

Changing Enzymatic Reaction Mechanisms by Mutagenesis: Conversion of a Retaining Glucosidase to an Inverting Enzyme

Qingping Wang,[†] R. W. Graham,[‡] D. Trimbur,[‡]
R. A. J. Warren,[‡] and S. G. Withers^{*,†}

Departments of Chemistry and
Microbiology and Immunology
University of British Columbia, 2036 Main Mall
Vancouver, BC, Canada V6T 1Z1

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Glycosidases employ two separate and distinct mechanisms.^{1–4} In one set of enzymes, direct displacement leads to net inversion of anomeric configuration (Scheme 1A). In the other set, anomeric configuration is retained via a double displacement mechanism involving a glycosyl–enzyme intermediate (Scheme 1B). While the two mechanisms are quite distinct, there are significant similarities: both involve oxocarbenium ion-like transition states,⁵ and both involve a pair of carboxylic acids, which have different roles in the two cases. In “inverters” one functions as an acid catalyst and the other as a base catalyst, whereas in “retainers” one functions as an acid/base catalyst and the other as a nucleophile/leaving group. Furthermore, the two residues are farther apart in the inverting than in the retaining glycosidases to allow the intervention of a water molecule. The average separation in retaining α - and β -glycosidases is 4.8 ± 0.5 and 5.3 ± 0.2 Å, respectively, but in the inverting α - and β -glycosidases it is 9.0 ± 1.0 and 9.5 Å, respectively.⁶ The similar transition states raises the possibility of converting an enzyme from one mechanism to the other by changing the separation by mutation. This paper describes such a conversion.

The active site nucleophile in Abg (a retaining β -glucosidase from *Agrobacterium faecalis*) is Glu 358.⁷ Mutation⁸ of Glu

Scheme 1

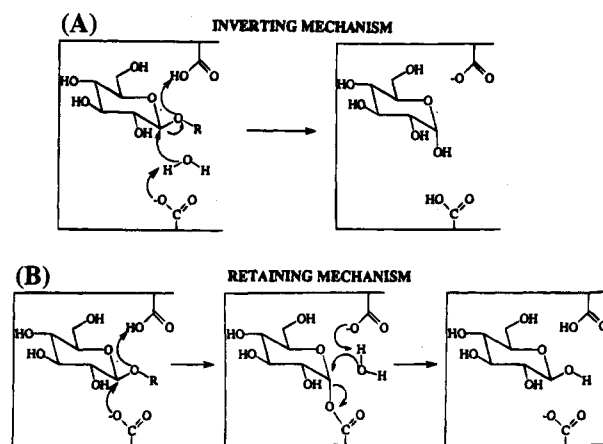


Table 1. Kinetic Parameters for Hydrolysis of 2',4'-Dinitrophenyl β -D-Glucopyranoside by *Agrobacterium* β -Glucosidase and Its Glu358Ala Mutant

enzyme + activators	k_{cat} (s^{-1})	K_m (mM)
native enzyme	89 ± 0.9	0.031 ± 0.001
E358A ^a	$(7.1 \pm 0.1) \times 10^{-6}$	0.10 ± 0.05
E358A ^a + 2 M azide ^b	1.10 ± 0.01	3.8 ± 0.5
E358A ^a + 4 M formate ^b	3.0 ± 0.1	1.1 ± 0.1

^a After treatment with 2',4'-dinitrophenyl 2-deoxy-2-fluoro- β -D-glucopyranoside. ^b Values are corrected for the small spontaneous rate observed with substrate and nucleophile but no enzyme.

358 to Asp increased the separation by approximately 1 Å and lowered the activity 2500-fold but did not change the mechanism. In the present experiment, the carboxylate was eliminated and the need for a general base catalyst was obviated by use of nucleophiles other than water.

The mutant Glu358Ala⁹ was constructed and purified as described previously for the Asp mutant.^{8,10} The activity of the mutant was some 10^6 -fold lower than that of the wild-type enzyme, but most of this activity could be shown to be associated with contaminating wild-type enzyme.¹¹ The true k_{cat} value for the mutant¹² was 10^7 -fold lower than that of the wild-type enzyme (Table 1), so direct attack of water is extremely inefficient. However, addition of azide or formate as alternative nucleophiles increased the k_{cat} 10^5 -fold, almost back to wild-type levels,¹³ as might be expected on the basis of their considerable reactivity in reactions *via* cationic transition states.¹⁴ Larger anionic nucleophiles did not function in this manner,¹⁵ presumably because they could not fit into the space created by removal of the carboxyl. The reaction must proceed *via* direct attack of azide (Scheme 2) since the product was

(9) A Glu358Gly mutant was constructed initially but was found to be contaminated with small but significant quantities of wild-type enzyme, presumably arising from translational misincorporation.

(10) The specific mutation of active site nucleophile Glu was carried out with a degenerate oligonucleotide primer: pTAC ATC ACC XXX AAC GCC TGC TGC (underline shows the location of mismatches), XXX = GCA for Glu358Ala. The fidelity of mutation was checked by nucleotide sequencing, and the mass of the mutant was confirmed to be 58 lower than that of the wild-type enzyme by electrospray mass spectrometry.

(11) Inactivation was time-dependent and resulted in a 93% reduction in activity for all inhibitor concentrations tested, even at concentration 0.5% that of the mutant, proving that the activity inhibited must arise from a contaminant. The 7% residual activity likely represents a true activity of the mutant, thus all further experimentation was performed with samples of mutant pretreated with 2',4'-DNP2FGlu.

(12) Kinetic studies were performed as described elsewhere.⁸

(13) Careful controls were performed to correct for azide-promoted nonenzymatic cleavage of 2',4'-dinitrophenyl- β -D-glucopyranoside; such spontaneous cleavage was much slower than the enzymatic process. Controls with native enzyme showed that addition of 65 mM azide caused a ~ 1.2 -fold increase in rate, but concentration > 100 mM caused inactivation.

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* To whom correspondence should be addressed. Tel.: (604) 822-3402. Fax: (604) 822-2847. e-mail address: withers@unixg.ubc.ca.

[†] Department of Chemistry.

[‡] Department of Microbiology and Immunology.

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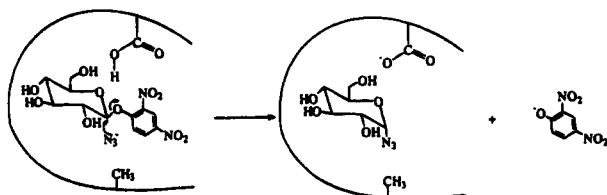
(5) Evidence includes secondary deuterium kinetic isotope effects, rate-retarding effects of electron-withdrawing substituents, and binding of transition state analogues, as reviewed in ref 2.

(6) Distances represent the averages of the four values measured between each pair of active site carboxylate oxygen atoms in the following enzymes: human pancreatic α -amylase,^{6a} *Aspergillus oryzae* (Taka) α -amylase,^{6b} *Aspergillus niger* α -amylase,^{6c} pig pancreatic α -amylase,^{6d} β -1,4-galactanase Cex from *Cellulomonas fimi*,^{6e} *Bacillus Circulans* xylanase,^{6f} hen egg white lysozyme,^{6g} glucoamylase from *Aspergillus awamori*,^{6h} β -amylase from soybean,⁶ⁱ and endoglucanase E2 from *Thermomonospora fusca*.^{6j} (a) Burk, D.; Wang, Y.; Dombroski, D.; Berhuis, A. M.; Evans, S. V.; Luo, Y.; Withers, S. G.; Brayer, G. D. *J. Mol. Biol.* 1993, 230, 1084. (b) Swift, H. J.; Brady, R. L.; Derewenda, Z. S.; Dodson, E. J.; Turemburg, J. P.; Wilkinson, A. J. *Acta Crystallogr.* 1991, B47, 535. (c) Brady, R. L.; Brzozowski, A. M.; Derewenda, Z. S.; Dodson, E. J.; Dodson, G. G. *Acta Crystallogr.* 1991, B47, 527. (d) Larson, S. B.; Greenwood, A.; Cascio, D.; Day, J.; McPherson, A. J. *Mol. Biol.* 1994, 235, 1560. (e) White, A.; Withers, S. G.; Gilkes, N. R.; Rose, D. R. *Biochemistry* 1994, 33, 12546. (f) Campbell, R.; Rose, D.; Wakarchuck, W.; To, R.; Sung, W.; Yaguchi, M. In *Proceedings of the second TRICEL symposium on Trichoderma reesei cellulase and other hydrolases*; Suominen, P., Reinikainen, T., Eds.; Helsinki Foundation for Biotechnical and Industrial Fermentation Research: Espoo, Finland, 1993; pp 63–72. (g) Imoto, T.; Johnson, L. N.; North, A. C. T.; Phillips, D. C.; Rupley, J. A. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1972; pp 666–668. (h) Aleshin, A.; Golubev, A.; Firsov, L. M.; Honzatko, R. B. *J. Biol. Chem.* 1993, 267, 19291. (i) Mikami, B.; Degano, M.; Hehre, E. J.; Sacchettini, J. C. *Biochemistry* 1994, 33, 7779. (j) Spezio, M.; Wilson, D. B.; Karplus, P. A. *Biochemistry* 1993, 32, 9906.

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Scheme 2



identified as α -glucosyl azide by $^1\text{H-NMR}$.¹⁶ This mutant clearly functions as an inverting enzyme.

A characteristic reaction of inverting glycosidases is the cleavage of glycosyl fluorides of the "wrong" anomeric configuration *via* a two-step mechanism involving an initial transglycosylation followed by hydrolysis between the two sugars in the normal manner.¹⁷ Glu358Ala cleaved α -glucosyl fluoride¹⁸ rapidly, with $k_{\text{cat}} = 5.5 \text{ min}^{-1}$ and $K_m = 53 \text{ mM}$.¹⁹ The reaction occurred at the active site because it was inhibited by the competitive inhibitor 1-deoxynojirimycin.²⁰ Further, the wild-type enzyme did not release fluoride under the same conditions.²¹

(15) Cyanide showed a ~ 2.5 times rate enhancement, and acetate, benzoate, sulfate, thiosulfate, thiocyanate, imidazole, pyridine, and Meldrum's acid gave no rate enhancement.

(16) $^1\text{H NMR}$ (400 MHz, D_2O): δ 5.52 (1 H, d, $J = 4.1 \text{ Hz}$), 3.7–3.9 (2 H, m), 3.58–3.69 (2 H, m), 3.55 (1 H, dd, $J = 6.5 \text{ Hz}$, $J = 11.8 \text{ Hz}$), 3.42 (1 H, t, $J = 9.2 \text{ Hz}$); identical to that reported for the authentic α -glucopyranosyl azide. Takeda, T.; Sugiura, Y.; Hamada, C.; Fujii, R.; Suzuki, K.; Ogiwara, Y.; Shibata, S. *Chem. Pharm. Bull.* **1981**, *29*, 3196.

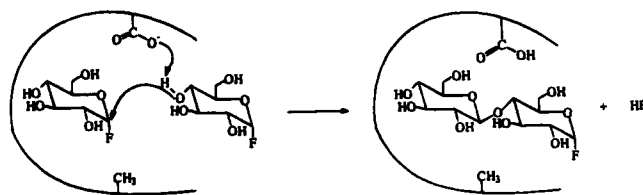
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(19) Fluoride ion analyses were performed essentially as described as in the paper of Liu et al. Liu, W.; Madsen, N. B.; Braun, C.; Withers, S. G. *Biochemistry*, **1991**, *30*, 1419.

(20) Addition of 1-deoxynojirimycin (0.53 mM) reduced the reaction rate for 12 mM α -glucosyl fluoride from 0.96 to 0.55 min^{-1} . The K_i of this transition state analogue would be expected to be high.⁸

Scheme 3



By analogy with other inverting enzymes, reaction is presumably occurring *via* transglycosylation (Scheme 3). A new product ($t_R = 5.79 \text{ min}$), in addition to the glucose arising from spontaneous hydrolysis, was detected by HPLC²² and identified as a cellobiose derivative (presumably α -cellobiosyl fluoride) by hydrolysis of the fluoride (100 $^\circ\text{C}$, 30 min) and detection of cellobiose ($t_R = 6.83 \text{ min}$) and glucose by HPLC.

It is clear that the mechanism of a glycosidase can be changed by mutation,²³ indicating that the transition states for the two mechanisms are quite similar, as suggested by Hehre's work¹⁷ and by the recent finding that sialidases of similar fold can have different mechanisms.²⁴ The potential use of such mutant glycosidases in oligosaccharide synthesis is currently being explored.

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(21) Some retaining glycosidases have been shown (see ref 17f) to hydrolyze the "wrong" glycosyl fluoride, but at very low rates.

(22) The HPLC analysis was performed on a Dynamax-60A amino (NH_2) analytical column module (4.6 mm i.d. \times 25 cm) as follows: 15 μL of reaction mixture was injected into the column, and the column was run with a solvent system of $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ 1:1 at a rate of 0.8 mL/min.

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