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Thioglycosynthases: double mutant glycosidases that serve as scaffolds for thioglycoside synthesis†

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A double mutant, retaining glycosidase that lacks both the catalytic nucleophile and the catalytic acid/base residues efficiently catalyzes thioglycoside formation from a glycosyl fluoride donor and thiosugar acceptors.

Glycosidases are highly effective catalysts, increasing rate constants for glycoside hydrolysis some 10^{17} fold through an active site that has evolved to bind and stabilize oxocarbenium ion-like transition states.[1] Two catalytic acids play key roles in this process for retaining glycosidases; one acts as the nucleophile, the other as the acid/base catalyst (Fig. 1, **A**).[2] In the first, glycosylation step the catalytic acid/base residue protonates the substrate's glycosidic oxygen while the catalytic nucleophile attacks at the anomeric position, and a covalent glycosyl enzyme intermediate is formed. In the second, deglycosylation step the catalytic acid/base residue now acts as a general base, deprotonating and thereby activating the acceptor (water in the case of hydrolysis, or an alcohol, *e.g.* a sugar, in the case of transglycosylation) for attack at the anomeric centre, releasing a product sugar of net retained anomeric configuration. With very few exceptions (sialidases using a tyrosine nucleophile,[3] myrosinase lacking the catalytic acid/base residue[4] and some hexosaminidases lacking the catalytic nucleophile[5]) all retaining glycosidases act according to this mechanism.[6]

However, when activated sugars are used as substrates, the mechanism is more plastic, and this property has been put to use in the generation of mutant glycosidases that are useful in the synthesis of glycosidic bonds. Such catalysts – alternative to glycosyl transferases – are synthetically useful since conventional chemical synthesis of oligosaccharides requires lengthy protecting group chemistry and provides limited stereochemical control, while use of the transglycosylation reaction of retaining glycosidases typically results in low yields. Two approaches using mutant glycosidases have been developed. One strategy, the glycosynthase methodology, uses enzymes with a mutation at the catalytic nucleophile position in conjunction with glycosyl fluoride donors with the opposite anomeric configuration to that of the natural substrates (Fig. 1, **B**).[7,8] Since they are missing the catalytic nucleophile glycosynthases cannot hydrolyze their products, thus transglycosylation yields are usually very high. The thioglycoligase methodology on the other hand employs acid/base mutants of retaining glycosidases to couple donors that contain reactive leaving groups that do not need acid catalysis with nucleophilic thiosugar acceptors (Fig. 1, **C**).[9] By using this strategy thioglycosidic linkages in oligosaccharides can be synthesized enzymatically with high yields under mild, aqueous conditions.

A logical extension is to attempt to combine the two approaches to generate a "thioglycosynthase" methodology, in which a double mutant (acid/base and nucleophile) of a retaining glycosidase might catalyze – in the fashion of a scaffold – the transglycosylation of a glycosyl fluoride with inverted anomeric stereochemistry onto a thiosugar acceptor (Fig. 1, **D**). The synthesis of thioglycosidic linkages in oligosaccharides by this method would have two major advantages over the thioglycoligase strategy. Firstly, nucleophile mutants are completely hydrolytically inactive, whereas acid/base mutants used in the thioglycoligase strategy can slowly hydrolyse the donor. Secondly, glycosyl fluoride donors are more easily synthesized than dinitrophenyl glycosides.

An obvious candidate to test the thioglycosynthase strategy was the β-glucosidase Abg from *Agrobacterium sp.*, as both the glycosynthase and the thioglycoligase methodology have been successfully applied to this enzyme.[7,9] The double mutant Abg E171A, E358G was therefore generated by site-directed mutagenesis using a megaprimer PCR method with three oligonucleotide primers. One primer contained the mutation at the acid/base position (E171), and the plasmid containing the nucleophile mutant (E358G) Abg gene served as a template.[10]† The nucleophile glycine mutant was chosen because previous experiments had revealed that this glycosynthase showed the highest transglycosylation activity.[11]

The exchange of two polar, charged carboxylates for two nonpolar, hydrophobic side chains in the active site could conceivably affect the folding and stability of the enzyme. However, circular dichroism spectra between 200 and 240 nm confirmed that the double mutant Abg E171A, E358G and the two single mutants Abg E171A and Abg E358G adopted essentially the same fold as the wild type Abg.† The thermal stability of the enzymes was evaluated by measuring melting curves by monitoring circular dichroism at 222 nm (Fig. 2). Interestingly, the wild type enzyme is the least thermally stable protein with a melting

† Electronic supplementary information (ESI) available: experimental details. See http://www.rsc.org/suppdata/cc/b3/b313155f/

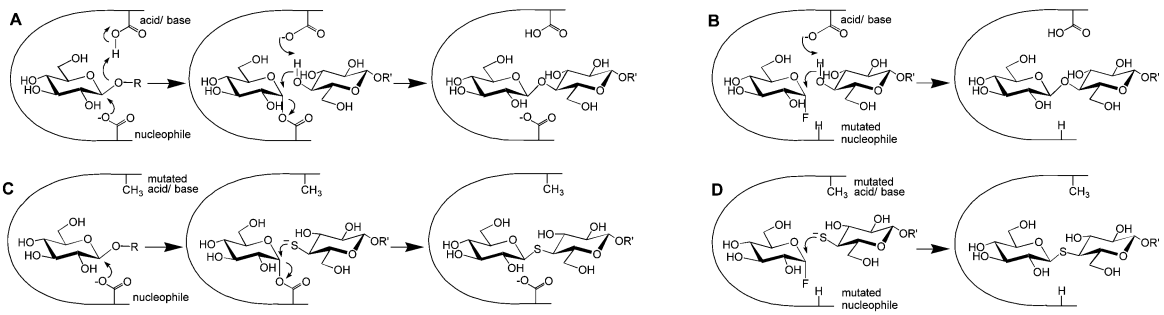

Fig. 1 Mechanism for wild type retaining glycosidase (**A**), glycosynthase (**B**), thioglycoligase (**C**) and thioglycosynthase (**D**).

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temperature of ~59 °C, whereas the double mutant (~65 °C) and the two single mutants (E171A: 66 °C, E358G: 68 °C) are considerably more stable. This is consistent with previous observations that the pK_a of the acid/base residue in retaining glycosidases is raised by ~2.5 units by the presence of the charged nucleophile, thereby constituting a form of structural destabilisation.[12] Removal of one charged group removes this electrostatic destabilisation, thereby stabilizing the protein.

Having established that the double mutant Abg E171A, E358G was correctly folded and stable at neutral pH the catalytic activity of the enzyme was tested. The readily synthesized potential donor α-glucosyl fluoride and acceptor pNP 4-deoxy-4-thio-β-D-glucopyranoside were incubated with the double mutant Abg E171A, E358G overnight at neutral pH (Fig. 3). TLC experiments revealed that indeed a new UV-active compound was formed, and ESI-MS experiments indicated that the new compound had the molecular weight of the expected thio-linked pNP disaccharide. The equivalent thioglycosylation event was observed when the acceptor was changed to methylumbelliferyl (MU) 4-deoxy-4-thio-β-D-glucopyranoside. The products were isolated by flash chromatography after standard acetylation, and characterized by one- and two-dimensional ^{1}H and ^{13}C NMR spectroscopy to unambiguously establish the regio- and stereoselectivity of the reactions. As anticipated, the products – in each case formed in ~45–50% yield – were the pNP and MU cellobiosides **1** and **2** (Fig. 3).[9,†] Control experiments revealed that omission of the double mutant Abg E171A, E358G resulted in no product formation, and that added wild type Abg did not cleave the thio-linked products. Importantly, in contrast to what is found with glycosynthases when using GlcF as donor, only a single transfer occurred, yielding a disaccharide. This is obviously a consequence of the requirement of this mutant for a thiosugar acceptor, thereby allowing closer control of the reaction.

Michaelis–Menten parameters for this process were determined with α-glucosyl fluoride as donor and pNP 4-deoxy-4-thio-β-D-glucopyranoside as acceptor using a fluoride-sensitive electrode. Surprisingly, the apparent K_M value for the thiosugar acceptor at various donor concentrations is extremely low (K_M < 50 μM), whereas in the absence of acceptor no enzymatic donor hydrolysis was observed. K'_M values for the donor are quite pH-dependent, as are k'_{cat} values. With saturating concentrations of acceptor (~200 μM) the apparent K_M value of the donor increased strongly with pH, presumably reflecting other active site ionizations: ~1 mM at pH 6, ~6 mM at pH 6.9 and ~37 mM at pH 8 (Fig. 4). The k'_{cat} value also increased considerably with pH: k'_{cat} ~0.5 min^{-1} at pH 6, ~1 min^{-1} at pH 6.9 and ~9 min^{-1} at pH 8.†

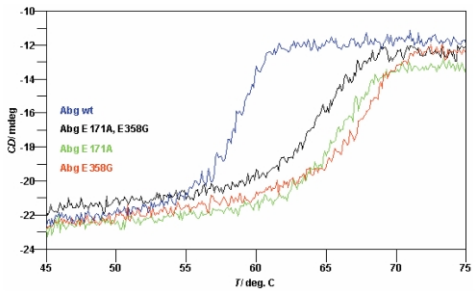

Fig. 2 Thermal stability of wild type and mutants of Abg. Shown is the decrease of the CD signal at 222 nm during temperature ramping. $\Delta T/\Delta t$ = 1 °C min^{-1}.

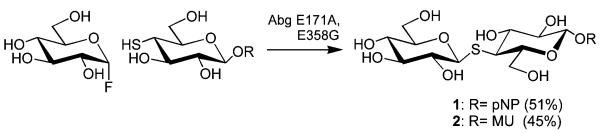

Fig. 3 Thioglycosynthase reaction. pNP: *para*-nitrophenyl; MU: methylumbelliferyl.

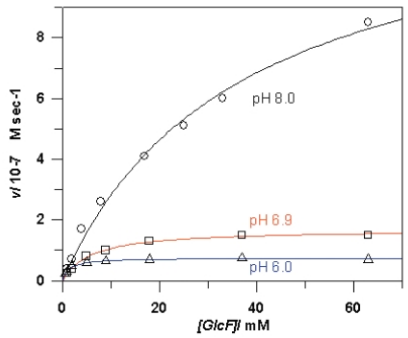

Fig. 4 Michaelis–Menten curves at different pH values. 200 μM pNP 4-deoxy-4-thio-β-D-glucopyranoside, 100 mM NaP_i, 145 mM NaCl, 27 °C.

The increase in k'_{cat} values with pH is consistent with reaction *via* the thiolate species, but unfortunately, at pH > 8 nonenzymatic background hydrolysis of the glucosyl fluoride donor complicates the determination of kinetic parameters, making the measurement of full pH profiles difficult. Comparison with previous studies indicates that the single mutants are somewhat more effective catalysts, the highest k'_{cat} value observed for the Abg E358G glycosynthase[11] being ~200 min^{-1}, and that for the Abg E171A thioglycoligase[13] being >50 min^{-1}. Presumably in the single mutant enzymes some chemical catalysis is still provided by the remaining active site carboxylate, at the very least in stabilizing the oxocarbenium ion. At this stage the double mutant thioglycosynthase cannot provide such assistance, serving primarily as a scaffold to position the reactants with some preferential stabilization of transition state conformations. However, it is quite possible that a different combination of mutations would yield a more effective catalyst: such studies are underway.

In summary we have shown that a retaining glycosidase that has been doubly mutated in the active site to remove both catalytically active amino acid residues can still provide useful catalytic activity in carrying out a synthetically valuable reaction. Further, the advantages of very low acceptor K'_M values and the absence of background donor hydrolysis make these thioglycosynthases attractive catalysts for applications with valuable acceptors.

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