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Enzymatic cleavage of glycosides: How does it happen?

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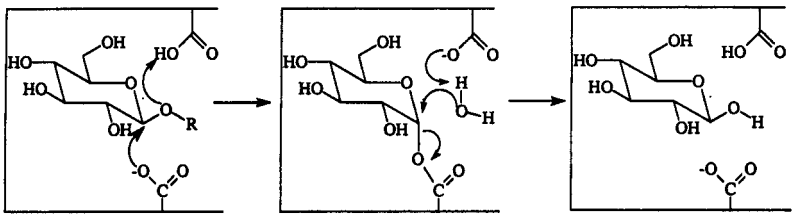
Abstract: Many glycosidases operate through a double-displacement mechanism in which a glycosyl-enzyme intermediate is formed and hydrolysed *via* oxocarbenium ion-like transition states with acid/base catalysis. The two key active site residues involved in this mechanism, the active site nucleophile and the acid/base catalyst have been identified by novel means. The nucleophile is identified using a mechanism-based inactivator which functions by formation of a stabilised glycosyl-enzyme intermediate. Identification of the labelled peptide from proteolytic hydrolysates is achieved using a new tandem mass spectrometric method. The acid/base catalyst is identified by detailed kinetic analysis of candidate amino acids chosen on the basis of sequence similarities.

Glycosidases are a class of enzymes responsible for the hydrolysis of glycosidic linkages[1-4]. They play important roles in a wide variety of medically important biological processes such as digestion, where such enzymes as α-amylase, maltase and lactase are responsible for the degradation of dietary polysaccharides and oligosaccharides to simple monosaccharides which are then absorbed into the bloodstream. Other important roles are in catabolism such as in the lysosomal degradation of glycolipids. Indeed, deficiencies in such enzymes result in serious, and often fatal genetic diseases such as Gaucher and Tay Sachs diseases. A third important role is in glycoprotein processing, where linkage-specific glycosidases are reponsible for the conversion of fully glycosylated precursor glycoproteins into the correct glycoform for their specific function. They are also important in a number of non-medical applications, particularly in biotechnology, where the efficient enzymatic degradation of cellulosic biomass has considerable potential for the generation of alternative energy sources. Another application currently gaining favour is the use of xylanases to assist in the bleaching of wood pulp by degradation of hemicellulose whose presence hinders the decolorisation of lignin contaminants. Two other significant uses of cellulases are as additives to laundry detergents to enhance removal of cellulose-based stains, and as reagents for the 'stone-washing' of denim fabrics!

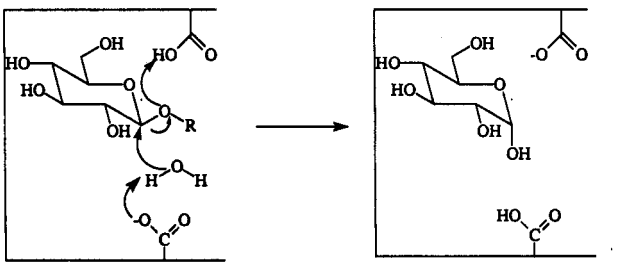
A detailed understanding of the mechanisms of these enzyme is important not only academically, but also for manipulation of their activities through protein engineering or for the design of new selective inhibitors to control their activities. This lecture will focus upon the structures and particularly the mechanisms of these enzymes, as well as upon methods devised for control of this activity. The focus will be upon enzymes currently under study in the author's laboratory, principally a β-glucosidase from *Agrobacterium faecalis*, which has been a "test bed" enzyme for the last 10 years for this group, a cellulase/xylanase from *Cellulomonas fimi* (Cex) and a xylanase from *Bacillus subtilis*. However, in a number of cases, other enzymes have been studied to similar extents, and equivalent results obtained, thus the conclusions are not necessarily specific to the enzymes mentioned, but general for the class.

Glycosidases fall into two major mechanistic categories: those which hydrolyse the glycosidic bond with net inversion of configuration (inverting enzymes) and those which do so with net retention of anomeric configuration (retaining enzymes) [3]. Likely mechanisms for these two enzyme classes were proposed by Koshland[5] over 40 years ago, and have largely stood the test of time. Although the two mechanisms are distinctly different, they do retain a number of features in common, as illustrated below. Inverting glycosidases are believed to function by a single step mechanism in which a water molecule effects a direct displacement at the anomeric centre as shown below. This displacement process is general acid/base catalysed, with one active site amino acid acting as the general base, helping to

RETAINING MECHANISM



INVERTING MECHANISM



deprotonate the nucleophilic water molecule, and the other amino acid acting as a general acid, protonating the departing oxygen atom in a concerted fashion as the bond cleaves. 3-Dimensional structures are available for several different inverting glycosidases, perhaps the best studied being the cellulases CbhII from *Trichoderma reesei*[6] and E2 from *Thermomonospora fusca* [7] and the glucoamylase from *Aspergillus awamori* [8,9]. In all cases studied it is apparent that the acid and base catalytic groups are the carboxylic side chains of aspartic or glutamic acids. Other studies, particularly those based upon kinetic isotope effects[10], Bronsted relationships[11], or effects of substitution by halogens[12], have provided evidence that the transition state for these enzymes has considerable oxocarbenium ion character.

Retaining glycosidases are generally believed to function through a double displacement mechanism in which a glycosyl enzyme intermediate is formed and hydrolyzed *via* oxocarbenium ion-like transition states as shown above. Again the reaction is facilitated by acid/base catalysis, but in this case it is probable that the same group plays both roles. 3-Dimensional structural information is available on several such enzymes including the "original" glycosidase structure hen egg white lysozyme[13], several α-amylases[14-16], cyclodextrin glucanotransferase[17] and *B. subtilis* xylanase[18,19]. In all cases it is apparent that the two residues involved, the nucleophile and the acid/base catalyst, are again the carboxylic side chains of glutamic and aspartic acid residues. However, in the absence of information on

the orientations of these residues relative to bound substrates or analogues it is often difficult to assign these roles to specific amino acids.

This lecture will focus upon strategies devised by the author's laboratory for the identification of the amino acids which play these particular roles in retaining glycosidases, in the absence of X-ray crystallographic insights. These results will be justified, where possible, by discussion in light of active site structures since determined by means of X-ray crystallography.

Identification of the active site nucleophile

The mechanism for retaining glycosidases involves the formation of a glycosyl-enzyme intermediate in which the sugar is covalently attached to the protein *via* the carboxylic side chain of a glutamic or aspartic acid. In order to identify the nucleophilic amino acid residue it was necessary to develop techniques for the trapping of this intermediate. This required differential manipulation of the rates of formation (glycosylation) and hydrolysis (deglycosylation) of the intermediate such that the rate constant for the deglycosylation step was slowed enormously from its typical values ($t_{1/2}$ around 1-10 ms). In the process it was possible that the rate constant for glycosylation could also decrease, but this step should not end up slower than the deglycosylation step or no intermediate would accumulate.

The desired effect was obtained through the use of 2-deoxy-2-fluoro glycosides with good leaving groups such as dinitrophenolate or fluoride[20,21]. The presence of the fluorine substituent at C-2 slows *both* the glycosylation and deglycosylation steps in two ways. One of these is due to the fact that the hydroxyl substituent at C-2 plays a crucial role in transition state stabilisation in glycosidases by making key interactions (worth more than 8 kcal/mol), probably predominantly hydrogen bonding interactions, with the enzyme active site[22]. Removal, or diminution, of these interactions by replacement of the hydroxyl with fluorine, a substituent of limited hydrogen bonding potential, destabilises both transition states significantly. Indeed this was the basis for the previous use by Legler of p-nitrophenyl 2-deoxy-glucoside to accumulate a glycosyl-enzyme intermediate on the *Aspergillus wentii* β-glucosidase[23]. He subsequently identified the labelled amino acid after denaturation trapping. However, such compounds only rarely function as effective inactivators, since reactivation frequently occurs at rates approaching that of inactivation. The second way in which the fluorine substituent slows the two steps is through inductive destabilisation of the two electron-deficient transition states. Fluorine is much more electronegative than a hydroxyl, thus the positive charge developed at the transition state will be significantly destabilised by the presence of this substituent, with consequent retarding effects upon the rates of both steps. The consequence of these two effects combined is a massive (up to 10^6 - 10^7 fold) reduction in rates of both steps[24,25]. Incorporation of a good leaving group (aglycones as 'reactive' as 2,4-dinitrophenolate or fluoride can be attached to sugar acetal centres and form reasonably stable species) speeds up the glycosylation step relative to the deglycosylation step with the effect that the intermediate is accumulated. Incubation of the enzyme with its corresponding 2-deoxy-2-fluoroglycoside results in time-dependent inactivation, *via* the accumulation of a relatively stable 2-deoxy-2-fluoroglycosyl-enzyme intermediate.

Supporting evidence for this mechanism has been obtained in several ways. Stoicheometric reaction of inhibitor and enzyme have been demonstrated by electrospray mass spectrometry and by measurement of the magnitude of the "burst" of dinitrophenolate released[25]. ^{19}F-NMR studies of the inactivated enzyme have demonstrated the formation of a covalent α-D-glycopyranosyl-enzyme intermediate[26]. The formation of a unique covalent linkage to a specific amino acid has been demonstrated by identification and sequencing of the peptide [27-32] (*vide infra*). Finally, and very

importantly, the catalytic competence of this intermediate has been demonstrated by measuring turnover of the inactivated enzyme *via* hydrolysis of the intermediate, to yield free sugar and enzyme[25,27]. This reactivation can be greatly accelerated by the inclusion of a suitable sugar acceptor into the reactivation mixture, such that turnover occurs *via* transglycosylation, a reaction typical of glycosidases.

Identification of the amino acid residue labelled has been achieved in two distinct ways. The first of these required the synthesis of a radiolabelled version of the inactivator to generate a radiolabelled enzyme. Standard methods of proteolysis, HPLC separation of the resultant peptide mixture, and purification and ultimately sequencing of the labelled peptide were then employed. This strategy has been used successfully in the identification of the active site nucleophiles of the first four glycosidases shown in Table 1.

Table 1. *Sequences of active site nucleophile-containing peptides in glycosidases*

Enzyme	Sequence	Reference
*A. faecalis*β-glucosidase	YITENGA	27
C.fimi exo-glycanase	VRITELD	31
E. coli β-galactosidase	ILCEYAH	28
C. thermocellum endo-glucanase	YCGEF	32
Human glucocerebrosidase	FASEA	30
B. subtilis xylanase	YGETRSPLIEY	29

A new, efficient strategy, which obviates the need for synthesis of radiolabelled versions of the inactivator, has been developed in the author's laboratory recently, and has proved extremely valuable in the rapid identification of the labelled peptides. Again, the inactivated enzyme is subjected to proteolysis and HPLC separation. However in this case the peak containing the labelled peptide is identified by electrospray tandem mass spectrometry, by monitoring for a fragmentation reaction that is specific to the sugar-peptide linkage. This is illustrated below and in Figure 1 using the identification of the catalytic nucleophile in *B. subtilis* xylanase by inactivation of the enzyme with 2',4'-dinitrophenyl β-xylobioside as an example[29].

Peptic hydrolysis of the 2-deoxy-2-fluoroxylobiosyl-labeled xylanase resulted in a mixture of peptides which was separated by reversed phase-HPLC. This chromatogram reveals a large number of peaks, which arise from every peptide in the mixture (Fig.1). The labelled peptide was then identified by using the tandem mass spectrometer in the neutral loss mode. In this method ions are subjected to limited fragmentation in the second quadrupole by collisions with an inert gas (Ar). Under these conditions, homolytic cleavage of the ester linkage between the sugar inhibitor and the peptide occurs, resulting in the loss of a neutral sugar residue of known mass (267), but leaving the peptide moiety with its original charge. Scanning of the two quadrupoles in a linked mode in which they are offset by the desired m/z ratio corresponding to the mass of the sugar lost, permits only those ions differing in mass by that of the lost sugar moiety (267) to pass through both quadrupoles and be detected. If the peptide may bear more than one charge it is also necessary to look for m/z differences of one half, or one third of the mass of the neutral species, depending upon the charge carried. In this case no signal was detected when the spectrometer was scanned in the neutral loss tandem MS/MS mode searching for the mass loss m/z 267. However, a single peak was observed when scanned for the mass loss m/z 133.5 (Fig.1b), yet no such peak was detected in the neutral loss spectrum of the unlabeled xylanase digest (Fig.1c). Thus a doubly charged peptide corresponding to the active site glycopeptide is being selectively detected. A m/z value of 825.5 (Fig.1d) was measured for this peptide, corresponding to a molecular weight of 1649 Da

{(825.5 X 2) - 2H}. Since the mass of the lost sugar is 267, the unlabelled peptide must have a molecular weight of 1383 (1649 - 267 + 1H).

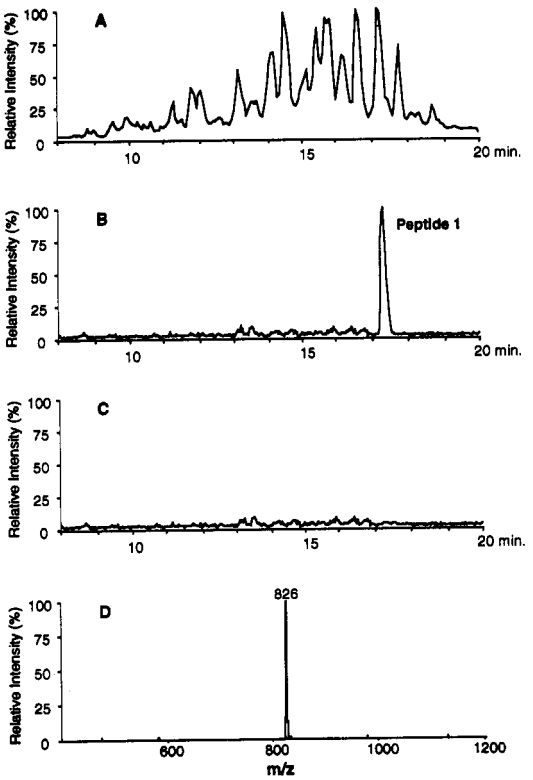


Figure 1. ESMS on xylanase proteolytic digest

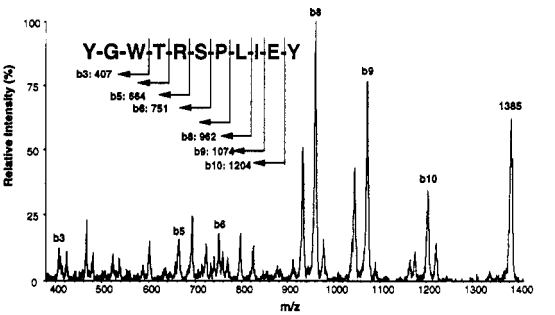


Figure 2. Tandem MS/MS daughter ion on peptide

Upon searching the amino acid sequence of the enzyme for all possible peptides with this mass, only three with a mass 1383 +/- 1 could be identified, namely YGWTRSPLIEY (69-79), GWTRSPLIEYY (70-80), and PLIEYYVVDSW (75-85). They all contain the same Glu^{78} residue and the third peptide also contains an aspartate (Asp^{83}). Absolute identification of the labelled peptide was obtained in a second experiment, without a need for purification, by further fragmentation of the peptide of interest in the daughter ion scan mode. After selection of the parent ion (*m/z* 825.5) in the first quadrupole it was subjected to collision induced fragmentation in the second quadrupole and the masses of the daughter ions were detected in the third quadrupole as shown in Figure 2. The peak at *m/z* 1385 arises from the unlabeled peptide ion in the singly charged state (MH^+) after loss of its sugar. Other peaks represent fragmentation essentially from the C-terminus; N-terminal fragments are not observed since the loss of the charged N-terminal amino acid produces undetected neutral peptides. The peak at m/z 1204 (b10) arises from loss of C-terminal tyrosine (*m/z* = 181) from the parent ion peak at *m/z* = 1385 while the other peaks (b9, b8, b6, b5, and b3) result from the respective losses of EY, IEY, PLIEY, SPLIEY, RSPLIEY, and TRSPLIEY fragments from the C-terminus. This sequence information is sufficient to unambiguously identify the labeled peptide as YGWTRSPLIEY (69-79), thereby indicating that the catalytic nucleophile in *B. subtilis* xylanase is Glu^{78}. This result was confirmed by isolation of the labelled peptide and conventional solid phase sequencing.

This approach has proved highly successful. In addition to confirming results with most of the glycosidases listed in Table 1 the catalytic nucleophiles in both the xylanase[29] and human β-glucocerebrosidase[30] have been identified in this way without ever needing to synthesise the radiolabelled reagent.

Identification of the acid/base catalyst

No reliable strategies have yet been developed for labelling of the acid/base catalyst in glycosidases.

We therefore developed an alternate strategy involving site-directed mutagenesis of conserved acidic residues and detailed kinetic analysis of the resultant mutants[33]. The approach is illustrated with the exo-glucanase/xylanase from *C.fimi* (Cex).

Cex belongs to a family of more than 20 enzymes capable of hydrolysing xylan and cellulose. Hydrolysis occurs with net retention of anomeric configuration[34], thus it is a 'retaining' glycosidase, following a double displacement mechanism[35]. Sequence comparisons permit the identification of a number of conserved amino acid residues in this family, among which are 6 glutamic and aspartic acids, the probable candidates for the acid/base catalyst as noted earlier. As expected, it is found that the catalytic nucleophile Glu233 identified using the fluorosugar approach[31] is indeed one of these conserved residues, thus establishing one of the others as the probable acid/base catalyst. The strategy proposed thus involves mutation of these conserved Glu and Asp residues to alanine, then detailed investigation of the kinetic properties of the mutants so generated. Such an investigation requires the application of several mechanistic tests, as described below.

As noted in previously, the residue in question will function as an acid catalyst in the first step (glycosylation) and as a base catalyst in the second (deglycosylation). Therefore deletion of this residue might be expected to slow down both steps. However if a substrate is used which has a very good leaving group not requiring acid catalysis for its departure, then in that case the first step may not be much compromised for the mutant, but the second step necessarily will be since this step is common for all substrates of a fixed sugar type. Mutants modified at Glu 127 do indeed show this behaviour as can be seen in Table 2.

Table 2: *Kinetic parameters for hydrolysis of various substrates by Cex and E127 mutants*

Enzyme	Substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($min^{-1}mM^{-1}$)
Native Cex	DNPC	419	0.06	6983
	PNPC	677	0.53	1278
	PBrPC	255	2.0	128
E127A	DNPC	2.4	0.0003	7742
	PNPC	2.3	0.025	92
	PBrPC	4.0×10^{-2}	1.9	2.1×10^{-2}

Rates of the first step, glycosylation, were assessed through k_{cat}/K_m measurements since in this system k_{cat}/K_m represents the first irreversible step, which is indeed glycosylation. Rates of deglycosylation were obtained from k_{cat} values of substrates for which deglycosylation was rate-limiting. As can be seen, for an excellent substrate such as 2',4'-dinitrophenyl cellobioside (pK_a of leaving 2,4-dinitrophenol = 4.0) the first step (k_{cat}/K_m) is not significantly slowed by mutation, whereas for a substrate requiring some acid catalysis such as 4'-nitrophenyl cellobioside (pK_a of 4'-nitrophenol =7.0) there is a significant rate reduction, and for substrates with leaving groups of very high pK_a such as 4-bromophenol (pK_a = 10) the rate was much lower again. These results are therefore completely consistent with the role of Glu127 as the acid/base catalyst.

A second consequence of removal of the acid/base catalyst is that a small cavity, possibly capable of accomodating an anion, will be generated at the β-face of the substrate adjacent to the anomeric centre. Such a site could permit the binding and attack of a nucleophilic anion at the anomeric centre. If a substrate with a good leaving group, such as DNPC, is employed, the second step (deglycosylation) will be rate-limiting, thus reaction of the intermediate with an anion more nucleophilic than water, and one not requiring general base catalysis, will result in an increase in the steady state rate. Such is indeed found to be the case, as is shown below in Figure 3. Increasing concentrations of sodium azide as exogenous nucleophile result in progressive increases in k_{cat} values. As can be seen, much greater overall rate increases are seen with DNPC (200-fold) than with PNPC (8-fold), rates reaching a limiting value at higher azide concentrations. This plateauing of rates is clearly due to a change in rate-determining step. As azide concentrations are increased, so the deglycosylation rate increases, and the steady state rate with it, up to the point at which the rate of the deglycosylation step becomes greater than that of the glycosylation step. Beyond this point no further rate increases are observed. The lower maximal rate observed with PNPC than with DNPC is therefore consistent with the smaller glycosylation rate constant for the substrate with the poorer leaving group.

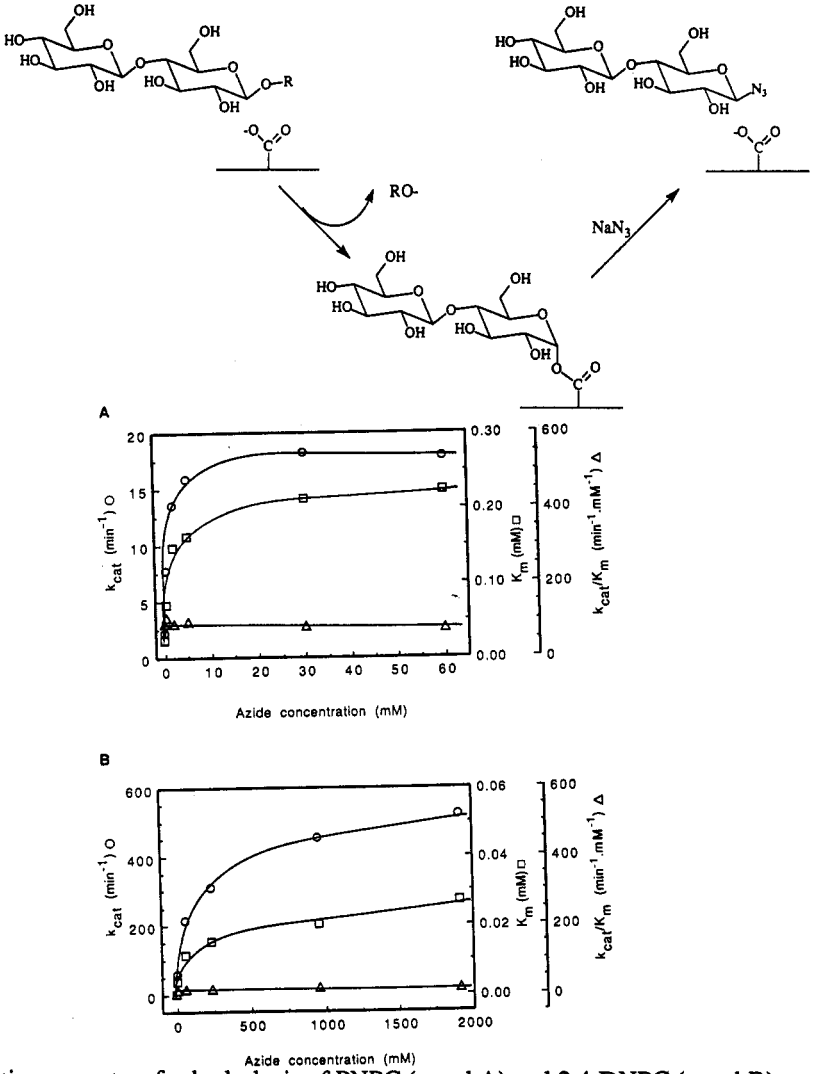


Figure 3. Kinetic parameters for hydrolysis of PNPC (panel A) and 2,4-DNPC (panel B) by Cex Glu127Ala in the presence of various concentrations of sodium azide

This mechanism (Fig 4) therefore requires that the reaction product in the presence of azide be β-cellobiosyl azide. This was clearly shown by chemical synthesis of β-cellobiosyl azide and comparison of its chromatographic behaviour and ^{1}H-NMR spectrum with that of the reaction mixture. The two were shown to be identical. Interestingly, as can be seen in Fig. 3, values of k_{cat}/K_m are esentially invariant with azide concentration. This is also completely consistent with the proposed mechanism since the parameter k_{cat}/K_m reflects the first irreversible step in catalysis, formation of the glycosyl-enzyme intermediate, and this step is not affected by nucleophilic competition with the water. Small effects due to stabilisation of a reactive conformation, after binding of the azide are possible, but are not apparent in this case.

Correlation with structural data

At the start of these studies no 3-dimensional information was available on any of the enzymes under study, thus these techniques were applied "blind" to each enzyme. Since then the 3-dimensional structures of three of these enzymes have been solved, those of the *B. subtilis* xylanase[18,19], the *E. coli* β-galactosidase[36], and the *C. fimi* exoglucanase (Rose, D. and White, A., Personal Communication). In only one of those cases, that of the *B. subtilis* xylanase, has the structure of an enzyme/substrate complex been solved[19]. In that case the residue identified as the nucleophile, Glu78, was found near the anomeric centre on the α-face of the reducing terminal sugar residue, and that suspected to be the acid/base catalyst was similarly positioned on the β-face. In the other two cases, *E.coli* β-galactosidase and *C. fimi* exoglucanase, the residues so identified (Glu 537 and Glu 461 in the former case; Glu 233 and Glu127 in the latter case for the nucleophile and acid/base catalyst respectively) were indeed found in a region of the protein (actually at the C-terminal end of an α/β barrel in each case) reasonably identifiable as the active site. Interestingly the two carboxylic acids are positioned some 5-5.5 Å apart in the xylanase and the exoglucanase, as well as in hen egg white lysozyme (this value as not yet available for the galactosidase). Presumably this separation is suitable for the correct formation of a covalent glycosyl-enzyme intermediate with efficient operation of acid/base catalysis.

Conclusion

The techniques described therefore provide reliable methods for the identification of the nucleophilc and acid/base catalytic groups in β-glycosidases in the absence of X-ray crystallographic insights. They will also be useful for the clarification of the roles of residues identified through crystallographic analyses since definitive assignments often cannot be made on the basis of structural data alone. As such they should be of interest to a range of people working on enzymes of this type.

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