). However, there is no β -deuterium effect. This is difficult to reconcile with reaction of the glycosyl-enzyme intermediate through the ⁴C₁ conformation of the sugar ring, since in this conformation the leaving carboxylate group (of Glu-408) and the β -C-²H bond are antiperiplanar, and hyperconjugative β -deuterium kinetic isotope effects should be maximized (see Fig. 1). One can argue from analogy with the experimentally determined conformations of aldono- δ -lactones (Nelson, 1979; Wałaszczek *et al.* 1982) and δ -valerolactone itself (Philip *et al.*, 1981) that classical boat conformations of glycopyranosyl cations should be comparably accessible to the ⁴H₃ (or ³H₄) half-chair conformations. Were the pyranose ring of the galactosyl-enzyme intermediate to adopt the B_{2,5} conformation (Fig. 1), then the absence of a β -deuterium kinetic isotope effect would become explicable, since the C₍₂₎-H bond and the C₍₁₎-O-Glu⁴⁰⁸ bonds would be close to orthogonal. Reaction through such a conformation would contravene the 'theory of stereoelectronic control' (Deslongchamps, 1983) for the second step of *ebg* enzyme action (although the first step would conform to it if the β -galactosyl derivative were bound initially also in the B_{2,5} conformation). It has elsewhere been argued that the 'theory of stereoelectronic control' represents an over-interpretation of small least-motion effects, which are particularly insignificant in acetal systems (Sinnott, 1988).

pH-dependence of steady-state kinetic parameters

Fig. 2 shows the dependence on pH of k_{cat} and k_{cat}/K_m for hydrolysis of *p*-nitrophenyl galactoside by *ebg*^a enzyme. As with the wild-type *ebg*^b enzyme, the variation with pH is non-classical, the fall in these parameters on the acid side of the pH optimum being

Fig. 1. α -Galactopyranosyl esters of a glutamate residue in the ¹C₄ and in the B_{2,5} conformations

Fig. 2. pH-dependence of k_{cat} and k_{cat}/K_m for hydrolysis of *p*-nitrophenyl galactoside by ebg^a enzyme

○, $\log k_{cat}$; ●, $\log(k_{cat}/K_m)$. The lines are calculated by BELL as the least-squares best fit to the two one-proton ionizations of a bell-shaped curve and clearly do not describe the experimental points: the fall on the acid side of the curve is steeper than a one-proton ionization. For discussion see the text.

steeper than would be predicted for a one-proton ionization (Burton & Sinnott, 1983).

Figs. 3, 4 and 5 show the variation with pH of these parameters for hydrolysis of *p*-nitrophenyl, 3,4-dinitrophenyl and 3-methylphenyl galactosides by ebg^b enzyme. Since the substrates do not ionize in the pH region studied, the variation of k_{cat}/K_m with pH, if classical, should represent the ionizations of free enzyme only. Indeed, the pK_a and pK_b values calculated by BELL (Cleland, 1979) from this variation are respectively 6.6 and 8.6, 6.5 and 8.7, and 6.7 and 8.3. The variation with pH is classical [root mean squares of the deviations of

Fig. 3. pH-dependence of k_{cat} and k_{cat}/K_m for hydrolysis of *p*-nitrophenyl galactoside by ebg^b enzyme

○, $\log k_{cat}$; ●, $\log(k_{cat}/K_m)$. The lines are the least-squares best fit to a single ionization and a bell-shaped curve respectively.

Fig. 4. pH-dependence of k_{cat} and k_{cat}/K_m for hydrolysis of 3,4-dinitrophenyl galactoside by ebg^b enzyme

○, $\log k_{cat}$; ●, $\log(k_{cat}/K_m)$. The lines are the least-squares best fit to a single ionization and a bell-shaped curve respectively.

$\log(k_{cat}/K_m)$ from the calculated line 0.036, 0.024 and 0.087 respectively, compared with 0.49 for the ebg^a enzyme data]. The value of k_{cat} for *p*-nitrophenyl and 3,4-dinitrophenyl galactosides at pH 7.5, and most probably at other pH values, represents k_{+3} . On this interpretation the variation of the degalactosylation rate with pH is sigmoid, with a pK_a calculated by HABELL as 6.2 and 6.5 respectively (root mean squares of the deviations of $\log k_{cat}$, 0.05 and 0.04).

The variation of degalactosylation with pH is thus as expected if degalactosylation proceeds by general base catalysis of the attack of water on α -galactosyl-Glu⁴⁰⁸. Microscopic reversibility suggests that the general base catalytic group is the conjugate base of the group that donates a proton to the leaving aglycone (microscopic reversibility indeed requires this to be so for the hydrolysis of methyl galactoside and the methanolysis of the galactosyl-enzyme).

We studied the variation with pH of the hydrolysis of 3-methylphenyl galactoside by ebg^b enzyme in the hope that this, the slowest-hydrolysed aryl galactoside, would

Fig. 5. pH-dependence of k_{cat} and k_{cat}/K_m for hydrolysis of 3-methylphenyl galactoside by ebg^b enzyme

○, $\log k_{cat}$; ●, $\log(k_{cat}/K_m)$. The lines are the least-squares best fit to a single ionization and a bell-shaped curve respectively.

Fig. 6. pH-dependence of k_{cat} and k_{cat}/K_m for hydrolysis of β -galatosyl-3-bromopyridinium ion by ebg^b enzyme

○, $\log k_{\text{cat}}$; ●, $\log(k_{\text{cat}}/K_m)$. The lines are the least-squares best fits to bell-shaped curves.

provide information on k_{+2} . In the event the variation of k_{cat} with pH is again sigmoid, with a pK_a of 5.9. It seems likely that the pK_a of the nucleophilic Glu⁴⁰⁸ will be in this region.

Fig. 6 shows the variation with pH of kinetic parameters for a representative pyridinium salt by ebg^b enzyme. It is probable that this variation with pH is non-classical: root mean squares of deviations calculated by BELL for $\log k_{\text{cat}}$ are 0.16 and for $\log(k_{\text{cat}}/K_m)$ are 0.078. Classical bell-shaped variations with pH are observed only when the enzyme reacts through only one protonated form of the enzyme, and it is chemically reasonable that cleavage of the C–N bond should take place whether or not the acid catalytic group is protonated. *lacZ* β -galactosidase, for example, shows a non-classical pH-dependence when it hydrolyses pyridinium salts (Withers *et al.*, 1978). Such a 'non-bottleneck' mechanism for the pyridinium ion would explain the difference in pK_a and pK_b calculated by BELL from k_{cat}/K_m for this substrate (7.1 and 9.1) and from the hydrolysis of aryl galactosides. Since the pyridinium salts do not ionize in the pH range studied, if both sets of substrates reacted through bottleneck mechanisms the pK values calculated from k_{cat}/K_m , representing ionizations of free enzyme, should be the same.

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REFERENCES

- Arraj, J. A. & Campbell, J. H. (1975) *J. Bacteriol.* **124**, 849–856
 Bachmann, B. J., Low, K. B. & Taylor, A. L. (1976) *Bacteriol. Rev.* **40**, 116–167
 Bennet, A. J. & Sinnott, M. L. (1986) *J. Am. Chem. Soc.* **108**, 7287–7294
 Burton, J. & Sinnott, M. L. (1983) *J. Chem. Soc. Perkin Trans. 2* 359–364
 Campbell, J. H., Lengyel, J. A. & Langridge, J. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1841–1845
 Cleland, W. W. (1979) *Methods Enzymol.* **63**, 103–138
 Cleland, W. W. (1982) *CRC Crit. Rev. Biochem.* **13**, 385–428
 Craze, G. A., Kirby, A. J. & Osborne, R. (1978) *J. Chem. Soc. Perkin Trans. 2* 357–368
 Deslongchamps, P. (1983) *Stereoelectronic Effects in Organic Chemistry*, p. 39, Pergamon, Oxford
 Fowler, A. V. & Smith, P. J. (1983) *J. Biol. Chem.* **258**, 10204–10207
 Hall, B. G. (1976) *J. Mol. Biol.* **107**, 71–84
 Hall, B. G. (1978) *Genetics* **90**, 673–691
 Hall, B. G. (1981) *Biochemistry* **20**, 4042–4049
 Hall, B. G. (1982) *Evol. Biol.* **15**, 85–150
 Hall, B. G. & Hartl, D. (1974) *Genetics* **76**, 391–400
 Hall, B. G. & Hartl, D. (1975) *Genetics* **81**, 427–435
 Hall, B. G. & Zuzel, T. (1980) *J. Bacteriol.* **144**, 1208–1211
 Hall, B. G., Murray, M., Osborne, S. & Sinnott, M. L. (1983) *J. Chem. Soc. Perkin Trans. 2* 1595–1598
 Herrchen, M. & Legler, G. (1984) *Eur. J. Biochem.* **138**, 527–531
 Jencks, D. A. & Jencks, W. P. (1977) *J. Am. Chem. Soc.* **99**, 7948–7960
 Knier, B. L. & Jencks, W. P. (1980) *J. Am. Chem. Soc.* **102**, 6789–6798
 Li, B. F. L., Osborne, S., Sinnott, M. L. & Yates, D. W. (1983) *J. Chem. Soc. Perkin Trans. 2* 365–369
 Nelson, C. R. (1979) *Carbohydr. Res.* **68**, 55–60
 Philip, T., Cook, R. L., Malloy, T. B., Allinger, N. L., Chang, S. & Yuh, Y. (1981) *J. Am. Chem. Soc.* **103**, 2151–2156
 Russell, A. J. & Fersht, A. R. (1987) *Nature (London)* **328**, 496–500
 Sinnott, M. L. (1984) in *The Chemistry of Enzyme Action* (Page, M. I., ed.), pp. 389–431, Elsevier, Amsterdam
 Sinnott, M. L. (1988) *Adv. Phys. Org. Chem.* **24**, 113–204
 Sinnott, M. L. & Souchart, I. J. L. (1973) *Biochem. J.* **133**, 89–98
 Sinnott, M. L., Withers, S. G. & Viratelle, O. M. (1978) *Biochem. J.* **175**, 539–546
 Stokes, H. W., Betts, P. W. & Hall, B. G. (1985) *Mol. Biol. Evol.* **2**, 469–477
 Van Doorslaer, E., van Opstal, O., Kersters-Hilderson, H. & De Bruyne, C. K. (1984) *Bioorg. Chem.* **12**, 158–169
 Wałaszczek, Z., Horton, D. & Ekiel, I. (1982) *Carbohydr. Res.* **106**, 193–201
 Withers, S. G., Jullien, M., Sinnott, M. L., Viratelle, O. M. & Yon, J. M. (1978) *Eur. J. Biochem.* **87**, 249–256
 Withers, S. G., Street, I. P., Bird, P. & Dolphin, D. (1987) *J. Am. Chem. Soc.* **109**, 7530–7531