A solvent-isotope-effect study of proton transfer during catalysis by *Escherichia coli* (*lacZ*) β -galactosidase

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1. Michaelis–Menten parameters for the hydrolysis of 4-nitrophenyl β -D-galactopyranoside and 3,4-dinitrophenyl β -D-galactopyranoside *Escherichia coli* (*lacZ*) β -galactosidase were measured as a function of pH or pD (pL) in both $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$. 2. For hydrolysis of 4-nitrophenyl β -D-galactopyranoside by Mg^{2+} -free enzyme, V is pL-independent below pL 9, but the V/K_m -pL profile is sigmoid, the pK values shifting from 7.6 ± 0.1 in $^1\text{H}_2\text{O}$ to 8.2 ± 0.1 in $^2\text{H}_2\text{O}$, and solvent kinetic isotope effects are negligible, in accord with the proposal [Sinnott, Withers & Viratelle (1978) *Biochem. J.* **175**, 539–546] that glycone–aglycone fission without acid catalysis governs both V and V/K_m . 3. V for hydrolysis of 4-nitrophenyl β -D-galactopyranoside by Mg^{2+} -enzyme varies sigmoidally with pL, the pK value shifting from 9.19 ± 0.09 to 9.70 ± 0.07 ; V/K_m shows both a low-pL fall, probably due to competition between Mg^{2+} and protons [Tenu, Viratelle, Garnier & Yon (1971) *Eur. J. Biochem.* **20**, 363–370], and a high-pL fall, governed by a pK that shifts from 8.33 ± 0.08 to 8.83 ± 0.08 . There is a negligible solvent kinetic isotope effect on V/K_m , but one of 1.7 on V , which a linear proton inventory shows to arise from one transferred proton. 4. The variation of V and V/K_m with pL is sigmoid for hydrolysis of 3,4-dinitrophenyl β -D-galactopyranoside by Mg^{2+} -enzyme, with pK values showing small shifts, from 8.78 ± 0.09 to 8.65 ± 0.08 and from 8.7 ± 0.1 to 8.9 ± 0.1 respectively. There is no solvent isotope effect on V or V/K_m for 3,4-dinitrophenyl β -D-galactopyranoside, despite hydrolysis of the galactosyl-enzyme intermediate governing V . 5. Identification of the ‘conformation change’ in the hydrolysis of aryl galactosides proposed by Sinnott & Souchart [(1973) *Biochem. J.* **133**, 89–98] with the protolysis of the magnesium phenoxide arising from the action of enzyme-bound Mg^{2+} as an electrophilic catalyst rationalizes these data and also resolves the conflict between the proposals and the ^{18}O kinetic-isotope-effect data reported by Rosenberg & Kirsch [(1981) *Biochemistry* **20**, 3189–3196]. It should be noted that the actual K_m values were determined to higher precision than can be estimated from the Figures in this paper. These are reported in Supplementary Publication SUP 50156 (2 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1990) **265**, 5.

INTRODUCTION

Although partial proton transfer from a group on the enzyme to the leaving aglycone has long been suggested to be a contributor to the catalytic efficiency of glycosidases (Thoma & Koshland, 1960; Vernon, 1967; Capon, 1971; Kirby, 1987), only recently has the first direct evidence for the assistance of aglycone departure from an *O*-glycoside by a glycosidase by means of general acid catalysis been produced (Selwood & Sinnott, 1988). We showed from a linear proton inventory that the pL- (i.e. pH- or pD-)independent solvent-kinetic-isotope effect on V of 1.33 for hydrolysis of 4-nitrophenyl α -L-arabinofuranoside by the α -L-arabinofuranosidase III of *Monolinia fructigena* arose from a single transferred proton.

We now extend these studies to the *lacZ* β -galactosidase of *Escherichia coli*. Like the α -L-arabinofuranosidase studied previously (Fielding *et al.*, 1981), this β -galactosidase hydrolyses its substrates with overall retention of the anomeric configuration, but for the β -galactosidase, unlike for the arabinofuranosidase, substrates are available for which hydrolysis of the intermediate glycosyl enzyme is rate-limiting (Sinnott & Viratelle, 1973). With this enzyme it is therefore possible to probe the role of proton transfer on the second chemical step of a retaining glycosidase. The glycosyl-enzyme is a covalent galactosyl derivative (Sinnott & Souchart, 1973) of Glu-461 (Herrchen & Legler, 1984). A further feature of this enzyme that makes it a suitable vehicle for investigation of proton transfer is that it requires Mg^{2+} ions for acidic or electrophilic catalysis to operate (Sinnott *et al.*, 1978;

Legler & Herrchen, 1983), and so solvent-kinetic-isotope-effect probes of catalytic proton transfer should give a null result with Mg^{2+} -free enzyme.

Although solvent deuterium kinetic isotope effects are the classic probe of proton transfers in non-enzymic reactions, their legitimate extension to an enzyme-catalysed reaction requires recognition of three complexities of enzyme systems (Venkata-subban & Schowen, 1984), as follows.

(1) Most exchangeable protons of a protein have fraction factors around unity (the protons of thiol groups, with fractionation factors around 0.5, are exceptional). However, the fractionation factor for the solvated hydron L_3O^+ is 0.69, so that ionizations of essential groups on the protein are shifted approx. 0.5 pK unit ($3 \times \log 0.69$) higher in $^2\text{H}_2\text{O}$. The pL behaviour of kinetic parameters in both $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ must therefore be determined.

(2) The contribution of rate constants for the chemical step(s) to observed steady-state parameters is often complex.

(3) The observed kinetic isotope effect may be merely a reflection of the consequences for protein conformation of exchange at a large number of sites on the protein. This will be revealed by measurements in mixtures of $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ (‘proton inventories’). In the general case, the variation of rate with isotopic composition is given by the Gross–Butler equation (Kresge, 1964; Gold, 1972; Alberly, 1975):

$$V_0/V_n = \Pi(1 - n + n \cdot \phi_T)/\Pi(1 - n + n \cdot \phi_R)$$

where ϕ_R are the fractionation factors for all the exchangeable

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protons in the ground state and ϕ_T the fractionation factors for the same protons in the transition state; therefore one only sees a kinetic effect from protons whose fractionation factors change. In the absence of participating thiols, then, the right-hand side of the above equation reduces to the numerator, and the determination of the number of protons involved in the transition state becomes relatively simple in that a single proton will produce a linear dependence of V on n (atom fraction of deuterium), two protons a quadratic, three a cubic and so on. An infinite number of protons produces an exponential dependence. In practice the precision of the data limits the models that may be distinguished, but one can usually differentiate among one-, two- and 'many'-proton models. Proton inventories are usually conducted at equivalent pL values (Schowen, 1978) at the plateau regions of the pL-rate profiles, to avoid necessarily imprecise corrections for isotope effects on essential ionizations.

MATERIALS AND METHODS

3,4-Dinitrophenyl β -D-galactopyranoside has been described elsewhere (Sinnott *et al.*, 1978). 4-Nitrophenyl β -D-galactopyranoside was purchased from BDH Chemicals (Poole, Dorset, U.K.). β -Galactosidase was purchased from Boehringer (Uxbridge, Middx., U.K.). The enzyme was further purified on a DEAE-Sephadex A-10 column by using a salt gradient [0.5–2.5% (w/v) NaCl in 0.1 M-Tris/HCl buffer, pH 7.0]; the enzyme was eluted at a salt concentration of about 1.1 M, and was stored as a precipitate in saturated $(\text{NH}_4)_2\text{SO}_4$ solution. When required, a portion of the precipitate was dialysed at 4 °C against 0.1 M sodium phosphate buffer, pH 7.0, containing 145 mM-NaCl and also, for studies with the Mg^{2+} -enzyme, 1 mM- MgCl_2 , or, for studies with the Mg^{2+} -free enzyme, 10 mM-EDTA. The buffer solution was changed three times during the 2-day dialysis to produce the Mg^{2+} -free enzyme, to ensure complete removal of Mg^{2+} .

Water was distilled, deionized and degassed. $^2\text{H}_2\text{O}$ (99.8% ^2H) was obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.) and used without further purification. Ethanol and 1,4-dioxan were distilled before use.

The following buffer systems were employed: Mes/NaOH (pH 5.0–6.5, pD 5.5–7.0); $\text{Na}_2\text{HPO}_4/\text{HCl}$ (pH 6.5–7.5, pD 7.0–8.0); $\text{Na}_4\text{P}_2\text{O}_7/\text{HCl}$ (pH 7.5–8.5, pD 8.0–9.0); $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ (pH 8.5–10.0, pD 9.0–10.5). The final buffer concentration in all cases was 33 mM and the ionic strength was maintained at 0.17 ± 0.02 by addition of 145 mM-NaCl. For the Mg^{2+} -enzyme 1 mM- MgCl_2 was added to all buffers of pH 6.6 or above. Below this pH 5–15 mM- MgCl_2 was added in an attempt to maintain saturation. In the Mg^{2+} -free experiments 10 mM-EDTA replaced the MgCl_2 .

The pH values of buffer solutions were measured with a Radiometer PHM Standard pH-meter equipped with a glass combination electrode. For buffers in $^2\text{H}_2\text{O}$ 0.4 was added to the pH-meter reading.

The proton inventory experiments were carried out in equivalent buffers corresponding to a pH of 6.64. The atom fraction of deuterium (n) was determined gravimetrically.

The hydrolysis of 3,4-dinitrophenyl β -D-galactopyranoside was monitored at 400 nm and that of 4-nitrophenyl β -D-galactopyranoside at 347.3 nm, the isosbestic point for the ionization of 4-nitrophenol ($\Delta\epsilon_{\text{H}_2\text{O}}^{(1)} 2904 \text{ M}^{-1} \cdot \text{cm}^{-1}$, $\Delta\epsilon_{\text{H}_2\text{O}}^{(2)} 2759 \text{ M}^{-1} \cdot \text{cm}^{-1}$), on a Pye-Unicam PU 8800 u.v.-visible spectrophotometer interfaced with a BBC microcomputer and fitted with a thermostatically controlled cell block maintained at 25.0 ± 0.05 °C. Molar absorption coefficients for 3,4-dinitrophenol at 400 nm were measured as a function of pL in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$, and found

to be described by the following equations, the units of ϵ being $\text{M}^{-1} \cdot \text{cm}^{-1}$.

$$\begin{aligned}\epsilon_{\text{H}_2\text{O}}^{(1)} &= (1.637 \pm 0.007) \times 10^4 / (1 + [\text{H}^+]/10^{-5.45 \pm 0.02}) \\ \epsilon_{\text{H}_2\text{O}}^{(2)} &= (1.46 \pm 0.01) \times 10^4 / (1 + [\text{H}^+]/10^{-5.80 \pm 0.03})\end{aligned}$$

These curves were measured by using the same 50 μl syringe to add portions of the same ethanolic stock solution of the phenol to buffer solutions of various pL values. Molar absorption coefficients at each pL were calculated from values of absorbance at three concentrations of 3,4-dinitrophenol. The solvent isotope effect of 12% on the molar absorption coefficient of 3,4-dinitrophenolate is therefore quite unequivocal.

Rate data were collected and rate constants evaluated by using the manufacturers' software. Michaelis–Menten parameters were calculated by using the program HYPER (Cleland, 1979). Initial-rate measurements for the proton inventory were made at a concentration of 0.183 mM and corrected to saturation assuming a linear change in the molar absorption coefficient on moving from $^1\text{H}_2\text{O}$ to $^2\text{H}_2\text{O}$. The programs HBBELL and HABELL (Cleland, 1979) were used appropriately to fit the pL-dependencies of V , V/K and the ionization of 3,4-dinitrophenol. The errors on pK values produced by these programs are standard deviations. The programs were adapted to run on a BBC Master microcomputer. Proton inventory data were analysed by using the SAS (1985) statistical package (Sas Institute, Cary, NC, U.S.A.). k_{cat} values are standardized on a k_{cat} value of 156 s^{-1} for hydrolysis of 4-nitrophenyl β -D-galactopyranoside by Mg^{2+} -enzyme (Sinnott & Souchard, 1973; Sinnott & Smith, 1978).

The α -deuterium kinetic isotope effect on V/K for hydrolysis of 4-nitrophenyl β -D-galactopyranoside by Mg^{2+} -enzyme at pH 7.0 was measured by direct comparison of 13 pairs of first-order rate constants for hydrolysis of 3.2 μM protiated and deuteriated substrate (approx. $0.1 \times K_m$) by the same enzyme solution. The deuteriated substrate is described by Sinnott & Souchard (1973).

RESULTS AND DISCUSSION

Galactosylation of Mg^{2+} -free enzyme

The $\log(k_{\text{cat}}/K_m)$ -pL rate profiles for the β -galactosidase-catalysed hydrolysis of 4-nitrophenyl β -D-galactopyranoside are displayed in Fig. 1. The lines are the least-squares fit of the data to a sigmoidal curve and are described by the following expressions, k_{cat}/K_m having units of $\text{M}^{-1} \cdot \text{s}^{-1}$.

$$\begin{aligned}\log(k_{\text{cat}}/K_m^{(1)}_{\text{H}_2\text{O}}) &= 4 + \log(19.7/1 + 10^{-7.6 \pm 0.1}/[\text{H}^+]) \\ \log(k_{\text{cat}}/K_m^{(2)}_{\text{H}_2\text{O}}) &= 4 + \log(20.7/1 + 10^{-8.2 \pm 0.1}/[\text{H}^+])\end{aligned}$$

Fig. 1. Dependence of k_{cat}/K_m for hydrolysis of 4-nitrophenyl β -D-galactopyranoside by the Mg^{2+} -free β -galactosidase of *E. coli* (*lacZ*) upon pL in $^1\text{H}_2\text{O}$ (○) and $^2\text{H}_2\text{O}$ (●) at 25 °C

Fig. 2. Dependence of k_{cat} for hydrolysis of 4-nitrophenyl β -D-galactopyranoside by the Mg^{2+} -free β -galactosidase of *E. coli* (*lacZ*) upon pL in $^1\text{H}_2\text{O}$ (O) and $^2\text{H}_2\text{O}$ (●) at 25 °C

Fig. 3. Dependence of k_{cat} for hydrolysis of 4-nitrophenyl β -D-galactopyranoside by the Mg^{2+} - β -galactosidase of *E. coli* (*lacZ*) upon pL in $^1\text{H}_2\text{O}$ (O) and $^2\text{H}_2\text{O}$ (●) at 25 °C

The profiles for $\log V$ (Fig. 2) are essentially horizontal, with a slight increase about pH 9. The $^1\text{H}_2\text{O}$ profiles are similar to those reported for phenyl β -D-galactopyranoside by Tenu *et al.* (1971).

Apart from the expected normal solvent isotope effects on essential ionizations, there is no significant solvent isotope effect on V or V/K . This is entirely in accord with our previous conclusion that the Mg^{2+} -free enzyme operated without the application of acid or electrophilic catalysis (Sinnott *et al.*, 1978), which received subsequent support from the work of Legler & Herrchen (1983). These authors discovered that basic *N*-glycosides such as *N*-benzylgalactosylamine were much more tightly bound than *O*-glycosides, but only in the presence of Mg^{2+} , and they advanced a model in which the tight binding arose from protonation (partial or complete) of the nitrogen atom by the catalytic acid group.

Galactosylation of Mg^{2+} -enzyme

The k_{cat} value for 4-nitrophenyl β -D-galactopyranoside (156 s^{-1}) is well below the degalactosylation rate (1300 s^{-1}), and nucleophilic competition with methanol (Sinnott & Viratelle, 1973) confirms that some step(s) before hydrolysis of the galactosyl-enzyme are rate-limiting.

The $\log k_{\text{cat}}$ -pL and $\log(k_{\text{cat}}/K_m)$ -pL rate profiles in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ for 4-nitrophenyl β -D-galactopyranoside are displayed in Figs. 3 and 4 respectively. The curves for V are described by the equations below, in units of s^{-1} :

$$\begin{aligned}\log k_{\text{cat}}(^1\text{H}_2\text{O}) &= \log\{156/(1 + 10^{-9.19 \pm 0.09}/[\text{H}^+])\} \\ \log k_{\text{cat}}(^2\text{H}_2\text{O}) &= \log\{89.7/(1 + 10^{-9.70 \pm 0.07}/[\text{H}^+])\}\end{aligned}$$

Fig. 4. Dependence of k_{cat}/K_m for hydrolysis of 4-nitrophenyl β -D-galactopyranoside by the Mg^{2+} - β -galactosidase of *E. coli* (*lacZ*) upon pL in $^1\text{H}_2\text{O}$ (O) and $^2\text{H}_2\text{O}$ (●) at 25 °C

Fig. 5. Proton inventory for V for hydrolysis of 4-nitrophenyl β -D-galactopyranoside by the Mg^{2+} - β -galactosidase of *E. coli* (*lacZ*) at 25 °C

The $\log(V/K)$ -pL profile is known to be non-classical since the low-pH fall arises from competition between Mg^{2+} and two protons (Tenu *et al.*, 1971). Fig. 4 accords at least qualitatively with this idea, since the low-pH fall occurs at pL values around a full unit higher in $^2\text{H}_2\text{O}$ than in $^1\text{H}_2\text{O}$. However, there is little solvent isotope effect on V/K at its maximum.

There is however a pronounced solvent isotope effect on V but not on V/K . Fig. 5 shows the proton inventory on V , carried out at a buffer ratio that gave a pH of 6.64. Fitting of the data to a straight line gave $\phi_1 = 0.544 \pm 0.002$; fitting to a parabola gave $\phi_1 = 0.45 \pm 0.1$ and $\phi_2 = 1.15 \pm 0.1$. The inventory is thus strictly linear, and the isotope effect arises from a single proton.

The molecular nature of the rate-limiting step in the glycosylation of the Mg^{2+} -enzyme is not clear from the literature, but any mechanism must accommodate the following pieces of evidence obtained in this laboratory in the middle 1970s (summarized in Sinnott, 1978).

1. Hydrolysis of aryl galactosides by the Mg^{2+} -enzyme shows no simple dependence on aglycone pK_a , and α -deuterium kinetic isotope effects on V are accounted for by limiting values of 1.00 for glycosylation and 1.25 for deglycosylation.

2. Hydrolysis of aryl galactosides by the Mg^{2+} -free enzyme is between 1 and 2 orders of magnitude slower, and is largely determined by aglycone pK_a . α -Deuterium kinetic isotope effects on V are accounted for by limiting values of 1.09 for glycosylation and 1.21 for deglycosylation.

Scheme 1.

3. Hydrolyses of galactosylpyridinium ions by both Mg^{2+} -enzyme and Mg^{2+} -free enzyme proceed at comparable rates and are governed exclusively by aglycone $\text{p}K_a$. α -Deuterium kinetic isotope effects on V are in the 1.15–1.20 range.

On the basis of these findings, we suggested that the enzyme could operate with or without acid catalysis, but that for acid catalysis to be operative a conformation change had to take place, and this could only happen with the Mg^{2+} -enzyme (Scheme 1). The direct action of the Mg^{2+} ion as an electrophilic catalyst, for which there is some precedent in model systems (e.g. Clark & Hay, 1973; Przystal & Fife, 1980), was discounted on the basis of n.m.r. relaxation measurements of the methyl protons of methyl β -D-1-thiogalactopyranoside and of β -D-galactopyranosyltrimethylammonium ion in the presence of Mn^{2+} -enzyme (Loeffler *et al.*, 1979), which apparently showed the Mn^{2+} to be 0.7–1.0 nm (7–10 Å) away from the mean position of the methyl protons of the bound inhibitor, and thus too far away to act directly as an electrophilic catalyst.

However, Rosenberg & Kirsch (1981) measured leaving-group ^{18}O kinetic isotope effects of $^{18}(V) = 1.022 \pm 0.002$ and $^{18}(V/K) = 1.014 \pm 0.003$ for 4-nitrophenyl β -D-galactopyranoside hydrolysis by the Mg^{2+} -enzyme. These measurements are undoubtedly experimentally correct (cf. Bennet *et al.*, 1987). They could (just) be reconciled with the mechanism above on the assumption that there was a virtual transition state governing V for 4-nitrophenyl β -D-galactopyranoside, to which loss of nitrophenolate without acid catalysis and the conformation change (i.e. k_{+2} and k_{+3}) both contributed (Sinnott, 1987). On this proposal, however, any solvent isotope effect should arise only from the contribution of the conformation change to the virtual transition state, and this should be the product of a large number of individually small contributions (i.e. many-proton catalysis). We now find that the solvent isotope effect on V arises from one proton.

The mechanism of Scheme 1 must therefore be revised. The alternative proposals of Rosenberg & Kirsch (1981), of reaction of aryl galactosides with good leaving groups via an $\text{S}_\text{N}1$ mechanism and aryl galactosides with poor leaving groups via an $\text{S}_\text{N}2$ mechanism, are unsatisfactory because they require the Mg^{2+} -free enzyme to react via an $\text{S}_\text{N}1$ mechanism with all aryl galactosides, and both Mg^{2+} -enzyme and Mg^{2+} -free enzyme to react via an $\text{S}_\text{N}1$ mechanism with the galactosylpyridinium ions, which are poor substrates.

Oxocarbenium-ion-like transition states are probably a general

feature of glycosidase catalysis, so the limiting α -deuterium kinetic isotope effect of 1.00 on V for aryl galactosides implies that there are no bonding changes at the glycone in the rate-determining step. The ^{18}O kinetic isotope effects for 4-nitrophenyl β -D-galactopyranoside reported by Rosenberg & Kirsch (1981) require no more than that there be bonding changes at the leaving-group oxygen atom. These bonding changes could in principle be protonation or co-ordination of 4-nitrophenolate: indeed, Rosenberg & Kirsch (1981) themselves report an equilibrium effect on the acid dissociation of 4-nitrophenol of $^{18}(K_a) = 1.018$. Co-ordination of 4-nitrophenolate to Mg^{2+} ions is expected to be subject to bigger isotope effects, since Mg^{2+} is heavier than the proton and therefore the effect of ^{18}O substitution on the reduced mass is greater. A suitable model for magnesium 4-nitrophenolate is the magnesium salts of 1-hydroxyanthraquinone and 1,4-dihydroxyanthraquinone studied by Kirszenbaum (1977), in which the Mg -O stretching frequency is about 450 cm^{-1} . If we approximate the Mg -O bond in an enzyme-bound magnesium 4-nitrophenolate by a hypothetical diatomic Mg -O with a stretching frequency of 450 cm^{-1} , then the difference in zero-point energies of ^{24}Mg - ^{16}O and ^{24}Mg - ^{18}O is large enough to give rise to a maximal kinetic isotope effect of 1.039 at 25°C .

It is therefore reasonable to tentatively re-assign the 'conformation change' first proposed in 1973 to the loss of the first product, the aglycone, from the complex with enzyme-bound Mg^{2+} in which it is first formed. Although the n.m.r. measurements have to be discounted (the reasons for so doing being discussed below), this assignment successfully rationalizes our previous kinetic findings (points 1–3, above) and the ^{18}O kinetic isotope effects. It also successfully accounts for the present proton inventory, since it is entirely reasonable that the magnesium 4-nitrophenoxide complex would have to be protonated by a single proton for the aglycone to leave the enzyme active site. The idea that this enzyme uses electrophilic rather than protic catalysis to assist aglycone departure also explains why the contribution to catalysis still operates even on leaving groups, such as 4-nitrophenol itself, that are deprotonated at the operating pH of the enzyme (Sinnott *et al.*, 1978). The mechanism of the Mg^{2+} -enzyme then becomes that of Scheme 2.

The results reported by Legler & Herrchen (1983) on the tight binding of galactosylamine inhibitors to the Mg^{2+} -enzyme are compatible with an electrophilic role for Mg^{2+} , since this ion shows some preference (but less than the proton) for co-ordination with amines over the structurally analogous oxygen

Scheme 2.

Fig. 6. Effects of methanol (○) and dioxan (●) on the k_{cat} value for hydrolysis of 3,4-dinitrophenyl β -D-galactopyranoside by the Mg^{2+} - β -galactosidase of *E. coli* (*lacZ*) at 25 °C and pH 7.0

ligands: in aqueous solution, for example, Mg^{2+} preferentially co-ordinates ammonia rather than water by a factor of 88-fold (Smith & Martell, 1976).

If loss of aglycone from its complex with enzyme-bound Mg^{2+} (k_{+3}) limits V , then glycone-aglycone bond fission (k_{+2}), a step that necessarily precedes this, could in principle limit V/K . There are two tentative indications that this might be so: the absence of a solvent deuterium kinetic isotope effect on V/K and the detectably different ^{18}O kinetic isotope effects on V (1.022 ± 0.002) and on V/K (1.014 ± 0.003). If bond fission dominates V/K for this substrate then a $^{2\text{H}}(V/K_m)$ effect should be observed of similar size to the $^{2\text{D}}(V)$ for the Mg^{2+} -free enzyme. In fact we now report a value of $^{2\text{D}}(V/K)$ 1.07 ± 0.03 for hydrolysis of 4-nitrophenyl β -D-galactopyranoside at pH 7.0, well in line with the value for the Mg^{2+} -free enzyme.

De-galactosylation of the Mg^{2+} -enzyme

Sinnott & Viratelle (1973) described nucleophilic competition experiments at pH 7 on 2,4-, 2,5- and 3,5-dinitrophenyl β -D-galactopyranosides that indicated that k_{cat} values for these substrates represented hydrolysis of the galactosyl-enzyme. The spontaneous hydrolyses of the 2,4- and 2,5-isomers are too fast for extended measurements at alkaline pH or of proton inventories. The 3,5-isomer is no longer readily accessible, since the starting 1,3,5-trinitrobenzene is now perceived as a safety hazard even when wet, and is no longer marketed. We were therefore constrained to repeat in outline the experiments of Sinnott & Viratelle (1973) with the 3,4-isomer (Fig. 6). The increase in k_{cat} with methanol concentration, but not with dioxan, is as observed with the other positional isomers of this substrate, and shows the hydrolysis of the glycosyl-enzyme intermediate is the slow step.

The variation with pL of $\log k_{\text{cat}}$ and $\log(k_{\text{cat}}/K_m)$ for 3,4-dinitrophenyl β -D-galactopyranoside in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ is given in Figs. 7 and 8. The lines are the least-squares fit to a single ionization and are described by:

$$\begin{aligned}\log k_{\text{cat}}(^1\text{H}_2\text{O}) &= \log\{1172/(1 + 10^{-8.78 \pm 0.09}/[\text{H}^+])\} \\ \log k_{\text{cat}}(^2\text{H}_2\text{O}) &= \log\{1089/(1 + 10^{-8.65 \pm 0.08}/[\text{H}^+])\} \\ \log(k_{\text{cat}}/K_m(^1\text{H}_2\text{O})) &= 5 + \log\{63.3/(1 + 10^{-8.7 \pm 0.1}/[\text{H}^+])\} \\ \log(k_{\text{cat}}/K_m(^2\text{H}_2\text{O})) &= 5 + \log\{77.6/(1 + 10^{-8.9 \pm 0.1}/[\text{H}^+])\}\end{aligned}$$

The difference in the V/K profiles obtained with this substrate and for 4-nitrophenyl galactopyranoside suggest that with the very good 3,4-dinitrophenyl leaving group the reaction is proceeding through more than one protonated form of the enzyme ('non-bottleneck mechanism'). There is a slightly inverse solvent isotope effect on V/K .

Fig. 7. Dependence of k_{cat} for hydrolysis of 3,4-dinitrophenyl β -D-galactopyranoside by the Mg^{2+} - β -galactosidase of *E. coli* (*lacZ*) upon pL in $^1\text{H}_2\text{O}$ (○) and $^2\text{H}_2\text{O}$ (●) at 25 °C

Fig. 8. Dependence of k_{cat}/K_m for hydrolysis of 3,4-dinitrophenyl β -D-galactopyranoside by the Mg^{2+} - β -galactosidase of *E. coli* (*lacZ*) upon pL in $^1\text{H}_2\text{O}$ (○) and $^2\text{H}_2\text{O}$ (●) at 25 °C

There is no solvent isotope effect on V , which with this substrate represents hydrolysis of the galactosyl-enzyme. This is consistent with our present proposal that electrophilic rather than acidic catalysis is operating in the glycosylation step, since this would require degalactosylation to proceed by nucleophilic attack of the Mg^{2+} -bound OH^- ion, a process in which there is no proton transfer. Moreover, it rationalizes the recent results obtained by J. P. Richard & J. Beard (personal communication), who found galactosyl-enzyme to transfer the sugar residue to trifluoroethanol more readily than to ethanol. The two alcohols have approximately the same size and shape, but trifluoroethanol has a pK_a of 12.4 and ethanol one of 16.0. Formation of an enzyme-bound magnesium alkoxide complex will therefore be much readier for trifluoroethanol than for ethanol, although this will partly be offset by its lower reactivity once formed. However, the absence of a solvent isotope effect for degalactosylation does not necessarily rule out general base catalysis of the attack of water, since Dietze & Jencks (1989) have discovered a model system in which such a reaction takes place without an appreciable solvent isotope effect.

Evidence against an electrophilic role for Mg^{2+}

Three sets of experiments at first sight argue against our present tentative proposal for an electrophilic role for Mg^{2+} . The first is the n.m.r. experiments of Loeffler *et al.* (1979), the second

the stereochemical experiments of Lehmann & Schlesselmann (1983) and the third site-directed mutagenesis experiments of Bader *et al.* (1988) and Ring *et al.* (1988).

Loeffler *et al.* (1979) replaced Mg^{2+} in the enzyme with Mn^{2+} , and studied the effect on the relaxation of the methyl protons of methyl 1-thio- β -D-galactopyranoside and β -D-galactopyranosyltrimethylammonium bromide as they bound to the enzyme. The conclusion, that the Mn^{2+} ion was 0.7–1.0 nm (7–10 Å) away from the mean position of the methyl protons of the bound inhibitor, depends on the assumptions that the Mg^{2+} and Mn^{2+} bind at the same site and that the inhibitors bind in the same way as substrate binds productively. At least in the case of the quarternary ammonium salt inhibitor, this cannot be so, since in the enzyme-inhibitor complex the aglycone nitrogen atom can make no contacts with any atom of the protein. The active site of the enzyme must be flexible, and indeed *o.r.d.* evidence for localized conformation changes during catalysis by β -galactosidase has been gathered (Viratelle & Yon, 1980). The necessary assumptions of our n.m.r. experiments, that the inhibitors bound to the same site and in the same way as the substrate bound productively, and that the Mn^{2+} bound at the same site as the Mg^{2+} , are probably incorrect.

Lehmann & Schlesselmann (1983) found that the enol ether (I) was hydrated efficiently by the enzyme, and that the proton in the product came from above the plane of the molecule as drawn. Given the different location of the exocyclic methylene carbon compared with an aglycone oxygen atom of a glycoside, however, it is not unreasonable to suggest that protonation of this substrate takes place through a water chain or even by a water molecule co-ordinated to the electrophilic Mg^{2+} .

Ring *et al.* (1988) replaced tyrosine-503, the residue originally suggested by Sinnott (1978) to function as an acid catalyst, with phenylalanine by the techniques of site-directed mutagenesis, and found that k_{cat} and k_{cat}/K_m for 2- and 4-nitrophenyl galactosides were decreased by between $2\frac{1}{2}$ and $3\frac{1}{2}$ orders of magnitude, depending on the substrate and whether k_{cat} or k_{cat}/K_m was considered. Although the work undoubtedly indicates that tyrosine-503 is in some way essential for catalysis, the precedents of both chemical model systems and other site-directed mutagenesis experiments suggest that the effect is too big to be attributed simply to the abolition of proton donation to a nitrophenolate leaving group. [Mutation of the nucleophilic glutamate residue to glutamine, in mechanistic terms a rather more drastic change, in fact has a marginally less pronounced effect (Bader *et al.*, 1988).]

Proton donation to a leaving nitrophenolate by a group of higher pK, such as the simple phenol moiety of a tyrosine residue,

necessarily cannot be complete, and must involve only hydrogen-bonding. In the model system (II) of Hibbert & Spiers (1989), where the pK_a values of the proton donor and the leaving group are matched, intramolecular proton transfer contributes a rate acceleration of only a factor of 40-fold.

In the case of enzymic systems, complete abolition of the histidine residue that donates a proton to the enolate intermediate in triose-phosphate isomerase (Nickbarg *et al.*, 1988) has a marginally smaller effect than the tyrosine-503/phenylalanine change in β -galactosidase, as does replacement by a serine residue of the carboxylic acid group that donates a proton to nitrogen in dihydrofolate reductase (Howell *et al.*, 1986). Alteration of the proton-donation mechanism of lactate dehydrogenase has little effect on simple steady-state parameters (Clarke *et al.*, 1988).

It seems therefore that, although tyrosine-503 plays a crucial role in the mechanism of the enzyme, it is unlikely to be as a simple acid catalyst of glycone-aglycone bond fission. Possibly it may act as the proton donor for product release of aglycone from its first-formed Mg^{2+} complex.

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