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Expansion of the glycosynthase repertoire to produce defined manno-oligosaccharides†

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Mutant endo-mannanases, in which the catalytic nucleophile has been replaced, function as glycosynthases in the synthesis of manno-oligosaccharides of defined lengths.

Mannan, one of the major plant cell wall polysaccharides, is a synthetically-challenging polymer, not least because the β-mannosidic linkages from which it is constituted are arguably the hardest glycosidic bonds to synthesize chemically.[1] Enzymatic synthesis *via* the transglycosylation reactions of retaining glycosidases is highly stereoselective but suffers from low yields due to hydrolysis of the products.[2,3] In response to this problem we have recently described the generation and use of "glycosynthases", retaining glycosidases that lack the catalytic nucleophile, for the enzymatic synthesis of various glycosidic linkages through use of glycosyl fluorides with inverted anomeric stereochemistry as glycosyl donors.[4] In our quest to expand the repertoire of glycosynthase reactions we desired the capacity to incorporate β-mannobiosyl moieties into a variety of oligosaccharides. The use of a mannan glycosynthase would allow the synthesis of manno-oligosaccharides of defined length. The necessary strategy would involve the deletion of the catalytic nucleophile of an endo-β-mannanase and the use of α-mannobiosyl fluoride as glycosyl donor.

β-Mannanases, classified into glycoside hydrolase families 5 and 26,[5] have been characterized both structurally and mechanistically, notably the β-mannanase Man26A from *Cellvibrio japonicus* (formerly *Pseudomonas cellulosa*).[6–8] High resolution analysis of the 3-D structures of complexes along the reaction pathway suggest an unusual $B_{2,5}$ transition-state for hydrolysis.[9] Mutants‡ of Man26A in which the catalytic nucleophile (Glu320) has been replaced by Gly or Ser were selected as potential glycosynthases on the basis of precedent with other glycosidases.[10–12]

Initial tests of the ability of nucleophile mutants of Man26A to catalyze glycosynthase reactions were performed by incubating potential acceptor sugars together with α-mannobiosyl fluoride‡ and Man26A Glu320Gly or Glu320Ser. TLC analysis and mass spectrometry revealed that a transglycosylation reaction had occurred with the acceptors pNP-Glc, pNP-Man, pNP-Xyl and pNP-Cellobioside. The time course of the reaction of α-mannobiosyl fluoride with pNP-Glc catalyzed by Man26A Glu320Gly (Figure 1) clearly reveals the formation of higher oligosaccharides. Using pNP-Glc as acceptor, a 59% isolated yield of mannooligosaccharides (36% tri-, 18% penta- and 5% heptasaccharide) was obtained, while use of pNP-Man as acceptor yielded pNP-mannotrioside in 35% isolated yield. Structural analysis of the purified, acetylated reaction products confirmed that exclusively β-(1,4) glycosidic linkages were formed, as expected given the substrate specificity of the wild type enzyme. The stereochemistry at the anomeric centre of mannosides cannot be unambiguously revealed by ^{1}H chemical shifts and coupling constants in NMR spectra. Therefore we established the β-(1,4) connectivity by demonstrating the degradation of the oligosaccharides with the exo β-mannosidase Man2A from *Cellulomonas fimi*.

As observed on other systems,[12] the glycine mutant glycosynthase is kinetically superior with an apparent rate constant, k'_{cat} of ~50 min^{-1} compared to ~10 min^{-1} for the serine mutant.‡ Mannobiosyl fluoride donor K'_M values are similar for both enzymes, ~0.8 mM for Glu320Gly and ~0.7 mM for Glu320Ser yielding k'_{cat}/K'_M values of 63 and 14 mM^{-1} min^{-1}, respectively. The donor K'_M values are comparatively low for a glycosynthase but reflect wild-type values,[13] whilst the approximate equivalence of the two is consistent with structural analysis (*vide infra*), which shows no specific interactions of the donor with the former nucleophile position. As with other glycosynthase variants, acceptor K'_M values are considerably elevated, with apparent K'_M values with PNP-Glc as acceptor of ~38 mM for Glu320Gly and ~7 mM for Glu320Ser. It is also interesting to note that the Man26A nucleophile mutants, unlike other glycosynthases derived from endoglycanases, do not utilise the donor disaccharyl fluoride as an acceptor. The reason is unclear but one possible explanation is that the +2 subsite cannot accommodate the α-anomer of mannobiosyl fluoride.

Determination of the pH profile for transglycosylation‡, to inform strategies for preparative applications, was performed over the range 4.5–8, Figure 2, revealing a pH-optimum in the neutral range. Base catalysis is required for the transglycosyla-

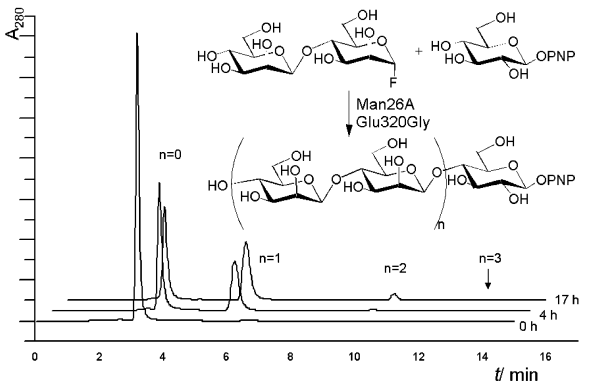

Fig. 1 Time course of the mannan-glycosynthase Man26A Glu320Gly catalyzed reaction of α-mannobiosyl fluoride with pNP-Glc. Shown are the HPLC chromatograms monitored at 280 nm.

† Electronic supplementary information (ESI) available: experimental details. See http://www.rsc.org/suppdata/cc/b3/b302380j/

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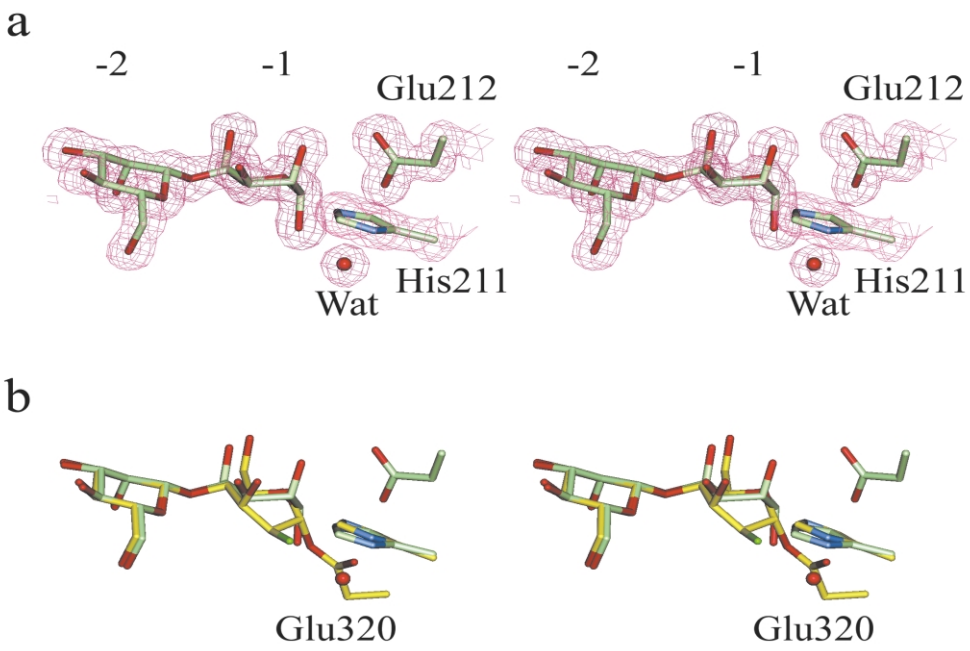

Fig. 3 (a) Electron density ($2F_{obs} - F_{calc}\ \alpha_{calc}$, at 0.6 eÅ^{-3} in divergent stereo) at the active-centre of the Man26A Glu320Gly glycosynthase (pale green) in complex with mannobiose, −2 and −1 subsites. (b) Overlap of the Man26A Glu320Gly glycosynthase variant mannobiose complex (pale green) with the distorted covalent glycosyl-enzyme intermediate of Man26A Glu212Gly (yellow).

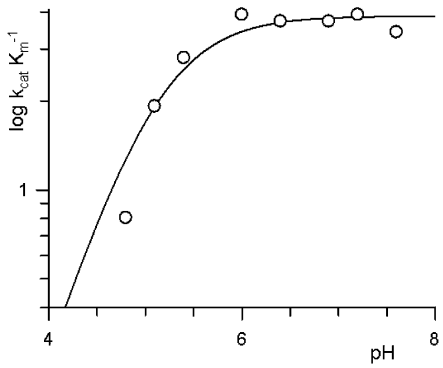

Fig. 2 Determination of the pH-optimum of the mannan-glycosynthase Man26A Glu320Gly.

tion step, which explains the drop of activity under acidic conditions. High background hydrolysis of the α-mannobiosyl fluoride donor occurs at pH-values above 8.

The 3-D structure of Man26A Glu320Gly was determined in complex with mannobiose at 1.4 Å resolution‡. Mannobiose lies in the −2 and −1 (donor) subsites, as its α-anomer, Figure 3, with both sugars in undistorted $^4C_1$ (chair) conformation. In the −1 subsite, Figure 4, the mannosyl moiety "stacks" above Trp360 which provides the hydrophobic platform common to carbohydrate-protein interactions. In common with Michaelis and intermediate complexes,[9] O6 interacts with the solvent and O3 *via* a hydrogen-bond to His143. The interactions at O2 are markedly different for the glycosynthase complex with its −1 site mannose moiety in the $^4C_1$ conformation, compared to the distorted $^1S_5$ and $^0S_2$ conformations of the Michaelis and covalent intermediates observed previously.[9] In the Glu320Gly complex the axial *manno*-O2 of the −1 sugar takes the place of the glycosidic oxygen/attacking water in the former complexes, Figure 3, hydrogen-bonding to Glu212, the catalytic acid/base. Furthermore, the axial O2 no longer interacts with His211 (distance 4.8 Å). This residue is important for catalysis, with the His211Ala mutant about 1000 times less active on mannotetraose,[7] and was observed to interact with the pseudo-equatorial 2-substituent of the distorted Michaelis and intermediate complexes. It is possible that structural reorientation of the ligand, together with a conformation potentially distant from that of the transition-state,[9] and the loss of transition-state stabilizing interactions, all contribute to the poor catalytic rates displayed by manno-glycosynthases.

In the mannobiose/Man26A Glu320Gly structure a single solvent molecule takes the place of the nucleophile and makes a 2.8 Å hydrogen bond to the α-anomeric O1 atom. Such a water molecule may play a role similar to that proposed for the hydroxyl of the serine glycosynthases in aiding fluoride departure. Indeed, the plasticity of solvent position may well facilitate better interaction with the departing fluoride than with a rigid serine side-chain and may account for the superiority of some glycine glycosynthases as catalysts.[12] An additional interaction of O1, with the hydroxyl of Tyr285, Figure 4, may further contribute to facile departure of the leaving-group.

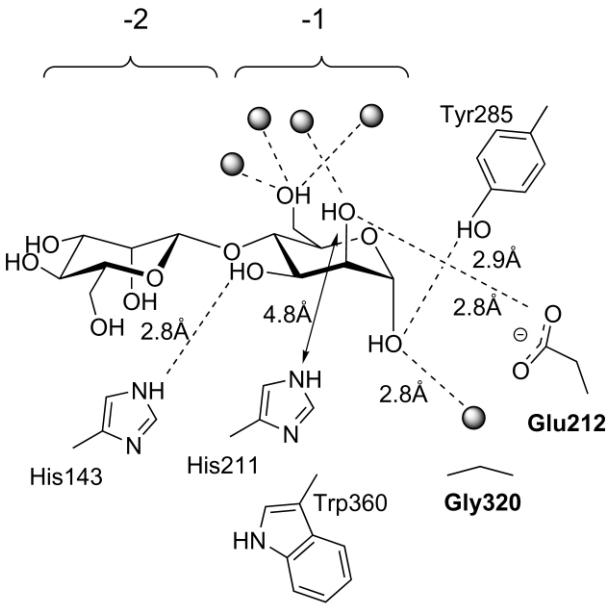

Fig. 4 Schematic diagram of the interactions of Man26A Glu320Gly with mannobiose. Only the −1 subsite interactions are included, with distances given where relevant. Water molecules are shown as shaded spheres.

In an attempt to expand further upon our collection of glycosynthases we generated Gly and Ser mutations at the nucleophile position of another family 26 mannanase, the Man26A from *Cellulomonas fimi*.[14] This enzyme has 40% sequence identity to Man26A from *Cellvibrio japonicus*, but did not catalyse glycosynthesis. The *Cellvibrio* enzyme is, perhaps, an unusual glycoside hydrolase in that it contains only four subsites and yet hydrolyses mannan efficiently, primarily because the −2 subsite binds very tightly to mannose as exemplified by the observation that the enzyme hydrolyses mannotriose ~5000 times more efficiently than do other mannanases.[13] The tight binding at the −2 subsite may explain why the *Cellvibrio* but not the *Cellulomonas* mannanase is effective as a glycosynthase.

In summary this study has shown that mannooligosaccharyl residues derived from naturally-occurring mannan can be incorporated into oligosaccharide structures using the glycosynthase approach. Use of this mutant enzyme should allow the assembly of defined manno-oligosaccharides as well as interesting mixed oligosaccharides with useful properties.

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Notes and references

‡ *Experimental*: Mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions, employing pDB1 as the template DNA and appropriate primers.[6] The donor for the glycosynthase reaction was synthesized by enzymatic hydrolysis of ivory nut mannan using wild type mannanase Man26A yielding predominantly the disaccharide. Acetylation with Ac_2O/pyridine gave an anomeric mixture of the octaacetates, which were converted into the α-fluoride by the action of HF/pyridine. Final deprotection with NH_3/MeOH gave α-mannobiosyl fluoride in an overall yield of 60%. Enzymatic synthesis of manno-oligosaccharides was performed by incubating potential acceptor sugars (30 mM) together with α-mannobiosyl fluoride (50 mM) and Man26A Glu320Gly or Glu320Ser (~0.3 mg ml^{-1}) in buffered solutions at neutral pH at room temperature. Kinetic analysis of the glycosynthase reactions of Man26A mutants was carried out by use of a fluoride ion selective electrode to monitor the coupling reaction. Apparent K'_M values for the donor (α-mannobiosyl fluoride) were determined by varying its concentration in the presence of a fixed concentration (70 mM) of the acceptor (pNP-Glc), and apparent K'_M values for the acceptor were determined at fixed concentrations (15 mM) of α-mannobiosyl fluoride. To determine the pH-optimum, reactions were monitored using a fluoride ion sensitive electrode, and a fit of the time course of fluoride release at low donor concentration (~0.05 mM) and high acceptor concentration (55 mM) to first order rate equations at different pH-values between 4.5 and 8 yielded a set of values of k'_{cat}/K'_M. 100 mM citrate and phosphate buffers were used to adjust the various pH-values. Crystals of Man26A E230G were grown as in Ref. 9 in the presence of 10 mM mannotetraose. Data, to 1.4 Å resolution, were collected from a single crystal at 100 K in a stream of N_2 gas on European Synchrotron Radiation Facility beamline ID14-EH2 and were processed with the HKL suite.[15] The structure was solved using AMoRe[16] and the structure refined with REFMAC[17]and programs from the CCP4 suite.[18] Data are 94% complete to 1.4 Å (100% to 1.6 Å) with Rmerge 0.036 (0.38 in outer resolution shell). The final structure has R_{cryst} 0.15 with R_{free} 0.18. Details of data and structure quality are provided as supplementary material.† Coordinates have been deposited with accession code 1odz.pdb.

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