Mechanistic Insight into Enzymatic Glycosyl Transfer with Retention of Configuration through Analysis of Glycomimetic Inhibitors**

James C. Errey, Seung Seo Lee, Robert P. Gibson, Carlos Martinez Fleites, Conor S. Barry, Pierre M. J. Jung, Anthony C. O’Sullivan, Benjamin G. Davis,* and Gideon J. Davies*  

One of the fundamental challenges in the field of glycobiology remains the dissection of the catalytic mechanism of nucleotide-sugar dependent glycosyltransferases (GTs), especially those that act with retention of anomeric configuration. For a class of enzymes that contains >20000 putative members in over 90 families it is remarkable that our understanding of the mechanistic origin of the notably high stereoselectivity of retaining GTs is so poor; to date only glimpses of trapped pseudo- (off pathway) intermediates,[3] mutational analyses[3] or unexpected inhibitor configurations[3] have given hints. None unambiguously support the origin of net retention being either through double inversion (SN2) or internal return (SNi) mechanisms. Theoretical analyses[4] have shown that both pathways are accessible and that the SNi pathway provides a lower energy manifold as result of active-site geometrical constraints.

A major barrier to the study of these enzymes is the lack of non-hydrolysable substrates or inhibitors, with affinities similar to that of the substrates, which would thus allow structural access to the ternary complex or something that resembles it. Glycosyltransferase inhibitors are rare; rarer still are those which do not harness portions of the nucleotide donor.[5,6] Only one bisubstrate analogue of a retaining GT has previously been described;[3] surprising inhibition profiles were observed, inconsistent with a double-displacement mechanism. Partially as a consequence of this lack of suitable compounds, there are no 3-D structures of intact ternary complexes with which to describe the catalytic centre and reveal geometry of the transfer process. Here we describe the synthesis and screening of pseudo-disaccharide inhibitors of the trehalose-6-phosphate synthase, a classical retaining glycosyltransferase of the “GT-B” fold. Enzyme kinetics in the absence and presence of different inhibitors revealed one compound with an affinity similar to that of the substrates that was subsequently used to obtain a ternary complex with this synergistic inhibitor.

The disaccharide trehalose (1, α-α-glucopyranosyl-α-α-glucopyranoside; Scheme 1) and its 6-phosphate (2) are non-reducing α,α-1,1 disaccharides with considerable importance in nature.[7,8] Given their absence in mammalian biology, trehalose synthesising and processing enzymes offer attractive inhibition targets. For example, trehalase is the target of the commercial agricultural fungicide validamycin,[9] and trehalose-6-phosphate synthase was chosen by Bayer scientists as a target for developing a new fungicide.[10] Trehalase is also central to many bacterial and fungal strategies to overcome environmental stress, and it is a major constituent of the unusual glycolipids of mycobacteria, including Mycobacterium tuberculosis.[11] Most success has been achieved with inhibitors of the hydrolytic trehalase enzyme. Compounds such as validoxylamine A (3) have proven to be tight-binding and structurally informative[12] trehalase inhibitors by virtue, in part, of the resemblance of the pseudodisaccharide scaffold of 3, for example, to 1.

The predominant[13] pathway for the biosynthesis of trehalose involves the initial formation of trehalose-6-phosphate (T6P, 2, the product of OtsA), and comparative pseudodisaccharide mimetics based on the α-glucopyranosyl moiety. Numbering shown is that of trehalose to aid comparison.

* Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200905096.
The trehalose-6-phosphate synthase, OtsA, was the first retaining nucleotide-sugar dependent glycosyltransferase structure solved of the “GT-B” fold (one of two glycosyltransferase topologies). The structure of *E. coli* OtsA features a fold similar to that seen for the PLKP-dependent enzyme glycogen phosphorylase,[16] with N-terminal acceptor and C-terminal UDP-Glc donor domains that act, with conformational flexibility, to achieve the synthesis of trehalose-6-phosphate (2).[15,17] Structures have subsequently been solved in complex with Glc-6-P and UDP and as the binary complex with a UDP-2FGlc donor but, as with most glycosyltransferase structural analysis, no ternary complex exists.

In order to generate potential disaccharide mimetics that would allow access to these first such ternary complexes, we prepared compounds 4–7 (Scheme 2, see Supporting Information for full details). These were based on the previously successful scaffold mimetic validoxylamine 3 and utilized features of transition state mimicry suitable for inhibition:[16] 1) bisubstrate mimicry; 2) flattening of transferred sugar mimic (modelling of the S NiT S[4] has suggested near-cyclo-

Scheme 2. a) PhCH(OMe)₂, DMF, TsOH, 39%; b) py, Ac₂O, 76%; c) AcOH (70% aq.), 60°C, 79%; d) TscI, py, 40°C, 97%; e) Ac₂O, py, 96%; f) AcOH (80% aq.), 63%; g) (Pr₂N)PO(Ph)₂, tetrazole, then MCPBA, CH₂Cl₂, 82%; h) TMSBr, iPr₂NEt, CH₂Cl₂, 95%; i) NH₃, MeOH, 47%; j) H₂NSO₂Cl, 98%; k) NH₃, MeOH, 30%; l) NH₃, MeOH, 94%; m) Br₂, Bu₃Si, NaH, 83%; n) AcOH (70% aq.), 90%; o) (Pr₂N)PO(Ph)₂, tetrazole, then MCPBA, CH₂Cl₂, 82%; p) Li, NH₃, 58%; q) H₂NSO₂Cl, 99%; r) Li, NH₃, 77%. DMF = dimethylformamide, Ts = p-toluenesulfonyl, py = pyridine, Trt = trityl, Bn = benzyl, MCPBA = meta-chloroperbenzoic acid, TMS = trimethylsilyl.

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Table 1: IC₅₀ values for compounds 4–7.

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OTsA “pseudo-single substrate” kinetics were performed using an assay in which UDP release was coupled through pyruvate kinase and lactate dehydrogenase to NADH formation. Under these conditions, OtsA yielded $k_{cat} = 34 ± 1 s^{-1}$, and $K_M$ values of 1.7 ± 0.3 mM (UDP-Glc) and of 7.3 ± 0.6 mM (Glc-6-P). An initial evaluation revealed that compounds 4, 5, and 7 (Table 1) all inhibited *E. coli* OtsA with IC₅₀ values between 5 and 28 mM. Consistent with intended structural analogy, better inhibition was observed for the 6-phosphomimetics 4 and 5 than for 7. Compound 6 showed only weak inhibition with slow onset.

Although less potent in numerical terms than other GT inhibitors, the 5 mM IC₅₀ values obtained for 4 were respectable when compared to the $K_M$ values of OtsA for its natural substrates. Initial screens (IC₅₀) also suggested inhibition by UDP and putative synergistic inhibition and prompted more detailed investigation of the inhibitory modes of OtsA using both 4 and UDP. Double reciprocal analyses (see Supporting Information, Figures S3 to S5) revealed that 4 alone competitively inhibited UDP-Glc ($K_M = 1.3 ± 0.2$ mM) and non-competitively inhibited Glc-6-P ($K_M = 4.2 ± 0.2$ mM). UDP alone competitively inhibited only UDP-Glc ($K_M = 140 ± 10$ µM) but not Glc-6-P. Together these suggested an ordered “bi-bi mechanism”[20] with UDP-Glc binding first followed by Glc-6-P and the effectiveness of 4 as a bisubstrate inhibitor likely operating through a direct inhibitory equilibrium between E and E′[20] Furthermore, addition of NDP (UDP) showed synergistic enhancement (Figure 1) of the inhibition of 4—approximately 100-fold improvement in the IC₅₀ value, to yield an IC₅₀ of 41 µM at 0.15 mM UDP (a concentration around UDP’s own $K_M$). It should be noted that in other than the leading examples of α-2,6-sialyltransferase inhibition[23,24] (which use NMP sugars), good inhibition of GTs has only been shown with a bisubstrate analogue towards...
the inhibition of inverting α1,3-fucosyltransferase[25] Such inhibition similarly required the synergistic presence of appropriate NDP (GDP). Such synergistic inhibition in the presence of NDP is, of course, of reduced potential clinical relevance since NDP concentration is not only uncontrollable in vivo but often unknown. However, here, in screening for a putative TS-like ligand this binding was highly encouraging.

Indeed, the inhibition, in the absence of UDP, shown here by 4 of OtsA is highly reminiscent of the weak binding of the inhibitor acarbose to glycogen and maltodextrin phosphorylases which also allowed access to the key ternary complex of that enzyme.[16] The structure of OtsA was therefore determined (statistics in Table S1) in a ternary complex with UDP and bisubstrate mimic 4. The structure, solved at 2.2 Å resolution, provides unambiguous electron density for both UDP and the pseudo-disaccharide 4.

The complex between OtsA and 4 illuminates the geometry present around the anomeric centre (Figure 2 and Scheme 3). This is especially important when formulating reaction mechanisms consistent with glycosyltransfer with net retention of anomeric configuration. These mechanisms remain very poorly understood, not least because a mechanism involving a double displacement with a covalent intermediate has little experimental support forcing enzymologists to consider “front face” mechanisms such as those akin to internal return “S_{Ni}” mechanisms[26] (discussed extensively in Ref. [27]). Recently, Goedl and Nidetzky have provided convincing evidence in a change in kinetic and chemical mechanism, from a double displacement to a “front side” mechanism in a sucrose phosphorylase (GH13) variant.[28]

A “front face” mechanism poses many problems. In particular, what is the catalytic geometry and what acts as the catalytic base to deprotonate the acceptor for nucleophilic attack at the donor anomeric carbon (in the direction of synthesis)? Intact nucleotide sugar glycosyltransferase ternary complexes are exceedingly rare and where they have been achieved, notably with the UDP-2FGal/3-deoxyxylate complex of the GT-A fold enzyme LgtC,[2] the geometry of the crucial phosphorous-C1-acceptor is undefined.

The 3-D structure of OtsA with 4 shows that the glucose-6-P mimicking portion binds essentially identically as observed in the Glc-6-P/UDP complex previously[15] (Figure 2). The flattened cyclohexenyl moiety of 4 binds in an approximate E conformation. Of particular interest is the H-bond (13 Å, bond c; Scheme 4) between the donor phosphate oxygen O3B (leaving group oxygen in the direction of synthesis and nucleophile in the reverse direction) to the “glycosidic” NH of the pseudo-disaccharide (Figure 2). This interaction shows, we believe for the first time on a UDP-sugar transferase of this kind, that the leaving-group phosphate oxygen indeed contacts the acceptor nucleophile (here the NH of the disaccharide inhibitor) in a manner consistent with the phosphate acting as a base to deprotonate the acceptor in the “internal return like” mechanism (Scheme 4), as semi-nally invoked by Sinnott and Jencks for the solvolysis of glycosyl fluoride by trifluoroethanol[26] and as predicted by modelling of retaining GTs (12, bond c, Scheme 4).[4] Indeed, the LgtC ternary structure is also completely consistent with such a mechanism if the absent 3-OH is simply modelled in its expected position. The more selective, concerted “internal-
Scheme 4. An “SNi-like” mechanism for retaining glucosyltransfer. The boxed ternary species 12 and 13 equate to that proposed for Sn2 and modelled by Tvaroska[26] and that seen experimentally in the OtsA complex with UDP and 4, respectively.

return-like[28] mechanism[29] is likely additionally favoured in a desolvated, less polar active centre, and involves the deprotonation of the incoming nucleophile by the departing phosphate oxygen.[30] The extent and timing of the making and breaking of bonds a–d in 12 (Scheme 4) is unclear and extremes as represented by 14 may be possible. Nonetheless, structure 13 determined here for the first time gives direct evidence of a bond geometry consistent with a TS such as 11.

Experimental Section
Trehalose-6-phosphate synthase activity was assayed spectrophotometrically by coupling the formation of UDP to the reactions of pyruvate kinase, and lactate dehydrogenase inhibition experiments were carried out at varying concentrations of both substrates and pyruvate kinase, and lactate dehydrogenase inhibition experiments were carried out at varying concentrations of both substrates and whilst varying the concentration of inhibitors. Initial velocity kinetic analyses, including data were then fitted using GRAFIT5 to allow full double reciprocal analysis, including data were then fitted using GRAFIT5 to allow full double reciprocal analysis, including data were then fitted using GRAFIT5 to allow full double reciprocal analysis, including data were then fitted using GRAFIT5 to allow full double reciprocal analysis, including Kₘ determinations or OtsA activity and inhibition by 4 and UDP. Rate measurement when UDP was used as a sole inhibitor or a synergistic inhibitor determined OtsA activity by coupling the formation of UDP to the reactions of pyruvate kinase and lactate dehydrogenase (PK/LDH) in a background/product-calibrated stopped assay format (see Supporting Information for further details). Structures of OtsA were crystallised from protein at 10 mgl⁻¹ in a buffer of 20 mm TrisHCl pH 8 and 200 mm NaCl with 10 mm UDP and 5 mm 5. Data, to 2.2 Å resolution were collected at 100 K at the European Synchrotron Radiation Facility. Structures were refined using the CCP4 suite. [31] Data and structure quality statistics are given in Table S1, other experimental information is provided in the protein data base (pdb no. 2WTX). Additional synthetic details are given in the Supporting Information.

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