Substrate and metal-ion promiscuity in mannosylglycerate synthase

Running title: metals and glycosyltransferases

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The enzymatic transfer of the sugar mannose from activated sugar donors, is central to the synthesis of a wide range of biologically significant polysaccharides and glycoconjugates. In addition to their importance in cellular biology, mannosyltransferases also provide model systems with which to study catalytic mechanisms of glycosyltransfer. Mannosylglycerate synthase (MGS) catalyzes the synthesis of α-mannosyl-D-glycerate using GDP-mannose as the preferred donor species; a reaction that occurs with net retention of anomeric configuration. Past work has shown that the Rhodothermus marinus MGS, classified as a GT78 glycosyltransferase, displays a GT-A fold and performs catalysis in a metal-ion dependent manner. MGS shows very unusual metal-ion dependences with Mg2+, Ca2+ and, to a varying extent, Mn2+, Ni2+, and Co2+, facilitating catalysis. Here, we probe these dependences through kinetic, and calorimetric analyses of wild-type and site-directed variants of the enzyme. Mutation of residues that interact with the guanine base of GDP are correlated with a higher $k_{cat}$ whilst substitution of H217, a key component of the metal-coordination site, results in a change in metal specificity to Mn2+.

Structural analyses of MGS complexes not only provide insight into metal coordination but also into how lactate can function as an alternative acceptor to glycerate. These studies highlight the role of flexible loops in the active centre and the subsequent coordination of the divalent metal-ion as key factors in MGS catalysis and metal-ion dependence. Furthermore, Y220, located on a flexible loop whose conformation is likely influenced by metal binding also plays a critical role in substrate binding.

Mannose-containing oligosaccharides, polysaccharides and glycoconjugates are ubiquitous across nature. The sugar is present in N-linked glycans and glycolipids, while mannose-containing polysaccharides represent key features of plant, fungal and bacterial cell walls. In higher organisms, glycan decorations on glycoproteins have the potential to modulate many integral cellular processes including protein trafficking, cell signalling, immune modulation and host-pathogen recognition (1,2). Reflecting their role in modulating many key cellular processes, mannose-processing enzymes, both glycoside hydrolases (GHs) and glycosyltransferases (GTs), have considerable potential as cellular targets to probe both healthy and diseased cells.
In order to survive in the harsh conditions of their environments, extremophiles attach mannose to small negatively-charged molecules such as glyceramide, glycerate, lactate, and phosphoglycerate; forming molecules which confer tolerance to osmotic stress and thermostability (3). These tolerance-conferring osmolytes function through balancing the ionic strength across membranes in saline rich environments, as well as stabilizing proteins by reducing backbone motions, resulting in overall protein rigidification and melting temperature elevation (4,5). Of these stabilizers, 2-O-α-D-mannosylglycerate (MG) appears to be both a potent and highly prevalent osmolyte in thermophilic and hyperthermophilic eubacteria and archaea (3). MG synthesis is catalysed by a GDP-Mannose dependent glycosyltransferase, termed mannosylglycerate synthase (MGS), an enzyme that is central to studies reported here.

Based on sequence similarities GTs have been grouped into approximately ninety families in the carbohydrate active enzyme database (6,7) (www.cazy.org), roughly nineteen of which contain members that transfer mannosyl groups, although other donor substrate specificities are evident in many of these families. In the majority of these families the donor substrate is GDP-Man (2, 4, 15, 26, 32, 33, 55, 62, 69, 71, 78, 81, 91), while in GT families 22, 39, 50, 58, 76 and 87 the mannosyl residue is transferred from an activated phospholipid to its donor molecule. Surprisingly, given the sequence diversity represented across GT families, predominantly only two structural folds are adopted by nucleotide sugar-dependent GTs; termed GT-A and GT-B (6,8) (A third fold class, GT-C, has recently been proposed for trans-membrane spanning GT family 66 which utilize lipid phosphate-activated sugars as the donor substrate (9,10)).

The majority of GT-A type enzymes employ divalent cations to assist catalysis whereas GT-B classified enzymes utilize positively charged side chains and/or hydroxyls and helix dipoles (reviewed in Ref. (11)). The presence of the metal in the catalytic cycle is therefore pivotal to the major distinction between the GTs; yet the variation of this metal and the implications for its role have been explored little.

The overall architecture of both GT-A and GT-B fold types is similar in that each consists of a pair of β/α/β Rossmann-like domains. In the GT-A fold the Rossmann-like domains are tightly associated, forming a central extended β-sheet architecture whose loops change conformation upon ligand binding, whereas in GT-Bs the two domains are more loosely associated “facing” each other and often moving as whole domains in response to ligand binding (11). Mechanistically, GTs may also be divided into whether the reaction leads to net retention or inversion of the stereochemistry of the anomeric carbon of the donor glycoside. Representatives of both inverting and retaining GTs exist in both fold families. Analogous to the mechanism of inverting glycoside hydrolases, inverting GTs most likely follow a single SN2-like displacement mechanism where the acceptor molecule, which is activated generally be a catalytic base, acts as a nucleophile, attacking the sugar donor at the anomeric carbon (reviewed in Ref. (11)). In contrast, the mechanistic details of retaining GTs are far less clear, although it has been proposed that these enzymes catalyze glycosidic bond formation through an SNi-like reaction of asynchronous ‘front-face’ departure and attack which is mechanistically analogous to the well known reaction of alkyl chloro sulphites (12) (recently discussed in a glycosyltransferase context in, for example, (11,13)). The continuing catalytic ambiguity of retaining GT highlights that, despite the importance of glycosyl transfer in nature and potential for commercial application, there is a paucity of
structural and mechanistic information on these enzymes.

The crystal structures have been reported for three mannosyltransferases which produce the protective osmolytes MG (family 78) or mannosyl-3-phosphoglycerate (family 55 and 81); (14-16). The choice of systems reflects, in part, the amenability of hyperthermophilic proteins for structural investigation, the excellent comparable models using relatively simple acceptor substrates but also their role in the synthesis of metabolites essential for survival. The GT family 78 mannosyltransferase, mannosylglycerate synthase (MGS), is expressed by the hyperthermophilic bacterium *Rhodothermus marinus*. MGS adopts a GT-A fold and catalyzes glycosidic bond formation with retention of anomeric configuration of the transferred α-mannosyl moiety (14), Figure 1a. Employing a high-throughput substrate screening strategy, MGS was shown to utilize GDP-α-mannose (and other GDP-sugars) as a donor substrate in a reaction in which the mannose is transferred not only to the C2-OH group of D-glycerate but also D-lactate or glycolate. Kinetically, MGS was shown to employ various divalent cations, with Mg$^{2+}$, Ca$^{2+}$ and, to a less extent, Mn$^{2+}$, Ni$^{2+}$, and Co$^{2+}$, facilitating catalysis.

Here we have used structural and biochemical approaches to probe the unusual variation in metal-ion specificity displayed by MGS, as well as its donor recognition and acceptor substrate plasticity. The data revealed the structural features that determine the relative preferences for acceptor substrates, provided evidence for GDP interaction while also shedding light on the functional significance of H217 in the selection of catalytic metal ions by the enzyme.

**METHODS AND PROCEDURES**

**Materials**

2-O-α-D-Mannosylglycerate for ITC and product inhibition studies was synthesized and purified as described in the Supplemental Methods to Ref. (14).

**Cloning, site-directed mutagenesis and expression of RmMGS**

pJF1 and pJF2 (full-length MGS and MGS with a 15 residue C-terminal truncation, respectively) were previously constructed by amplification of MGS from *Rhodothermus marinus* genomic DNA as described (14). Mutants for kinetic studies were constructed by using the QuikChange site-directed mutagenesis kit (Stratagene) with pJF1 as template and primers listed in Supplemental Table S1. pJF2 was used for crystallization complexes with lactate. A modified pJF2 mutant was used for crystallisation of other complexes (see crystallization section). MGS wild-type and mutants were expressed and purified as described (14). Briefly, *Escherichia coli* strain Tuner transformed with appropriate plasmid was grown to OD ~ 0.6 in 1 L LB media with 50 µg/mL ampicillin, then incubated at 16 °C before induction of MGS expression with 0.2 mM isopropyl β-D-thiogalactopyranoside. Cell-free extracts were heat-treated at 65 °C for 20 min and MGS was purified by immobilized metal-ion affinity chromatography (IMAC). Proteins for kinetic studies were sufficiently pure after IMAC purification. When analyzing the influence of metals on enzyme function, MGS was treated with Chelex-100 (Biorad) to chelate and exchange unwanted metals. For crystallography MGS was further purified by anion exchange.

**MGS Surface Entropy Minimization Engineering**

Large, three-dimensional crystals of MGS readily develop in an array of crystallization conditions. However, the maximum resolution obtained during diffraction experiments of complex structures was of moderate quality (2.45-2.95 Å) (14). In an attempt to ameliorate MGS surface properties for crystallization, MGS was analysed with SERp (http://nihserver.mbi.ucla.edu/SER) to
suggest segments of residues which make substantial surface entropy contributions (17). SERp highlighted three putative clusters at positions E89/E90, E115/E116, and Q253/E254. An additional site was selected at Q201/Q202 through manual structural inspection, Figure 1b. Each cluster was evaluated for solvent exposure and contribution to intra- and intermolecular contacts using known MGS structures. Based on this analysis, E89A/E90A and Q201A/Q202A MGS mutants were generated by site-directed mutagenesis of which only the Q201A/Q202A MGS surface variant expressed to an appreciable amount.

Crystallization Data Collection and Structural Determination
Recombinant MGS-Tr-Q201A/Q202A surface variant (MGS-Sv) was expressed and purified as described previously for MGS-Tr (14) with the exception that MGS-Sv was buffer exchanged into 25 mM Tris-HCl, 500 mM NaCl, pH 8.0 prior to crystallization. MGS-Sv crystals developed after ~3 days in hanging drop setup at 19 °C in equal volumes of protein (15 mg/mL) and reservoir solution consisting of 4% (v/v) ethylene glycol, Bis-Tris propane, pH 5.5, supplemented with either magnesium formate, manganese formate, or sodium malonate between 0.2-0.5 M, where appropriate. MGS-lactate crystals were grown in 0.3 M sodium β-lactate, 0.1 M sodium acetate trihydrate, pH 4.6, 40% (v/v) 2-methyl-1,3-propanediol which acted as a cryoprotectant. GDP and GDP-mannose complexed crystals were obtained by soaking apo-crystals in mother liquor supplemented with 10 mM ligand for ~10 min and subsequently cryoprotected in mother liquor supplemented with 35% (v/v) ethylene glycol.

Diffraction data for were collected at beamlines ID-29 of the European Synchrotron Radiation Facility (lactate and Mg²⁺ + GDP), IO3 of Diamond Light Source (Mn²⁺ + GDP) and the York Structural Biology Laboratory home source (Mg²⁺ + GDP-mannose). In each case, MGS crystals were evaluated to belong to the space group $P\overline{3}21$ and to contain two MGS molecules in the asymmetric unit. Data were processed with either the HKL2000 suite (18) or iMosflm/Scala (19,20) and structural solutions were obtained by molecular replacement using PHASER (21). Ligands were subsequently incorporated followed by cycles of maximum-likelihood refinement using REFMAC (22) interspersed with manual corrections of protein models using COOT (23). Overall, the surface entropy minimization of the MGS Q201A/Q202A mutant together with the identification of novel crystallization conditions resulted in a slight improvement to diffraction. Other computing used the CCP4 suite (24) unless otherwise stated. Data processing and refinement statistics are presented in Table 1. Structural figures were drawn with PyMol (DeLano Scientific LLC).

Mass-spectrometric activity screening through direct detection of products
Initial rates were determined using a quantitative mass-spectrometry based assay as described previously (14,25). Briefly, a calibration curve for mannosylglycerate was constructed by measuring the ratios of the total ion count (TIC) of mannosylglycerate at varying concentrations (1 μM, 3 μM, 5 μM, 7 μM and 9 μM) to the TIC of pseudo-internal standard UPDP (100 μM) in 1 mM Tris-HCl buffer, pH 7.8. Enzyme-catalysed reactions were performed in 1 mM Tris-HCl buffer, pH 7.8 at 37 °C, using final concentrations of 100 μM GDP-Man, 100 μM D-glycerate, 50 μM Ca²⁺ and 1 μM enzyme. Initial rates were determined from mannosylglycerate concentrations calculated every 30 min over 480 min from TIC measurements of a 10 μL aliquot mixed with 10 μL of UDP (100 μM). Initial rates determined by MS methods were within error of those determined by linked methods thus eliminating the possibility of artefacts from the latter.
Continuous enzyme-linked assay
Initial rates were also determined using indirect detection of GDP by coupling the release of GDP to NADH oxidation via pyruvate kinase and lactate dehydrogenase developed from (26). Assays were performed in final concentrations: 10 mM HEPES, pH 7.0, 50 mM KCl, 0.1% (w/v) BSA, 0.15 mM NADH, 0.7 mM phosphoenolpyruvate potassium salt, 6 U/mL pyruvate kinase, and 30 U/mL lactate dehydrogenase (both Sigma, rabbit muscle - type II, ammonium sulphate suspension) with 75-500 nM enzyme at 23 °C. NADH oxidation was monitored by the decrease in absorbance at 340 nm. 2 mM MgCl₂ or 2 mM MnCl₂ were added to each assay to test the effect of the metal-ion. For each variant of MGS assayed the reaction was carried out in the absence of acceptor and showed no GDP release. These experiments confirmed that there was no enzyme-mediated hydrolysis of the donor substrate.

For pseudo-single substrate kinetics the fixed substrate concentration used was >3 x \(K_m\) value. \(k_{cat}\) and \(K_m\) were calculated by fitting the Michaelis-Menten equation to the initial rates (GraphPad Prism version 4.03, GraphPad Software, San Diego, CA). Bi-substrate kinetics substrate kinetics used concentrations of each substrate corresponding to \(\frac{1}{2}, 1, 2, 4, \) and 10 times the \(K_m\)-value determined in the pseudo-single substrate kinetics. Data were analyzed for a general bi-substrate system using Equation 1:

\[
v = \frac{v_{max} [A][B]}{K_{ia} K_B + K_B [A] + K_A [B] + [A][B]}
\]

(Eq. 1)

\(K_A\) and \(K_B\) are the limiting Michaelis constant for A(acceptor) and B(donor), respectively, when the other substrate is saturating. \(K_{ia}\) and \(K_{ib}\) are the dissociation constants between enzyme and acceptor or donor respectively. \(K_A, K_B, K_{ia}, \) and \(K_{ib}\) were calculated as described in (27).

Metal-ion dependence and product inhibition analysis
The metal-ion dependences of MGS wt and the H217A variant were determined by adding 0.001-0.5 \( \mu M \) enzyme at 37 °C (final concentration) to 100 \( \mu M \) d-glycerate and 500 \( \mu M \) GDP-mannose in 13 mM HEPES, pH 7.0, 1 mM EDTA, 0.1% (w/v) BSA with 3 mM of either CaCl₂, CoCl₂, MnCl₂, or NiCl₂. Aliquots were mixed with EDTA (final concentration 10 mM) to stop the reaction. Product inhibition reactions were carried out in 10 mM HEPES, pH 7.0, 50 mM KCl, 2 mM CaCl₂, 0.1% (w/v) BSA and 0.1 \( \mu M \) enzyme at 23 °C. The concentration of d-glycerate was fixed at 500 \( \mu M \), while the concentration of GDP-Man covered a range from 50-4000 \( \mu M \). GDP was added to give a final concentration of 10-100 \( \mu M \). Aliquots were mixed with the same volume of 10 mM EDTA as a metal chelator to inactivate MGS.

Direct measurement of GDP release
The release of GDP was determined by HPLC (Gemini C18, 5 \( \mu m \), 250 x 4.60 mm column (Phenomenex), Dionex ICS-3000 HPLC, 100 \( \mu L \) aliquots) equilibrated with buffer A (50 mM KH₂PO₄, 5 mM tetrabutylammonium hydrogen sulphate, 2% (v/v) acetonitrile). GDP and GDP-Man were eluted using a linear gradient (0 to 20% of buffer B (100% (v/v) acetonitrile)) and monitored by UV-detection (260 nm) at 25 °C (Dionex AD20 Absorbance detector).

Isothermal titration calorimetry
Isothermal titration calorimetry (ITC) measurement was performed on a VP-ITC MicroCalorimeter from MicroCal, Inc, (Northampton, USA). All titrations were carried out at 25 °C in 20 mM Tris-HCl, pH 8.0. The concentrations of ligand (GDP-Man, d-glycerate (calcium salt), GDP and mannosylglycerate) used was 0.5-1 mM and MGS wild-type and H217A mutant in the reaction cell were at 40-55 \( \mu M \). All protein was dialyzed in Chelex-treated buffer and stock solutions of GDP, CaCl₂, MgCl₂, and...
MnCl₂ were prepared in the same dialysis buffer. Background titrations with GDP or GDP-Man into buffer were subtracted from the raw data of titrations of MGS with GDP or GDP-Man, respectively. The determined $K_A$ and $\Delta H$ were used to derive $\Delta S$ from standard thermodynamic equations.

RESULTS
The general structure of MGS is composed of a mixed $\alpha/\beta$, GT-A fold catalytic domain (residues 2-262), and a six-helix C-terminal domain (residues 263-381) thought to be important for oligomerization (MGS is a tetramer) (14), Figure 1b. In previous work, we provided a preliminary indication that perhaps the active centre coordination of the nucleotide portion of the sugar donor species was as important as the nature of the donor sugar itself (14). Here we have probed the donor and acceptor subsites, primarily through kinetic analysis of enzyme-variants.

Three-dimensional structure of MGS and ligand complexes
In the previous report on the crystal structure of MGS there were six molecules in the asymmetric unit making structure determination difficult. In this report we have used a combination of site-directed mutagenesis and modification to the original crystallization conditions to generate crystals of MGS in the space group P3₂₁ with two molecules in the asymmetric unit, Table 1, which was associated with a high solvent content (~75 %). The description of the crystal structures of MGS in complex with various ligands is based partly on data described previously by Flint and colleagues (14) (GDP-Man/Mn²⁺, GDP/Mn²⁺, and Mn²⁺/glycerate) and also new complexes (GDP-Man/Mg²⁺, GDP/Mg²⁺, GDP/Mn²⁺/malonate, and lactate) that are reported here. Together these survey structures in which both hard and soft metals are bound.

The active centre is located almost in the protein core, with residues shown to make important donor/acceptor interactions contributed from various interlacing loops of the catalytic domain. Due to the high crystal solvent content (previous crystal structures and new structures reported here), the MGS structures have high molecular mobility. While this characteristic has the effect of diminishing crystal order and therefore diffraction resolution limits, it does provide an opportunity to characterise some of the highly dynamic regions of MGS. B-factor analysis suggests residues in regions 1-66, 100-140, 215-222, and 250-270 have high mean atomic displacement. Many of these residues are located in the active centre. Interestingly, these regions remain flexible even when MGS is in complex with donor or acceptor substrates and products, suggesting that protein flexibility is important for all stages along the reaction coordinate.

Donor Substrate Recognition
The MGS structure(s) revealed that the guanine base of GDP is housed in a hydrophobic pocket between the side chains of K9 and Y37 on one face, and the aliphatic portion of the side chain of K76 on the opposing face, with Q66 accepting hydrogen bonds from the endocyclic N1 and exocyclic N2, Figure 1c,d. To explore the functional significance of guanine recognition the properties of the alanine mutants K76A, Y37A, K9A and Q66A were evaluated, Table 2. Determination of kinetic parameters in the presence of excess co-substrate revealed that mutation of Lys76 to alanine resulted in ~3- to 4-fold increase in $k_{cat}$ coupled with an elevation in $K_m$ for D-glycerate and GDP-Man of 15- and 3-fold, respectively. Similarly, the Y37A and K9A mutations also cause a significant increase in $k_{cat}$ (4- to 10-fold) in the presence of saturating co-substrate. Notably, the two mutations had little effect on the $K_m$ for GDP-Man. The $K_m$ towards the acceptor substrate glycerate for the K9A mutant was also little affected, whilst the
Y37A mutant showed an 8-fold increase in the $K_m$ compared to wild type MGS, Table 2. As a consequence, K9A has a catalytic efficiency ($k_{cat}/K_m$) that is almost an order of magnitude higher than wild type enzyme for both substrates. The Q66A mutation resulted in a substantial increase in $k_{cat}$ (up to 72-fold) and $K_m$ (~30-fold) for both the acceptor and donor substrates, Table 2. The mutation data described above suggest that altered binding to the guanine base leads to an increase in the turnover rate of the enzyme. The elevation in $k_{cat}$ through mutation of regions remote from the catalytic residues (and hence less likely to directly influence the transition state of transfer) suggests that the rate of nucleotide product release limits the turnover rate of the reaction. Mutations that weaken the interaction of GDP with the enzyme facilitate product departure and, in so doing, increase the catalytic rate. It should be emphasised that the actual rate limiting step is unclear. It may comprise the direct release of the nucleotide product, although it is also possible that the turnover rate is limited by conformational changes required as a prelude to ejecting GDP from the active site.

Ribose recognition in the donor subsite is provided through interaction with the side chain of E11, the main chain amides of K9 and A101, and main chain carbonyl of P7. R218 and Y220 interact with the $\alpha$- and $\beta$- phosphates of GDP-Man, respectively, and have highly mobile side chains across MGS structures Figure 1. Previous mutational analysis (14) showed that the E11A mutation caused a modest increase in $k_{cat}$ and $K_m$ (for GDP-Man). By contrast the R218A substitution results in a significant increase in $k_{cat}$ and $K_m$ for GDP-Man, but had only a modest influence on the $K_m$ for the acceptor substrate, Table 2. The functional significance of mutants of Y220 is discussed with respect to acceptor recognition below.

The mannosyl moiety of GDP-Man is observed in $^{4}{C}_1$ conformation and is coordinated in a pocket with M229 and W189 providing a hydrophobic platform below the pyranoside ring, and side chains of K76, D100 and D192, together with the main chain carboxyl of L163, interacting with O3 (K76 and D100), O4 (K76 and D192), O6 (D192) and O2 (L163), Figure 1. A hydrogen-bonding network from D192 via K76 to D100 is established in a system analogous to the UDP-galactosyl transferase LgtC (28). Previous mutational analysis showed that D100 plays a critical role in substrate binding as the D100A mutant was completely inactive (14). The importance of polar interactions with mannose is reinforced by the observation that D192A is also completely inactive, Table 2. It is somewhat surprising that the K76A mutation did not have a detrimental effect on activity as the N$_{\zeta}$ of K76 appears to make hydrogen bonds with O3 and O4, while also making electrostatic interactions with D192. It would appear that these polar contacts are less important than those mediated by the carboxylate side chains. Indeed, we have previously speculated that a basic residue in a position equivalent to K76 was a conserved feature of GT-A glycosyltransferases, however, the functional significance of this amino acid is rather opaque. It is possible that its contribution to the guanine binding pocket may be more important than its polar contact with the donor sugar. The minor influence of the M229A mutation on catalytic performance suggests that the methionine also does not play an important role in substrate recognition despite its suggested role in the hydrophobic ‘base’ of the donor site. The W189A mutant displayed, respectively, a 3- and 7-fold increase in $k_{cat}$ and $K_m$ for d-glycerate, compared to the wild-type enzyme, but did not influence utilization of GDP-Man. Thus the hydrophobic platform-mediated mannose binding under these conditions does not appear critical, which is in contrast to many carbohydrate proteins (both catalytic and non-catalytic) where apolar interactions with sugar
rings represents an important binding mechanism.

**MGS acceptor site**

Previously (14), two MGS complexes with molecules in the acceptor-binding-site were obtained; MGS with the natural substrate D-glycerate and substrate analogue citrate. In both cases ligand coordination featured main-chain interactions complemented with the side chains of only Arg131 and Thr139. To investigate further interactions at the mannose-acceptor-site we obtained complexes of MGS with an additional substrate D-lactate, **Figure 2**. Comparison of this structure with the D-glycerate complex highlights that coordination of the acceptor carboxylate effectively anchors each molecule at an equivalent position in the acceptor site. Arg131 sits “below” the acceptor position, with the side chain guanidino NH₂ groups forming two polar interactions with one of the carboxylate oxygens. This oxygen makes an additional interaction with the hydroxyl side chain of Thr139. The partnering carboxylate oxygen forms hydrogen bonds with backbone amide hydrogens of Met137, Ile138 and Thr139 in an overall pyramidal arrangement. Additionally, the C3-OH of D-glycerate forms a hydrogen bond with the backbone amide of Ala136, stabilizing the substrate and helping to correctly orient the C2-OH for mannose addition. Overall, with the exception of the orientation of the Arg131 side chain, the MGS acceptor site undergoes very little change across all the obtained structures. To investigate the importance of the contribution of T139 to the acceptor site, the activity of T139A was analyzed (previously the R131A variant was shown to be >1,000 less active than wild-type MGS (14)). The data showed that the T139A mutation resulted in a modest decrease in \( k_{\text{cat}} \), but a 1500-fold increase in \( K_m \) for D-glycerate, **Table 3**. The mutant, similar to the wild type enzyme, was ~500 less active when D-lactate, rather than D-glycerate, was the acceptor substrate.

D135, although positioned in the vicinity of the C3-OH of glycerate, does not appear to make any direct interactions with either D-glycerate or D-lactate. However, D135 does form an ion pair with one of the guanidino NH₂ groups of R266 in structures examined, potentially contributing to the stability of the structure and helping to preserve the topology of the acceptor site. D135A had an extremely high \( K_m \) for D-glycerate (5600-fold higher than wild-type) and displayed a slight increase in \( k_{\text{cat}} \) (two-fold) compared to wild-type enzyme, **Table 3**. Structural alignment of the MGS ligand structures of acceptor (D-glycerate, PDB 2BO6) and donor (GDP-Man/Mn²⁺, PDB 2BO8) suggest that Y220 could also contribute to the binding of C2-OH of the acceptor. Tyrosine 220 is part of the highly mobile flexible loop that forms part of the active centre. Exact delineation of its position in crystal structures has proved extremely difficult with at best poor, and often "non-existent", electron density. Yet, the 500- and 1500-fold increase in \( K_m \) for D-glycerate of Y220A and Y220F in the presence of saturating GDP-Man, respectively, strongly suggest that Y220 constitutes an essential part of the acceptor binding site. A role of Tyr220 in acceptor binding perhaps comes from the GDP-Man (donor) complex published previously; here the loop and the whole of the Tyr220 side-chain become ordered with the Tyr220 hydroxyl interaction with the beta phosphate. In this position, it is highly likely that Tyr220 would interact with glycerate bound in the acceptor site providing a rationally-compelling argument for both the importance of GDP-Man binding in defining the acceptor site and thus also for the importance of Tyr220 to glycerate binding reflected in the kinetic analyses.

**Substrate binding order**

Inhibitors that compete with substrates, typically products of the reaction, can be used to probe the binding order of reactants in reactions catalyzed by glycosyltransferases.
GDP inhibited MGS by competing with GDP-Man with a $K_i$ of $\sim 36 \, \mu M$ **Supplemental Figure S1.** Pseudo-single substrate kinetics with Mn$^{2+}$ showed significant substrate inhibition with $\delta$-glycerate, which excluded the possibility of a rapid equilibrium random ordered mechanism (29). $\delta$-glycerate can only function as a substrate inhibitor if it is able to bind to MGS-GDP, forming a stable non-productive complex. This is consistent with the observation that $\delta$-glycerate will bind to MGS in the presence of GDP and a metal-ion ($K_D \sim 20 \, \mu M$ measured by ITC), but not in the absence of the nucleotide diphosphate (data not shown). These results support a compulsory order binding mechanism in which GDP-Man and $\delta$-glycerate bind successively to the enzyme. Mannosylglycerate was also evaluated as a product inhibitor; however, no inhibition was observed in the mM-range and ITC analysis revealed no significant binding of the molecule. By contrast ITC showed that GDP-Man and GDP binds to MGS, in the absence of $\delta$-glycerate, with $K_D$ values of 200 nM and 500 nM, respectively. These data are consistent with the view that the conjugated reaction product binds weakly to the enzyme and is likely to depart the active site prior to the other reaction product GDP. The relatively tight binding to GDP is consistent that departure of the nucleotide diphosphate limits the turnover rate of the enzyme.

**The role of metal ions in substrate recognition**

A key feature of GT-A glycosyltransferases, including MGS, is the role of metals in substrate recognition. Thus, notably, with GDP and the transition-series metals Co$^{2+}$ or Mn$^{2+}$, the divalent cation is coordinated by one of the imidazole nitrogens of H217 and the second aspartate of the DxD motif, D102 (11). The DxD motif is highly conserved in GT-A glycosyltransferases and is implicated in divalent metal-ion binding, which coordinates with the donor substrate. These residues aid in the positioning of the metal between O2 and O1 of the $\alpha$ and $\beta$-phosphates of GDP. Unusually, MGS displays a much higher $k_{cat}$ when group II metal-ions (Ca$^{2+}$ and Mg$^{2+}$) function as co-factors, compared to when transition series metal-ions fulfil this role, **Table 4 and Supplemental Figure S2.** The contribution of D102 and H217 to ion selection was explored through the characterization of the mutants D102A and H217A. The data, **Table 4**, show that while D102A was completely inactive in the presence of all metals tested, H217A displays a significant change in metal preference. Thus, the H217A mutant was essentially inactive in the presence of Ca$^{2+}$ (specific activity >1000 fold lower than wild-type MGS), while $k_{cat}$ was reduced 23-fold in the presence of Mg$^{2+}$ (compared to wild type enzyme). In the presence of Mn$^{2+}$, the removal of the imidazole side chain had the opposite effect; the $k_{cat}$ increased 16-fold. The $K_m$ for $\delta$-glycerate ($K_a$), increased 34- and >1600-fold for Mg$^{2+}$ and Mn$^{2+}$, respectively, and a similar increase was observed for the dissociation constant of $\delta$-glycerate ($K_i,a$) in the presence of Mg$^{2+}$. The kinetic parameters $K_a$ and $K_i,b$ ($K_m$ for GDP-Man) for wild-type MGS in the presence of Mn$^{2+}$, could only be estimated as the values are $>1 \, \mu M$. Thus, $K_i,a$ and $K_i,b$ (dissociation constant for GDP-Man) could not be calculated for wild-type MGS when Mn$^{2+}$ was the co-factor. To summarize, the H217A mutation causes a substantial reduction in the affinity for the acceptor substrate $\delta$-glycerate in the presence of both Mg$^{2+}$ and Mn$^{2+}$. While the mutation did not influence donor substrate binding in the presence of Mg$^{2+}$, the extremely tight interaction between the enzyme and GDP-Man in the presence of Mn$^{2+}$ was greatly reduced in the H217A mutant. Direct comparison of the activity of H217A in the presence of both metals also highlights that its binding of GDP-Man is increased with Mn$^{2+}$.

Together these data suggest that H217 may...
act as a functional ‘trigger’ of hard versus soft metal usage.

**Crystal structure of MGS bound to different metals**

To explore the structural basis for the metal preference of MGS, the crystal structure of the enzyme was determined in complex with GDP/Mg$^{2+}$ and GDP-Man/Mg$^{2+}$, Figure 2. Diffraction data were collected for three different GDP/Mg$^{2+}$ crystals but, in each case, side chain density corresponding to H217 is absent, even though magnesium was present, consistent with a poor, indeed absent, interaction between nitrogen and these “harder” cations Figure 2. Whether cause or effect, the presence of a manganese-ion positions GDP and GDP-mannose “deeper” in the transfer centre with most of the active centre side chains translated slightly to accommodate this association. This translation positions the O1 of the donor sugar 0.94 Å closer to the O2 of glycerate compared to the GDP-Man/Mg$^{2+}$ complex. The alpha-carbon positions of the flexible loop, comprising residues 215 to 222, vary in all four structures, making it difficult to evaluate if loop positions are directly affected by different cations. However, this inherent plasticity, even in substrate loaded forms, emphasizes the flexibility of MGS which is hypothesized to be important for substrate recognition and product release. As demonstrated in multiple GDP/Mg$^{2+}$ crystal structures, cation coordination does not require H217, potentially mimicking the H217A mutant, Figure 2. In the GDP-Man/Mg$^{2+}$ structure, cation coordination with D102 results in the magnesium ion being pulled away from the transfer centre relative to Mn$^{2+}$ coordinated with H217, Figure 2. This could result in the donor sugar being less tightly packed in the active centre, potentially facilitating product release in a proposed rate limiting step as discussed below.

**Calorimetric studies of nucleotide binding**

To explore the influence of metal-ions on nucleotide binding the capacity of MGS to bind GDP and GDP-Man in the presence of different metals was explored. Titration of GDP into MGS without addition of metal-ion showed weak enthalpies and poor affinity (data not shown), suggesting that a cation was critical for GDP binding. GDP, however, bound to MGS in the presence of Ca$^{2+}$, Mg$^{2+}$ or Mn$^{2+}$, with binding driven by both enthalpic and entropic processes, Figure 3 and Table 5. The affinity ($K_D$) of MGS for GDP was in the low µM to high nM range (10- to 100-fold tighter binding than observed for α3GT (30)). MGS showed the strongest affinity towards GDP-Mn$^{2+}$ of the three tested cations (Ca$^{2+}$, Mg$^{2+}$, or Mn$^{2+}$), Table 5, which could correlate to the more buried position for GDP in the transfer centre in MGS within the GDP/Mn$^{2+}$ complex structure, compared to those obtained with Mg$^{2+}$ present. Metal-ion coordination in the H217A variant displays an approximately 3-fold decrease in binding of GDP/Mn$^{2+}$ and GDP/Mg$^{2+}$, while the mutation had no significant effect on affinity for GDP/Ca$^{2+}$.

To explore the influence of the donor sugar on metal-ion coordination, binding of GDP-Man in the presence of the three metals was determined by ITC, Figure 3. GDP-Man bound 2- to 10-fold tighter to wild-type MGS than GDP, Table 5 & Supplemental Table S2, suggesting that mannose makes a contribution to donor substrate binding. Addition of Mn$^{2+}$ resulted in a higher $K_A$-value for GDP-Man, compared to when the donor substrate was titrated with the enzyme in the presence of Mg$^{2+}$ or Ca$^{2+}$, however, the difference was smaller than with GDP. Similar to the data obtained for the GDP-metal complexes, the H217A mutation significantly reduced affinity for GDP/Mn$^{2+}$ and GDP/Mg$^{2+}$, but not for GDP/Ca$^{2+}$, Table 5.
The ITC data indicate that while H217 contributes to GDP/Mn$^{2+}$ and GDP/Mg$^{2+}$ metal coordination, the relatively modest reduction in affinity for these complexes in the H217A mutant suggest that the two metals can adopt alternative coordinate bonds between the protein and nucleotide. This is consistent with the observation that in the GDP/Mg$^{2+}$ crystal structure the metal makes a bidentate coordination with D102, while H217 is highly disordered. Indeed the similar affinity of GDP/Ca$^{2+}$ for wild-type MGS and the H217A mutant suggests that the histidine does not contribute to calcium coordination with the nucleotide product. While it is possible that the metals are making nucleotide independent contact with the enzyme, this is unlikely as titration with the metal in the absence of GDP or GDP-Man revealed no binding (data not shown).

**DISCUSSION**

This study shows that the introduction of the H217A mutation into MGS induces a shift in metal preference form the hard group II metals, which are typically less commonly used by GTs, to softer transition metals. Structural data reveal subtle yet clear differences in the position adopted by the donor substrate in complex with different metals, which may influence catalytic efficiency. The metal coordination observed here, in which H217, D102 (the second aspartate in the DxD motif) and the $\alpha$ and $\beta$ O of the diphosphate are ligands, is a common feature of GT-A enzymes, although examples of enzymes in which both acid residues of the DxD motif bind the metal co-factor have been observed in blood group enzymes (31). It is possible that the H217A mutation alters the coordination of the group II metals such that they are unable to induce the conformational changes required for catalysis. By contrast, the transition metals may be able to participate in different co-ordination chemistries, possibly involving both carboxylates in the DxD motif, which may facilitate the formation of a ternary complex.

Such changes in metal preference in glycosyltransferases are unusual, although the work reported here has considerable resonance with a study by Qasba and colleagues on bovine $\beta$-1,4-galactosyltransferase (32). The normal metal in this GT-A inverting GT is Mn$^{2+}$, with no detectable activity with Mg$^{2+}$. In this case, mutation of a methionine that made an unusual coordinate bond with Mn$^{2+}$, to histidine generated a variant whose loops were able to close (allowing access to a "closed" 3-D structure) whilst reversing the metal-ion specificity from Mn$^{2+}$ to Mg$^{2+}$. (32). This differential use of metals and their different coordination and activation also has strong resonance with chemical methods for the activation of glycosyl donors. In these chemical systems the use of the appropriate Lewis acid is a common strategy for ‘tuning’ reactivity of a given glycosyl donor to higher or lower levels. It may be that in a similar manner, in the case of diphosphate nucleotides as leaving groups then nature has evolved the appropriate combination of amino acids and metal to achieve ‘activation’. Thus, harder metals, such as Ca(II), which in its binding to GDP-Man here was little affected by His217, may be capable of direct activation of the phosphate in MGS with the need for only D102 as a stabilizing/coordinating ligand (and hence lead to higher activity). In such a scenario the DxD motif may act as a ‘soft’ motif, while the D102 alone acts as ‘hard’; here His217 may then be playing the role of a metal mimic to engage the second D. On the other hand, increasingly softer metals Mg(II) and then Mn(II) may require additional activation, perhaps via coordination of His217 that might tune the metal reactivity through both sigma donation and pi back-bonding. In this way, and through such hard-soft tuning methods, if harder (Ca(II)) metals were environmentally unavailable, softer metals could be used as surrogates. Over time a greater abundance of softer metals may have led to GTs evolving to use Mn(II) without the need for such
additional ‘Lewis acidity tuning’ or, indeed, this may have reinforced the DxD motif. MGS, an archeal enzyme, may therefore represent a historically early ‘Ca(II)-affluent ancestor’ on this progression.

Donor substrate binding is associated with a significant change in the conformation of a highly flexible loop extending from P212 to G221. How this flexible loop confers acceptor binding and catalysis is central to understanding how retaining GT-A glycosyltransferases mediate glycosyl transfer. Within this loop the phenolic ring of Y220 adopts a different orientation in complexes where MGS is bound to GDP and GDP-Man, respectively. In one conformation the phenol hydroxyl makes polar interactions with the α-phosphate of GDP-Man, while in its other conformation (adopted in the MGS-GDP complex) it is pointing into the acceptor site and likely makes a hydrogen bond with O₂ of glycerate. The observed, substantial, increase in $K_m$ for glycerate when the tyrosine is mutated, which is not mirrored by a change in affinity for GDP-Man, is consistent with its role within the acceptor site and suggests that the latter interaction with O₂ of glycerate is more functionally relevant than the former interaction with phosphate. Thus, we propose that the primary mechanism by which the donor substrate facilitates glycerate binding is through conformational changes in the highly mobile loop. The interaction of Y220, in this loop, with the donor, results in the presentation of the hydroxyl of this residue into the acceptor binding site.

To conclude, this study reveals the importance of metal-ion coordination not only in donor substrate binding but also in the assembly of the ternary complex, while leaving group departure defines the maximum catalytic rate. This report provides a foundation for manipulating the activity of glycosyltransferases by engineering active site metal-ion recognition and may reflect an intriguing mechanism by which GTs utilize available metals to tune their Lewis acid hardness to make them suitable activators for nucleotide diphosphate leaving groups in a manner akin to strategies used in synthetic chemical glycosylation chemistry (33).

**Acknowledgements**

Monica Palcic (Carlsberg Laboratory) is thanked for assistance and advice with kinetics. The Biotechnology and Biological Sciences Research Council of the UK is thanked for funding. MDLS is an European Molecular Biology Laboratory long term fellowship holder and GJD and BGD are Royal Society Wolfson Research Merit Award recipients.
REFERENCES
<table>
<thead>
<tr>
<th>Data collection</th>
<th>MGS + Lactate</th>
<th>MGS Mg(^{2+}) + GDP</th>
<th>MGS Mn(^{2+}) + GDP</th>
<th>MGS Mg(^{2+}) + GDP-Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P3(_{21})</td>
<td>P3(_{21})</td>
<td>P3(_{21})</td>
<td>P3(_{21})</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td>a, b, c (Å)</td>
<td>149.0, 149.0, 155.0</td>
<td>149.6, 149.6, 154.1</td>
<td>149.8, 149.8, 154.7</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>α, β, γ (°)</td>
<td>90, 90, 120</td>
<td>90, 90, 120</td>
<td>90, 90, 120</td>
</tr>
<tr>
<td>R(_{merge})</td>
<td>L / σL</td>
<td>0.10 (0.37)</td>
<td>0.072 (0.52)</td>
<td>0.075 (0.59)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.7 (96.8)</td>
<td>100 (100)</td>
<td>99.9 (100)</td>
<td>96.8 (98.8)</td>
</tr>
<tr>
<td>Redundancy (%)</td>
<td>2.6 (2.6)</td>
<td>6.1 (6.3)</td>
<td>10.9 (10.7)</td>
<td>5.4 (5.3)</td>
</tr>
<tr>
<td>Refinement</td>
<td>R(<em>{work}) / R(</em>{free})</td>
<td>0.20/0.22</td>
<td>0.20/0.23</td>
<td>0.19/0.22</td>
</tr>
<tr>
<td>R.m.s deviations</td>
<td>Bond lengths (Å)</td>
<td>0.017</td>
<td>0.015</td>
<td>0.016</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.58</td>
<td>1.52</td>
<td>1.67</td>
<td>1.62</td>
</tr>
<tr>
<td>PDB Codes</td>
<td>2y4j</td>
<td>2y4k</td>
<td>2y4l</td>
<td>2y4m</td>
</tr>
</tbody>
</table>
Table 2 Kinetic parameters of MGS variants containing mutations in the donor substrate binding region

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Varied substrate: glycerate</th>
<th>Varied substrate: GDP-Man</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ Min$^{-1}$</td>
<td>$K_M$ µM</td>
</tr>
<tr>
<td>Wild type</td>
<td>1.9 (± 0.2)</td>
<td>2.2 (± 0.3)</td>
</tr>
<tr>
<td>W189A</td>
<td>4.6 (± 0.1)</td>
<td>15 (± 1.9)</td>
</tr>
<tr>
<td>M229A</td>
<td>8.1 (± 1.8) x $10^{-1}$</td>
<td>7.8 (± 1.4)</td>
</tr>
<tr>
<td>D192A</td>
<td>Inactive$^a$</td>
<td>Inactive</td>
</tr>
<tr>
<td>K76A</td>
<td>4.5 (± 0.05)</td>
<td>30 (± 3.4)</td>
</tr>
<tr>
<td>E166A</td>
<td>1.4 (± 0.1)</td>
<td>32 (± 2.1)</td>
</tr>
<tr>
<td>Q66A</td>
<td>1.4 (± 0.09) x $10^2$</td>
<td>70 (± 5.6)</td>
</tr>
<tr>
<td>Y37A</td>
<td>11 (± 0.06)</td>
<td>17 (± 3.9)</td>
</tr>
<tr>
<td>K9A</td>
<td>6.9$^b$ (± 0.4)</td>
<td>1.4$^d$ (±1.0)</td>
</tr>
<tr>
<td>R73A</td>
<td>3.3 (± 0.5)</td>
<td>3.8 (± 0.3)</td>
</tr>
<tr>
<td>R73G</td>
<td>3.6 (± 0.02)</td>
<td>4.1 (± 0.3)</td>
</tr>
<tr>
<td>R218A</td>
<td>15 (± 0.6)</td>
<td>5.8 (± 1.2)</td>
</tr>
<tr>
<td>H217A</td>
<td>16 (± 0.6)</td>
<td>1.9 (± 0.3) x $10^3$</td>
</tr>
</tbody>
</table>

When glycerate was the varied substrate GDP-mannose was at 500 µM except for the Q66A mutation where GDP-Man was at 2 mM. When GDP-mannose was the varied substrate glycerate was at 100 µM except for Q66A, K9A and H217A where glycerate was at 1 mM, 10 µM and 10 mM, respectively.

$^a$Activity less than 2.8 x $10^{-4}$ min$^{-1}$,

$^b$High degree of substrate inhibition observed.
Table 3 Kinetic parameters (Mn$^{2+}$ as metal) of MGS variants containing mutations in the acceptor substrate binding region

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>[D-glycerate] (mM)</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$K_M$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type$^a$</td>
<td>1.9 (± 0.2)</td>
<td>2.2 (± 0.3)</td>
<td>0.1</td>
<td>1.1 (± 0.1)</td>
<td>3.1 (± 0.9) x 10$^{-1}$</td>
</tr>
<tr>
<td>Wild-type$^b$</td>
<td>1.0</td>
<td>7.2 x 10$^2$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T139A$^a$</td>
<td>1.0 (± 0.1)</td>
<td>3.3 (± 0.5) x 10$^3$</td>
<td>6</td>
<td>5.1 (± 0.1) x 10$^{-1}$</td>
<td>1.1 (± 0.8) x 10$^{-1}$</td>
</tr>
<tr>
<td>T139A$^b$</td>
<td>-</td>
<td>&gt;100 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Y220A$^a$</td>
<td>4.0 (± 0.1)</td>
<td>1.1 (± 0.06) x 10$^3$</td>
<td>100</td>
<td>2.5 (± 0.04)</td>
<td>2.9 (± 0.8)</td>
</tr>
<tr>
<td>Y220F$^a$</td>
<td>4.2 (± 0.1)</td>
<td>3.8 (± 0.2) x 10$^3$</td>
<td>100</td>
<td>2.2 (± 0.05)</td>
<td>6.5 (± 0.2)</td>
</tr>
<tr>
<td>D135A$^a$</td>
<td>3.9 (± 0.02)</td>
<td>1.3 (± 0.05) x 10$^4$</td>
<td>35</td>
<td>2.7 (± 0.2)</td>
<td>6.3 (± 2.1)</td>
</tr>
</tbody>
</table>

$^a$The varied acceptor substrate was glycerate.

$^b$The varied acceptor substrate was lactate.

$^c$When the acceptor substrates were varied the concentration of the donor substrate (GDP-Man) was 500 µM.
Table 4 Bi-substrate kinetics of wild type and variants of MGS

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Metal-ion</th>
<th>( k_{\text{cat}} ) ( \text{min}^{-1} )</th>
<th>( K_a ) ( \mu\text{M} )</th>
<th>( K_b ) ( \mu\text{M} )</th>
<th>( K_{i,a} ) ( \mu\text{M} )</th>
<th>( K_{i,b} ) ( \mu\text{M} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Mg(^{2+})</td>
<td>115 ± 17</td>
<td>3.9 ± 7</td>
<td>59 ± 16</td>
<td>5 ± 1</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Wild type</td>
<td>Mn(^{2+})</td>
<td>2 ± 0.4</td>
<td>&lt;1(^c)</td>
<td>&lt;1(^c)</td>
<td>-(^d)</td>
<td>-(^d)</td>
</tr>
<tr>
<td>Wild type</td>
<td>Ca(^{2+})</td>
<td>36</td>
<td>166</td>
<td>-</td>
<td>157</td>
<td>-</td>
</tr>
<tr>
<td>H217A</td>
<td>Mg(^{2+})</td>
<td>5 ± 1</td>
<td>1322 ± 438</td>
<td>46 ± 19</td>
<td>230 ± 31</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>H217A</td>
<td>Mn(^{2+})</td>
<td>35 ± 2</td>
<td>1610 ± 286</td>
<td>14 ± 1</td>
<td>159 ± 87</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>H217A</td>
<td>Ca(^{2+})</td>
<td>No activity detected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y220A</td>
<td>Mn(^{2+})</td>
<td>4 ± 0.1</td>
<td>1990 ± 340</td>
<td>&lt;1(^c)</td>
<td>-(^d)</td>
<td>-(^d)</td>
</tr>
</tbody>
</table>

\( a \) = D-glycerate (acceptor)
\( b \) = GDP-mannose (donor)
\(^c\) Value below detection limit.
\(^d\) Can not be calculated because \( K_a < 1 \mu\text{M} \) or \( K_b < 1 \mu\text{M} \).

The kinetics parameters in the presence of calcium were determined under pseudo-single substrate conditions where the reaction product was quantified by HPLC.
Table 5 The use of ITC to measure binding of GDP and GDP-Man to wild type and variants of MGS in the presence of metals

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ligand</th>
<th>Metal-ion</th>
<th>( K_A ) (M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>GDP-Man</td>
<td>Ca(^{2+})</td>
<td>9.0 (± 0.1) x 10(^5)</td>
</tr>
<tr>
<td>Wild type</td>
<td>GDP-Man</td>
<td>Mg(^{2+})</td>
<td>2.9 (± 0.3) x 10(^6)</td>
</tr>
<tr>
<td>Wild type</td>
<td>GDP-Man</td>
<td>Mn(^{2+})</td>
<td>5.4 (± 0.6) x 10(^6)</td>
</tr>
<tr>
<td>Wild type</td>
<td>GDP</td>
<td>Ca(^{2+})</td>
<td>1.5 (± 0.3) x 10(^5)</td>
</tr>
<tr>
<td>Wild type</td>
<td>GDP</td>
<td>Mg(^{2+})</td>
<td>2.8 (± 0.3) x 10(^5)</td>
</tr>
<tr>
<td>Wild type</td>
<td>GDP</td>
<td>Mn(^{2+})</td>
<td>2.0 (± 0.4) x 10(^6)</td>
</tr>
<tr>
<td>H217A</td>
<td>GDP-Man</td>
<td>Ca(^{2+})</td>
<td>1.4 (± 0.4) x 10(^5)</td>
</tr>
<tr>
<td>H217A</td>
<td>GDP-Man</td>
<td>Mg(^{2+})</td>
<td>1.0 (± 0.1) x 10(^5)</td>
</tr>
<tr>
<td>H217A</td>
<td>GDP-Man</td>
<td>Mn(^{2+})</td>
<td>6.3 (± 0.8) x 10(^5)</td>
</tr>
<tr>
<td>H217A</td>
<td>GDP</td>
<td>Ca(^{2+})</td>
<td>1.2 (± 0.02) x 10(^5)</td>
</tr>
<tr>
<td>H217A</td>
<td>GDP</td>
<td>Mg(^{2+})</td>
<td>8.9 (± 0.1) x 10(^4)</td>
</tr>
<tr>
<td>H217A</td>
<td>GDP</td>
<td>Mn(^{2+})</td>
<td>6.1 (± 0.7) x 10(^5)</td>
</tr>
</tbody>
</table>

\*n was fixed to 1.00 due to low affinity.
FIGURE LEGENDS

Figure 1. Anatomy of Mannosylglycerate Synthase
a. The reaction catalysed by mannosylglycerate synthase is the transfer of mannose, from GDP-Mannose to d-glycerate to generate α-D mannosyl 2-O d-glycerate in a metal-ion dependent manner
b. Composite diagram of Mg\(^{2+}\) + GDP-Mannose + d-glycerate donor and acceptor substrates shown in ball-and-stick. The flexibility of residues 215-222 in the MGS the complex structures presented in (14) of Mn\(^{2+}\) + d-glycerate, citrate, Co\(^{2+}\) + GDP, Mn\(^{2+}\) + GDP-mannose (PDB 2BO4, 2BO6, 2BO7, 2BO8, respectively) are highlighted in red, blue, yellow, and magenta, respectively, overlapped with Mg\(^{2+}\) + GDP-mannose presented herein. This stretch of residues includes H217 shown to be important for cation coordination in the active centre. Positions of Q201 and Q202 variants, used to lower surface entropy of MGS are shown in blue.

c. Composite diagram (divergent “wall-eyed” stereo) of the MGS active centre with Mg\(^{2+}\) + GDP-mannose and d-glycerate shown in ball-and-stick, colored gray. Some of the active centre residues mutated and subsequently analyzed by kinetic methods, and in some cases biophysical methods (H217 and Y220), are shown in green.

d. Composite schematic diagram of the MGS active centre based upon known GDP-Mannose and acceptor complexes. Tyr220 is present in a highly mobile loop with past crystal structures representing just one possible interpretation. The key structural feature is that Tyr220 becomes well ordered in the GDP-Man complex (blue) and then lies in a position where it would likely interact with the glycerate acceptor (colored red); consistent with kinetic data of variants presented here.

Figure 2. Electron density for diverse MGS complexes
a. MGS active centre complex with the mannose acceptor d-lactate shown (as a composite with Mg\(^{2+}\)-GDP-mannose). 2F\(_{o}\) - F\(_{c}\) electron density is presented for d-lactate shown at 1.0 σ. Helix 222-240 is omitted for clarity. The coordinates of glycerate from PDB 2BO6 are shown in cyan for reference
b. As (a) but with electron density shown for malonate (glycerate shown in blue)
c. GDP-divergent metal complexes (divergent “wall-eyed” stereo) with both Mg\(^{2+}\) (green/grey) and Mn\(^{2+}\) (purple). Cation coordinating active centre residues D100 and H217 are shown. With Mg\(^{2+}\) as the divalent metal, His217 is disordered in the GDP complexes and does not interact with the metal.

Figure 3. ITC. Analysis of ligand binding by ITC
In the example titrations shown the reaction cell contained wild type MGS at 50 μM in 20 mM Tris/HCl, pH 8.0, containing MnCl\(_{2}\) at 1 mM. The title of the different panels identity the titrant used, which was at 2 mM in the syringe. In the panel entitled glycerate (GDP) the nucleotide was included in the reaction cell. The top half of each panel shows the raw ITC heats; the bottom half, the integrated peak areas fitted using a one single model by MicroCal Origin software.
FIGURE 1

a

GDP-mannose (donor) + D-glycerate (acceptor) → 2-O-α-D-mannosyl-D-glycerate

b

flexible loop(s)
surface variants

c

d

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Figure 3.
Substrate and metal-ion promiscuity in mannosylglycerate synthase
Morten M. Nielsen, Michael D. L. Suits, Min Yang, Conor S. Barry, Carlos Martinez-Fleites, Louise E. Tailford, James E. Flint, Claire Dumon, Benjamin G. Davis, Harry J. Gilbert and Gideon J. Davies

J. Biol. Chem. published online February 2, 2011

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