Review Letter

IONS, ION-PAIRS AND CATALYSIS BY THE *lacZ* β-GALACTOSIDASE OF *ESCHERICHIA COLI*

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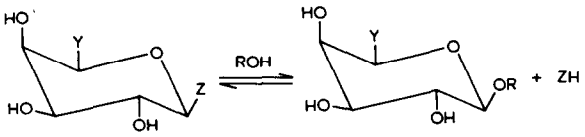
Received 21 June 1978

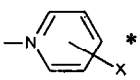
1. Introduction

Any realistic prospect of understanding the catalytic mechanism of an enzyme requires the conjunction of three favourable circumstances: a well-defined enzyme protein, well-defined substrates, and a reaction which is chemically well understood. These three circumstances obtain for the β-galactosidase coded for by the *lacZ* gene of *Escherichia coli*.

This enzyme is a tetramer; the amino acid sequence of the monomer is known [1]. It is a metalloenzyme, binding 1 Mg^{2+}/protomer [2,3]. Its substrates are β-D-galactopyranosyl O-, N- or S-glycosides [4,5], but this specificity is not absolute, and α-L-arabinopyranosyl derivatives are hydrolysed with only marginally less efficiency [6]. All reliable evidence indicates that the enzyme, acting on rigorously purified substrates, obeys strictly Michaelian kinetics, and that the active sites of the tetramer act independently. Since the enzyme exerts its full catalytic activity against monosaccharide derivatives, the complexities encountered with catenases are absent.



Y = CH_2OH(β-D-galactopyranosyl), H(α-L-arabinopyranosyl), CH_2Cl, CH_3

Z = O-alkyl, O-aryl, S-aryl, N_3,  , F

R = H, alkyl, aryl

Scheme 1

The reaction catalysed by the enzyme (scheme 1) is formally a nucleophilic substitution at saturated carbon.

Such reactions have been intensively investigated for many years, and the classical Ingold scheme of unimolecular and bimolecular pathways for fission of a C–X bond [7] has been amplified by the introduction of the concept of a reversibly-formed ion pair [8]. These species are not involved in nucleophilic substitutions at centres which give unstable carbonium ions, but a range of phenomena involving stabilised secondary and tertiary carbonium ions can be neatly rationalised by the use of the following scheme (scheme 2).

$$R{-}X \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} R^+X^- \overset{k_s}{\rightleftharpoons} R^+//X^- \rightarrow \text{products}$$

Scheme 2

The species $R^+//X^-$ is a solvent-separated ion pair, and is identical with the loosely associated complexes proposed to explain the colligative properties of solutions of stable ions. By R^+X^- is meant an intimate ion-pair; the chemical bond is substantially broken, although little solvent reorganisation has taken place [9].

The analogies between the Winstein solvolysis scheme of scheme 2, and the Marcus model for proton transfer, which also involves discrete bond-

breaking and solvent reorganisation steps, has been noted [10].

Since the reaction carried out by β-galactosidase results in retention of configuration about the reaction centre, at least one galactosyl-enzyme intermediate must be involved. The question of the mechanism of action of the enzyme then resolves itself into three component questions:

(i) What is the nature of the galactosyl enzyme?
(ii) What are the number and nature of the molecular events leading to it?
(iii) Why is the formation of the galactosyl enzyme faster than the hydrolysis of galactosyl derivatives in free solution?

2. Nature of the galactosyl-enzyme

The first requirement for the investigation of the galactosyl enzyme is substrates for which degalactosylation is rate-limiting. In principle this could be detected by a 'burst' of aglycone, but k_{cat} values for the faster aryl galactosides hydrolysed by Mg^{2+}-enzyme are around $10^3\ s^{-1}$, beyond the limits of resolution of conventional stopped-flow equipment. However, Viratelle et al. [11] were able to show that with 2- and 3-nitrophenyl galactosides as substrates, degalactosylation was partly rate-limiting: there was an increase in both k_{cat} and K_m for these substrates only, in accord with scheme 3 when methanol is a better nucleophile than water and $k_{+2} \approx k_{+3}$. It was a simple matter to make $k_{+2} \gg k_{+3}$ by adding nitro-groups to the aglycone [12]. The conclusions from nucleophilic competition with methanol were subsequently justified by the observation of a 'burst' under special conditions where it was predicted by this technique: with the product of a point-mutated *Z*-gene [13], at very low temperature [14], and with 2,4-dinitrophenyl galactoside with Mg^{2+}-free enzyme at 4°C [15].

Two independent lines of evidence indicate that the galactosyl enzyme cannot be a single species.

The α-deuterium kinetic isotope effect for degalactosylation (at 25°C) is 1.25 ± 0.04 for Mg^{2+}-enzyme and 1.21 ± 0.03 for Mg^{2+}-free enzyme [16,17]. It is empirically well-established that substitution of hydrogen by deuterium at the reaction centre decreases the rate if there is a change in the geometry about this centre from tetrahedral to trigonal, increases it if there is a change from trigonal to tetrahedral, and has little effect if there is an S_N2 reaction (or no covalency change at all) [18]. The isotope effect on degalactosylation then indicates a decrease in the coordination number of C-1 at the transition state. However, the dependence of the rate of degalactosylation upon the concentration of added nucleophile (methanol) implies an increase in this coordination number. The only way this paradox can be resolved is by a scheme of the following type*:

Galactosyl enzyme (C-1 trigonal) $\xrightarrow{k_{+6}}$ products

k_{-5} ⇅ fast k_{+5}

Galactosyl enzyme (C-1 tetrahedral)

Scheme 4

*It may be possible to reconcile the isotope effect for hydrolysis of the galactosyl-enzyme with the galactosyl-enzyme being only one species if a very 'S_N1-like S_N2' transition state for the hydrolysis of the galactosyl-enzyme is assumed. However, the isotope effect for methanolysis of the galactosyl-enzyme is higher (1.34 ± 0.04) than that for its hydrolysis: i.e., on reaction with a better nucleophile the isotope effect increases [18]. This change is in the wrong direction for an 'S_N1-like S_N2' transition state, since incorporation of better nucleophiles in this transition state should lead to a decrease in S_N1-like character. However, it is readily explained by scheme 4: reaction of the better nucleophile with the C-1 trigonal form of the galactosyl-enzyme will be subject to a lower inverse isotope effect, since the transition state will be more reactant-like. The overall isotope effect, the product of this inverse isotope effect and the equilibrium isotope effect, will then increase

$$E + Gal\,OR \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot Gal\,OR \xrightarrow{k_{+2}} E \cdot Gal \begin{cases} \xrightarrow{k_{+3}} E + Gal\,OH \\ \xrightarrow[k_{+4}]{[MeOH]} E + Gal\,OMe \end{cases}$$

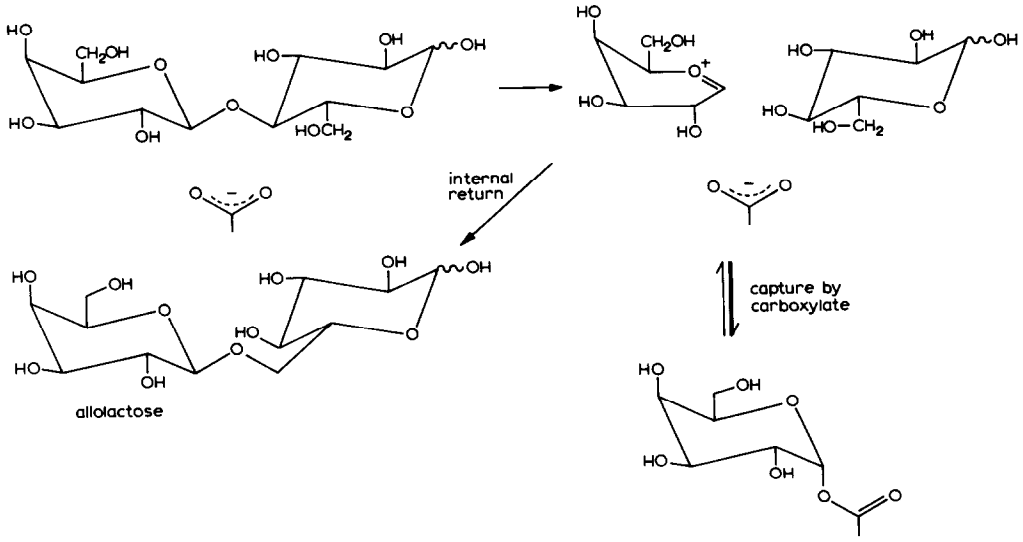
Scheme 3

Evidence that the galactosyl enzyme is really a mixture of species in which more than one type of bond joins C-1 of the galactosyl moiety to the protein can be obtained by consideration of the β-galactosidase-catalysed isomerisation of lactose (4-*O*-(β-D-galactopyranosyl)-D-glucose) to allolactose (6-*O*-(β-D-galactopyranosyl)-D-glucose) (scheme 5). Genetic experiments (allolactose, rather than lactose, is the in vivo inducer of the lactose operon) showed that in vivo the production of allolactose occurred by isomerisation of lactose rather than galactosylation of endogenous glucose [19]. Studies with purified enzyme confirmed that during the isomerisation the glucose portion of the molecule did not become free of the enzyme [20].

β-Galactosidase can also be made to synthesise allolactose by generating the galactosyl enzyme from an aryl galactoside, and adding glucose : allolactose is the predominant, but not exclusive, product. The dissociation constant governing the binding of glucose to the galactosyl enzyme is $10^{-1.5}$ M and the galactosyl-enzyme/glucose complex gives allolactose at a rate around $10^{2.5}$ s^{-1} [21]. If, as is usual, binding of glucose to the galactosyl-enzyme is diffusion controlled, the 'on' rate will be $\sim 10^8$ M^{-1} s^{-1}, and the 'off' rate consequently $10^{6.5}$ s^{-1}. Therefore, in the galactosyl enzyme–glucose complex the glucose will leave $\sim 10^4$ times more rapidly than allolactose is synthesised. This result is incompatible with the direct studies of the conversion of lactose to allolactose, unless the rate constants describing the reactions of the galactosyl enzyme are in fact composite quantities. According to scheme 4 this is the case, the rate being $k_{+6}k_{-5}/k_{+5}$. If the equilibration process is that between the covalent and ion-paired form of an α-galactosyl enzyme, then the lactose–allolactose interconversion is an enzymic example of an 'internal return' process, characterised by k_{-b} in scheme 2.

The other partner in the ion-pair is probably a carboxylate group: ionised carboxylate groups, correctly disposed to act as counterions to glycosyl cations, have been identified in the active sites of a number of glycosidases [22] – including, tentatively, β-galactosidase. β-D-Galactopyranosyldiazomethane also alkylates an active-site carboxylate group [23].

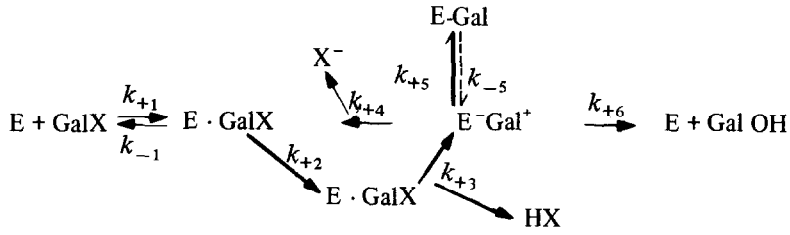
Such ion-pair chemistry is well precedented: ion-pair phenomena have been widely invoked in discussion of kinetic [24] and preparative [25] aspects of reactions at C-1 of pyranosyl derivatives.



Scheme 5

3. Events leading to the galactosyl enzyme

These can be summarised by scheme 6:



Scheme 6

Departure of aglycone can take place from the first ES complex without acid catalysis, or, after a rate-determining conformation change, with acid catalysis. For this conformation change, Mg^{2+} is required: with apo enzyme only the direct pathway is available. Evidence for this kinetic mechanism comes from structure–reactivity correlations and α-deuterium kinetic isotope effects for two sets of substrates, the aryl galactosides and the β-galactosyl pyridinium salts [26].

The molecular structure of these latter substrates precludes the operation of any acid catalysis of the departure of aglycone. With or without Mg^{2+}, their hydrolysis by the enzyme takes the k_{+4} route. As a consequence their hydrolysis is little affected by Mg^{2+} and good correlations of log k_{cat} and aglycone pK_a are observed. A galactosyl cation intermediate, rather than a direct displacement mechanism, is indicated by the identity of α-deuterium kinetic isotope effects for the enzymic hydrolysis (1.13–1.18) and for the S_N1 reaction in free solution [27], and the gradient (β_{lg}) of the log k/pK_a plots. β_{lg} for an S_N1 reaction in free solution is 1.26 ± 0.13 [27], but for an S_N2 displacement of pyridines is 0.4 [28].

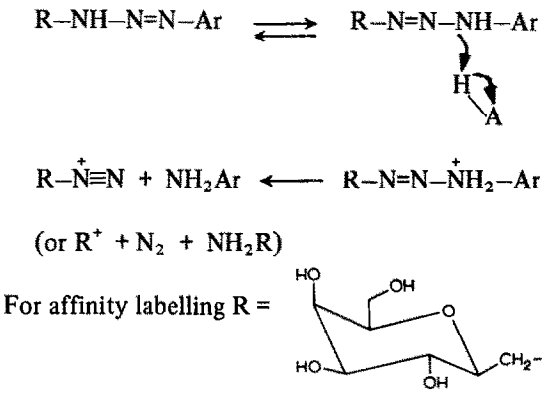
In the absence of Mg^{2+} a fair correlation of log k_{cat} with the pK_a of the aglycone of aryl galactosides is observed. A limiting* α-deuterium kinetic isotope effect of 1.09 for the slowest aryl galactosides is observed, and a β_{lg} value of ~0.6. These values are compatible with a simple S_N1 departure of phenolate anion, as anticipated for the k_{+4} route [17]. In the presence of Mg^{2+}, however, the hydrolysis of aryl galactosides shows little dependence on aglycone acidity [16] (correlation coefficients of –0.6 between log k_{cat}/K_m and aglycone pK_a, compared to a value of –0.05 for the k_{+2} step, are probably a consequence of departure of very acidic dinitrophenolate aglycones by the k_{+4} pathway). The limiting α-deuterium kinetic isotope effect is 1.00 [16]. These data indicate that the rate limiting process does not involve any covalency change. Our initial proposal that this was a protein conformation change [18] has been verified by studies of the interaction of Mg^{2+}-β-galactosidase with an aryl galactoside at very low temperature [29]. The considerably faster hydrolysis of aryl galactosides by Mg^{2+}-enzyme in contrast to the somewhat slower hydrolysis of pyridinium salts requires that some additional catalysis is being applied after the conformation change. The simplest assumption is that this is the operation of an acid catalyst.

If the mechanical requirements for substrate binding are considered, then the model of scheme 6 seems quite unremarkable. The active site is envisaged as a cavity defined by C-4, C-3, C-2 and C-1 of the pyranose ring and an aglycone binding region, with the counterionic group correctly placed. Such a cavity must have some access to bulk solution. If an acid-catalytic group were already in the correct position to donate a proton to the exocyclic oxygen atom, it would quite simply get in the way of approaching substrate molecules.

Some clues as to the nature of the acid catalytic group which we suppose to be put in place by the conformation change were obtained by affinity labelling the enzyme with β-D-galactopyranosyl-

* Because k_{cat} values for aryl galactosides approach the degalactosylation rate, the isotope effect on this will contribute to observed isotope effects on k_{cat}

methyl *p*-nitrophenyl triazene [30]. Alkyl aryl triazenes decompose to give reactive alkyl cations or alkyl diazonium ions [31].



Scheme 7

As active-site reagents they thus owe all their selectivity to the first non-covalent recognition of the active site, and none to the intrinsic selectivity of the alkylating species. Such intrinsic selectivity can be a problem with affinity labelling with, e.g., bromoacetyl derivatives and probably can account for the similar bimolecular rates of inactivation of β-galactosidase by *N*-bromoacetyl glucosylamine and *N*-bromoacetyl galactosylamine [32]: β-D-glucopyranosylmethyl *p*-nitrophenyl triazene has no effect on β-galactosidase [33].

When both Mg^{2+}- and Mg^{2+}-free β-galactosidase are reacted with β-D-galactopyranosyl-[^{14}C]methyl *p*-nitrophenyl triazene, 0.9 mol ^{14}C are incorporated into each 124 000 mol. wt protomer. However, for every apoenzyme active site alkylated 4 molecules of triazene are decomposed; for every 4 Mg^{2+}-enzyme active sites alkylated, 5 molecules of triazene are decomposed [34]. Therefore even in the active site the galactosylmethyl cation is being captured to some extent by water or buffer components. (Both inactivation of enzyme, and catalysis of triazene decomposition, are quantitatively protected against by competitive inhibitors.)

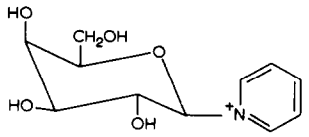
The site of attachment of the galactosylmethyl moiety is exclusively the sulphur atom of Met 500 in both apoenzyme and Mg^{2+}-enzyme. (During work-up the resulting sulphonium salt loses methyl by S_N2 attack at the least hindered position to give a stable *S*-galactosylmethyl homocysteine residue [34,35].) This methionine is presumably the one labelled by Naider et al. [36] and which these authors showed to be catalytically inessential. If it is not, the only remotely plausible function is to replace carboxylate as the group which captures the galactosyl cation during catalysis.

D-Galactal is an inhibitor of β-galactosidase, but binding and release of product are slow [37,38] and the products are 2-deoxy-β-D-galactopyranose, and, in the presence of acceptors, 2-deoxy-β-D-galactopyranosides in which the equatorial proton at C-2 is derived from solvent [39]. Following Hehre [40] we suggest that the pK_a of the counterionic carboxylate group is raised in the presence of the hydrophobic olefinic linkage, and that protonation of the olefin by this group occurs *trans* to the 3-OH. The resultant 2-deoxy-galactosyl cation then is captured by bound acceptor or, with <100% efficiency, by the sulphur of Met 500. Sulphides are however good leaving groups in S_N1 reactions [41], and the stable [42] 2-deoxygalactosyl cation is regenerated slowly.

During decomposition of galactosylmethyl *p*-nitrophenyl triazene in the active site, an electrophilic site is generated in exactly the position occupied by the aglycone oxygen atom. It is to this oxygen atom that any acidic catalysis must be applied, yet the acid-catalytic group is not captured by the electrophile. If it has to be placed in position in a subsequent step, then this is explicable.

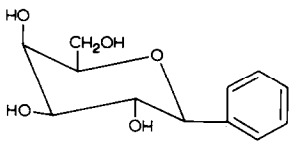
Residue 501 is Tyr, and the precedent of carboxypeptidase [43] makes it an attractive candidate for the role of acid catalyst. Examination of Dreiding models of the dipeptide Met–Tyr indicates that the maximum distance between the methionine sulphur and the phenolic hydroxyl is ~12 Å, but this can be achieved only with an extended conformation with largely hydrocarbon residues of low 'effective dielectric constant' between the two sites. Therefore, since the sulphur atom of Met 500 lies close to the glycone-proximal atom of the aglycone, if this atom carried a positive charge the ionisation of Tyr 501 must influence kinetic parameters.

The influence can be qualified by a study of the isosteric ligands I and II. (The pyridinium salt is a substrate, but a slow one, so $K_i \equiv K_m = K_s$.) Any difference in K_i values must be purely electrostatic in origin [44].



I

K_i^+



II

K_i^0

The ratio K_i^+/K_i^0, measured against Mg^{2+}-enzyme, varies as expected for a single ionisation of pK 8 in the complex with cationic inhibitor and 9.34 in the complex with neutral inhibitor. The change in the pK of the ionisation can also be expressed as a 24-fold decrease in K_i^+/K_i^0 on deprotonation of the whole system. The ionisation of Tyr 501 must influence K_i^+/K_i^0, and only one ionisation is in fact observed. Therefore Tyr 501 has a pK ~9.3 in the EI complex of neutral inhibitor and ~8 in the EI complex of cationic ligand [45]. Ascription of an ionisation in the pH 8–9 region to the acid catalyst would rationalise the differing behaviour of glycosyl pyridinium salts and aryl galactosides in this region: increasing pH somewhat increases k_{cat} values for pyridinium salts [46], but sharply decreases these values for aryl galactosides [47].

If the motion of the acid catalytic group, thought to be Tyr 501, requires Mg^{2+}, then that Mg^{2+} should be close enough to modulate the conformation change, but too far away to participate directly in catalysis. This is the case. Mg^{2+} in proteins can be located by the now standard technique of replacement of Mg^{2+} by the similar paramagnetic Mn^{2+}, and NMR relaxation measurements [48]. It was found that both T_1 and T_2 for the methyl protons of methyl thiogalactoside and of the galactosyl trimethylammonium ion were shortened in the presence of Mn^{2+}-enzyme (in D_2O) more than in the presence of Mg^{2+}-enzyme (in D_2O). Since lines narrowed with increasing temperatures, chemical exchange was fast compared with $1/T_1$ and $1/T_2$. Therefore an approximate distance (8–9 Å) for the distance between the protons in question and the Mn^{2+} could be obtained fairly straightforwardly from the Solomon-Bloembergen equations [46,49].

4. Sources of catalysis

4.1. *Acidic assistance to aglycone departure*

General acid catalysis of aglycone departure has often been proposed as a contributor to catalysis by glycosidases [50]. The ratio of k_{+3} to k_{+4} (scheme 6) should give some idea of the magnitude of this contribution. α-Deuterium kinetic isotope effects give reason for thinking that k_{cat} is close to the rate of aglycone departure for hydrolysis of 2-naphthyl galactoside [16] and galactosyl azide [26] by Mg^{2+}-enzyme. In both cases the ratio of k_{cat} values for hydrolysis by Mg^{2+}- and Mg^{2+}-free enzyme is $\sim 10^2$. The importance of general acid catalysis will increase as the pK_a of the leaving group increases [51], so rate-enhancements similar to those observed in model systems [52] will probably operate with alkyl glycosides.

4.2. *Nucleophilic or electrostatic catalysis*

There is no evidence that there is significant nucleophilic assistance to aglycone departure: the high β_{lg} value for the pyridinium salts and α-deuterium kinetic isotope effects argue against it. This is unsurprising; the galactopyranosyl ring is in some approximation to the $^4C_1$ conformation when it is bound [3] and nucleophilic assistance to the departure of equatorial substituents in a 6-membered ring which is held in the chair conformation is sterically prohibited [53].

The electrostatic stabilisation of the galactosyl cation by a carboxylate group – as proposed for lysozyme [54] lacks chemical precedent. Only insignicant 'electrostatic' stabilisation of the generation of an α-oxocarbonium ion could be detected in a carefully-designed model system [55]. It is more-

over not clear, on the Phillips 'electrostatically stabilised' carbonium ion model [54], what is holding the two charges apart; an unprecedented rigidity of the protein seems to be implied. The electrostatic attraction of two point charges 3 Å apart decreases with separation at 0.17 mdyne/Å, which is of the order of bond bending force constants.

On our model the function of the counterionic carboxylate is rather to capture the highly-reactive glycosyl cation and enable the aglycone to diffuse away.

4.3. *Non-covalent interactions*

Non-covalent interactions account for the major part of the catalytic power of this enzyme. The galactosyl pyridinium salts are substrates against which the whole of the catalytic action must arise by non-covalent interactions, since their chemical structure precludes the operation of an acid catalyst and the available evidence is that a nucleophile is not involved in the rate-determining step. Their enzymic hydrolysis is in fact an enzyme-catalysed S_N1 reaction, the rate of which is given by k_{cat} since these substrates adopt the k_{+4} pathway. The observation [27] of the spontaneous S_N1 hydrolysis of the same compounds makes possible direct measurement of the lowering of the free energy barrier to C-N bond breaking on formation of the ES complex. The lowering of $\Delta G^{\ddagger}$ in the ES complex is huge – in the case of the galactosyl 3-chloropyridinium ion, $10^{10.3}$ in rate or 14 kcal/mol in $\Delta G^{\ddagger}$ at 25°C. This rate enhancement, being the ratio of the rates of 2 unimolecular processes, is truly dimensionless (i.e., $\Delta G^{\ddagger}$ is independent of the choice of standard state). It increases as the leaving group ability declines, i.e., the β_{lg} value for the spontaneous reaction (1.26) is greater than that for the enzymic process (0.9). The system is exhibiting simple Hammond behaviour – as it becomes more reactive, it is less selective [56]. This effect is not very large, but does show that direct comparisons of structure–reactivity parameters for enzymic and non-enzymic reactions should be made with caution.

The absolute rate enhancement brought about by non-covalent forces on aryl galactosides seems similar to that experienced by galactosyl pyridinium salts: the rate of spontaneous, S_N1 hydrolysis of 3,4-dinitrophenyl galactoside is the same as that of the galactosyl 3-chloropyridinium ion [57], and their k_{cat} values for hydrolysis by Mg^{2+}-free-enzyme are also the same.

What are the origins of these non-covalent contributions to catalysis? Steric clashes between the enzyme and the C-5 hydroxymethyl group of bound substrate, which force the pyranose ring to adopt an unfavourable conformation, cannot be important, since k_{+4} and deglycosylation are only insubstantially different with α-L-arabinopyranosyl substrates [6]. Similar clashes, once thought to play a role in lysozyme catalysis [54] now seem not to do so [58,59].

It has been shown that the non-enzymic hydrolyses of those acetals (such as glycosides) which give unstable oxocarbonium ions are unlikely to pass through solvent-equilibrated intermediates: one possibility advanced was rate-determining diffusion away of the leaving alcohol from the reaction centre [60]. Such a mechanism was subsequently shown to obtain for departure of PhS^- from PhCH(OEt)SPh [61].

In an ES complex the substrate necessarily does not have the highly-structured solvent cage it has in free solution. Our current working hypothesis is that the simple fact of the removal of this cage will both decrease internal return, and lower the energy-barrier between intimate and separated ion-pairs, and that this is the primary source of the non-covalent rate-enhancements achieved by glycosidases. This model accounts for the higher leaving group secondary deuterium isotope effects, and higher $\Delta S^{\ddagger}$ values in non-enzymic than in β-galactosidase-catalysed hydrolyses of galactosyl pyridinium salts [27]. Most persuasively, however, it can account for non-covalent rate-enhancements of 10^9 achieved by a glycosidase (the β-glucosidase B of sweet almonds) which is non-specific with respect to aglycone, C-5 and C-4 [62].

Acknowledgements

I thank the United Kingdom Science Research Council and the William Briggs Fund of the Chemical Society for financing this work, and the Royal Society for an equipment grant-in-aid. It is a pleasure to acknowledge the many contributions of my associates, especially Dr P. J. Smith, Mr I. J. L. Souchard and Dr S. G. Withers, the helpful collaboration of Drs A. V. Fowler (UCLA), B. D. Sykes (University of Alberta)

and O. M. Viratelle (Université de Bordeaux II) on various aspects of this work, the advice of Professors H. Gutfreund, W. P. Jencks, M. C. Whiting and J. M. Yon, and information prior to publication from Professor J. Lehmann.

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