Substrate-Assisted Catalysis in Glycosidases

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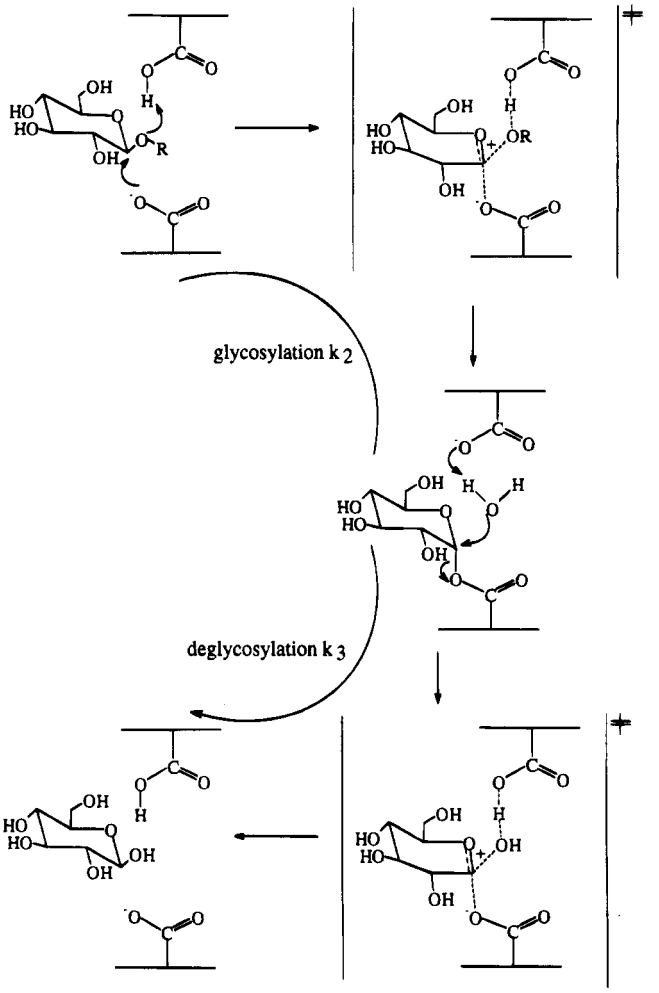
Agrobacterium β-glucosidase hydrolyzes β-glucosides with net retention of anomeric configuration through a double displacement mechanism,[1] as shown in Scheme 1. Formation of the α-glucosyl−enzyme intermediate (glycosylation step, k_2) requires general acid catalytic assistance, while breakdown of the intermediate (deglycosylation step, k_3) requires general base catalytic assistance, presumably from the same residue in the active site. This active site acid/base catalyst has been identified as Glu170 through kinetic analysis of mutants modified at conserved aspartic and glutamic acid residues. Mutation of Glu170 to Gly slows the glycosylation step 10^6-fold for substrates such as phenyl β-D-glucopyranoside which have relatively poor leaving groups needing acid assistance, but very little for substrates such as DNPGlu which have no such needs. The deglycosylation step, k_3, which requires general base catalytic assistance, is slowed 10^3-fold for all substrates. These observations opened the possibility of restoring activity to such mutants by use of substrates into which is incorporated the missing acid catalytic group, thereby providing enzymes of unique specificity. In this communication, we describe the first examples of such substrate-assisted catalysis[2] in a glycosidase.

Suitable substrates for this study require a carboxylic acid functionality incorporated into their structure at a suitable position to allow delivery of a proton at the glycosidic oxygen. The sensitivity of this enzyme to substitution at C-2 and C-6 of the substrate[1] necessitated incorporation of the carboxyl group into the aglycone, suitable substrates for such a study being the salicyl glucosides, which have been shown previously to undergo spontaneous hydrolysis with intramolecular general acid catalytic assistance.[3,4]

The carboxyphenyl β-D-glucosides **1**−**6** were synthesized according to the procedure described by Capon[3] with some modifications.[5] Kinetic parameters were determined with both the wild-type and the mutant enzyme,[6] and the results are summarized in Tables 1 and 2. Values of k_{cat} for hydrolysis of these substrates by wild-type β-glucosidase are all quite similar, comparable to that for PNPGlu,[1d] and consistent with borderline

Scheme 1. Mechanism of *Agrobacterium* β-Glucosidase



rate-limiting deglycosylation. Values of K_m, however, differ widely, being particularly high for substrates containing ortho carboxyl groups (**1** and **4**), presumably due to electrostatic repulsion between the substrate carboxylate and the active site carboxylates, but in the normal range for those with amide substituents at this position.

Inspection of data for Glu170Gly reveals that removal of the acid catalyst reduces the k_{cat} values enormously for the 2-car-

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(2) For the first description of engineered substrate-assisted catalysis, see: Carter, P.; Wells, J. A. *Science* **1987**, *237*, 394.

(3) (a) Capon, B. *Tetrahedron Lett.* **1963**, *14*, 911. (b) Capon, B.; Smith, M. C.; Anderson, E.; Dahm, R. H.; Sankey, G. H. *J. Chem. Soc. (B)* **1969**, 1038.

(4) (a) Anderson, E.; Fife, T. H. *J. Am. Chem. Soc.* **1973**, *95*, 6437. (b) Fife, T. H.; Przystas, T. J. *J. Am. Chem. Soc.* **1977**, *99*, 6693. (c) Fife, T. H.; Przystas, T. J. *J. Am. Chem. Soc.* **1979**, *101*, 1202. (d) Piszkiewicz, D.; Bruice, T. C. *J. Am. Chem. Soc.* **1968**, *90*, 2156.

(5) 2-Carboxyphenyl β-D-glucopyranoside (**1**), 4-carboxyphenyl β-D-glucopyranoside (**3**) and 2-carboxy-4-nitrophenyl β-D-glucopyranoside (**4**) were prepared as described,[3b] except that the final products were purified by ion-exchange chromatography as follows: ~100 mg of crude product was applied to a 40 mm × 100 mm DE-52 column and eluted with a 0−100 mM NH_4HCO_3 gradient. 2-Carbamoylphenyl β-D-glucopyranoside (**2**) and 2-carbamoyl-4-nitrophenyl β-D-glucopyranoside (**5**) were made by reaction of the corresponding methyl esters with ammonia in methanol. Detailed procedures for synthesis of **2**, **5**, and **6** will be published separately. All the compounds were characterized by NMR, mass spectrometry, and elemental analysis.

(6) Kinetic studies were performed at 37 °C in a 50 mM sodium phosphate buffer, pH 7.0, containing 0.1% bovine serum albumin. Reactions were initiated by addition of an appropriate amount of enzyme into the substrate solution in a 1 cm cuvette located in the thermostated block of the spectrometer. Due to the extremely high K_m and high ϵ for **4**, a 1 mm cuvette was used in that case. Hydrolysis was monitored at the following wavelengths, 315 nm, $\Delta\epsilon = 1.008$ mM^{-1} cm^{-1} (**1**); 320 nm, $\Delta\epsilon = 1.77$ mM^{-1} cm^{-1} (**2**); 400 nm, $\Delta\epsilon = 0.29$ mM^{-1} cm^{-1} (**4**); 395 nm, $\Delta\epsilon = 15.59$ mM^{-1} cm^{-1} (**5**); 405 nm, $\Delta\epsilon = 3.14$ mM^{-1} cm^{-1} (**6**), using a PU-8800 UV−vis spectrometer. Rates of hydrolysis of **3** were measured using a stopped assay as follows. To substrate (90 μL) in the same buffer as above was added enzyme (10 μL), and the reaction run for 3 min and then stopped by the addition of 900 μL of 50 mM sodium triphosphate, pH 12.0, and the absorbance at 280 nm was determined. Rates were calculated using a value of $\Delta\epsilon = 1.78$ mM^{-1} cm^{-1}. Since the hydrolysis of 4-carboxyphenyl β-D-glucopyranoside (**3**) by Glu170Gly is extremely slow, rates were determined at just two substrate concentrations (12.4 and 29.4 mM) using high concentrations (15 mg/mL) of enzyme, in both the presence and the absence of 200 mM azide, using a coupled assay[7] to measure the glucose released. Rates were determined at substrate concentrations ranging from $0.14K_m$ to $7K_m$ whenever possible and corrected for spontaneous hydrolysis when necessary. Values of k_{cat} and K_m were calculated using the computer program GraFit.[8]

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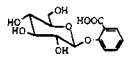
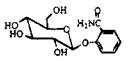
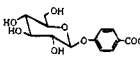
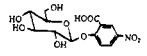
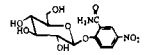
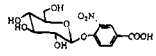
Table 1. Michaelis–Menten Parameters of *Agrobacterium* β-Glucosidase and Its Glu170Gly Mutant in the Presence or Absence of Azide[a]

Aryl β-D-glucosides	Enzyme	Azide (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	Relative rate with Glu170Gly[e]
	WT[b]	0	60	97	1.63	kcat(1)/kcat(1)
	E170G	0	0.92	0.056	0.061	= 1.0
1	E170G	200	26	16.0	0.61	
	WT	0	0.28	55	196.8	kcat(1)/kcat(2)
	E170G	0	0.31	0.0039	0.013	= 4000
2	E170G	200	0.56	0.0025	0.0045	
	WT	0	7.8	45	5.8	kcat(1)/kcat(3)
	E170G[c]	0		1.3×10^{-5}		$= 10^7$
3	E170G[d]	200		1.1×10^{-6}		

[a] All data are corrected for spontaneous hydrolysis and are accurate to within ±5% unless mentioned specifically. [b] Substrate concentrations used were $0.15K_m - 2K_m$; data have ±10% error. [c] Due to the extremely slow rate of hydrolysis, full determination of K_m and k_{cat} was not possible. The value reported has an accuracy of ±20%. [d] Value has an accuracy of ±50%. [e] In the presence of 200 mM azide.

Table 2. Michaelis–Menten Parameters of *Agrobacterium* β-Glucosidase and Its Glu170Gly Mutant in the Presence or Absence of Azide[a]

Aryl β-D-glucosides	Enzyme	Azide (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	Relative rate with Glu170Gly[d]
	WT[b]	0	45	62	1.4	kcat(4)/kcat(4)
	E170G	0	0.058	0.064	1.1	= 1.0
4	E170G	200	1.57	20.9	13.3	
	WT	0	0.093	87	931	kcat(4)/kcat(5)
	E170G[c]	0	0.00016	0.028	175	= 2.9
5	E170G	200	0.023	7.2	313	
	WT	0	0.22	101	459	kcat(4)/kcat(6)
	E170G	0	0.015	0.035	2.3	= 8.9
6	E170G	200	0.27	2.34	8.8	

[a] All data are corrected for spontaneous hydrolysis and are accurate to within ±5% unless mentioned specifically. [b] Substrate concentrations used were $0.15K_m - 2K_m$; data have ±10% error. [c] Due to the extremely low K_m, special care was taken in measurements. The value has an error of 10%. [d] In the presence of 200 mM azide.

bamoylphenyl (**2**) and 4-carboxyphenyl (**3**) glycosides (10^4- and 10^6-fold, respectively). This is consistent with a need for acid catalytic assistance in the hydrolysis of substrates with poor leaving groups. However, incorporation of this carboxyl group into the ortho position of the phenol results in a dramatic increase in the rate of the glycosylation step. This is not immediately apparent upon inspection of the data for **1**, since, as is suggested by the depressed K_m value, the deglycosylation step, k_3, has now become rate-limiting. However, the deglycosylation step can be accelerated by the addition of exogenous azide at concentrations sufficient to re-establish rate limiting glycosylation.[9] Addition of 200 mM azide increased the rate almost 300-fold to a value approaching that of the wild-type enzyme. Such azide stimulation is not seen for **2** and **3**, suggesting that glycosylation is already the rate-limiting step, a fact corroborated by the similarity of the K_m values for these substrates with the mutant and wild-type enzymes.

2-Carboxyphenyl β-glucoside **1** is a 10^7-fold better substrate for this mutant in the presence of azide than the electronically equivalent 4-carboxyphenyl derivative **3**. This massive rate difference is a consequence of substrate-assisted catalysis arising from intramolecular proton donation, as also seen in the 280-fold rate difference in the spontaneous hydrolysis study, but to a far greater extent. Indeed, this study provides a direct validation of the use of such substrates as models for enzymatic catalysis. Presumably, the greater rate difference seen in the enzymatic case is a consequence of the maintenance of the preferred orientation of the carboxyl group at the active site by local binding interactions.

As a further probe of this effect, and to ensure that the rate increases seen are due to increased acid catalysis and not some form of ground state destabilization associated with an ortho carboxyl group, a second series of substrates was prepared, into which a nitro group was incorporated, thereby eliminating the need for acid catalysis. Data from this study are provided in Table 2. As is apparent, rate increases of only 3–9-fold are seen upon introduction of the ortho carboxyl group into such substrates, even when measured in the presence of azide. This confirms that the "substrate assisted catalysis" seen for **1** was, indeed, a consequence of intramolecular proton transfer.

The substrate assisted-catalysis observed in **1** is the first such example in a glycosidase and represents the largest rate increase due to this phenomenon observed to date for an engineered enzyme/substrate system.[12] Interestingly, it is possible that substrate-assisted catalysis might have evolved naturally in the case of some glycosidases, as follows. Alignment of amino acid sequences for enzymes in β-glycosidase family 1 shows[16] that in two members of this family, both of which are thioglucosidases,[17] the acid/base catalytic residues are replaced by glutamine residues. This difference is likely an evolutionary consequence of the fact that the natural substrates for these enzymes, glucosinolates, contain an anionic sulfate group in their aglycon. Such substrates would not bind well to an enzyme containing a potentially anionic acid/base group, thus mutations to remove the charged residue from this position have occurred. It is quite likely that the sulfate group itself serves as the acid/base catalyst in these enzymes, in nature's own version of substrate-assisted catalysis.

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(9) Previous studies on acid catalyst mutants of the *Cellulomonas fimi* exoglycanase[10] and the *Escherichia coli* β-galactosidase[11] revealed that in the absence of charge screening from the enzymic carboxylate, anions such as azide function as excellent nucleophiles, competing with water for reaction with the glycosyl–enzyme intermediate. Progressive addition of azide results in rate increases up to a plateau value at which the glycosylation step has become rate-limiting.

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(12) A rate increase of 400-fold was seen for a serine protease mutant missing its key histidine residue when a peptide substrate incorporating a histidine was employed.[5,13,14] Similarly, a rate increase of 100-fold was seen for a β-lactamase[15] in which the loss of a positive charge was partly compensated by use of a substrate incorporating such a charge.

(13) Wells, J.; Cunningham, B.; Graycar, T.; Estell, D.; Carter, P. *Cold Spring Harbor Symp. Quant. Biol.* **1987**, *52*, 647.

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(17) (a) Xue, J.; Lenman, M.; Falk, A.; Rask, L. *Plant Mol. Biol.* **1992**, *18*, 387. (b) Falk, A.; Xue, J.; Lenman, M.; Rask, L. *Plant Sci.* **1992**, *83*, 385.