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Cavalier, M., Yim, Y., Asamizu, S., Neau, D., Almabruk, K., Mahmud, T., & Lee, Y. (2012). Mechanistic Insights into Validoxylamine A 7'-Phosphate Synthesis by VldE Using the Structure of the Entire Product Complex. *PLoS ONE, 7*(9) https://doi.org/10.1371/journal.pone.0044934

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Mechanistic Insights into Valdoxylamine A 7′-Phosphate Synthesis by VldE Using the Structure of the Entire Product Complex

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Abstract

The pseudo-glycosyltransferase VldE catalyzes non-glycosidic C-N coupling between an unsaturated cyclitol and a saturated aminocyclitol with the conservation of the stereochemical configuration of the substrates to form valdoxylamine A 7′-phosphate, the biosynthetic precursor of the antibiotic validamycin A. To study the molecular basis of its mechanism, the three-dimensional structures of VldE from Streptomyces hygroscopicus subsp. limoneus was determined in apo form, in complex with GDP, in complex with GDP and valdoxylamine A 7′-phosphate, and in complex with GDP and trehalose. The structure of VldE with the catalytic site in both an “open” and “closed” conformation is also described. With these structures, the preferred binding of the guanine moiety by VldE, rather than the uracil moiety as seen in OtsA could be explained. The elucidation of the VldE structure in complex with the entirety of its products provides insight into the internal return mechanism by which catalysis occurs with a net retention of the stereochemical configuration of the donated cyclitol.

Introduction

Glycosyltransferases comprise one of the most numerous and diverse groups of enzymes in nature. They are responsible for the formation of oligo/polysaccharides, glycoproteins, glycolipids, and many other glycosylated natural products by transferring a sugar moiety from an activated donor sugar to a sugar (or non-sugar) acceptor. This abundant group of proteins consists of 92 families encoded by more than 83,400 genes [1]. However, only a fraction of those genes has actually been functionally characterized. Our comparative bioinformatics studies suggest that among those reported as glycosyltransferases are also pseudo-glycosyltransferases (such as VldE, EC 2.x.x.x), which do not recognize sugars as substrates but rather catalyze the formation of non-glycosidic C-N bonds in the biosynthesis of C7N-aminocyclitol-containing natural products such as acarbose and validamycin A (Figure 1) [2–4]. Acarbose, an α-glucosidase inhibitor, has been proven useful in the treatment of type II insulin-independent diabetes, whereas validamycin A, a natural trehalase inhibitor, is an antifungal antibiotic that has long been used to protect crops from soil borne diseases such as rice sheath blight and the damping-off of cucumber seedlings [5–8].

Validamycin A is a pseudo-trisaccharide whose structure is comprised of valdoxylamine A and glucose (Figure 1). The final step in validamycin A biosynthesis is the attachment of glucose to the precursory valdoxylamine A by the action of the glycosyltransferase VldK (ValG) [2,9]. Valdoxylamine A is generated through the dephosphorylation of valdoxylamine A 7′-phosphate by VldH while validamycin A 7′-phosphate (VDO) results from a condensation of GDP-valienol and validamine 7-phosphate (both are pseudo-sugars) by the pseudo-glycosyltransferase, VldE [3,4,10–14].

The mechanism by which non-glycosidic C-N bond is formed by a pseudo-glycosyltransferase is not entirely understood. However, because of the structural similarity of valdoxylamine A 7′-phosphate to trehalose 6-phosphate (Figure 1), it has been speculated that the mechanism of the pseudo-glycosyltransferase VldE is similar to that of the glycosyltransferase, trehalose 6-phosphate synthase (OtsA, EC 2.4.1.15) [4]. VldE and Escherichia coli OtsA only share a modest 19% sequence identity (29% homology) (Figure 2), but they are both catalogued as members of the GT20 glycosyltransferase family by the CAZy database (www.cazy.org) [15]. OtsA mediates the transfer of glucose moiety from UDP-glucose to glucose 6-phosphate to form trehalose 6-phosphate (Figure 1). Similar to VldE, the product of OtsA conserves the anomeric configuration of the donor moiety. Glycosyltransferases have been shown to both retain and invert the anomeric state of the carbon C-1 of the donor moiety. The inversion of the anomeric center by glycosyltransferases has been well explored and is known to be carried out by a simple
nucleophilic substitution. However, the underlying catalytic mechanism of glycosyltransferases that retain the anomeric configuration of the donated moiety within the product is not as well understood. Catalysis by a retaining glycosyltransferases is thought to occur through either a double displacement (SN2 X2) or internal return mechanism (SNi) [16]. For a double displacement nucleophilic substitution reaction to occur, a nucleophilic catalytic base must be available in close vicinity to the sugar anomeric carbon to form a covalent intermediate. OtsA apparently lacks such a residue in the catalytic site and analyses of theoretical energy profiles along with recent studies of kinetic isotope effects have substantiated this unusual enzymatic reaction mechanism within OtsA [17–19]. Lastly, structural studies of OtsA using a bi-substrate inhibitor as a product mimic place the atoms involved in catalysis in an orientation favorable for an internal return mechanism and show that the leaving phosphate acts as a general base to deprotonate the incoming nucleophile of the acceptor group [20]. Within the SNi mechanism of OtsA, a carbocation is developed upon the detachment of the nucleotide phosphate which is stabilized by resonating into an oxonium ion-like transition state [18,20]. However, no oxonium ion formation is possible in the VldE-catalyzed non-glycosidic C-N coupling reaction. Alternatively, it is proposed that the olefinic moiety of GDP-valienol may play a critical role in facilitating the coupling reaction [4]. Coupling reactions involving an allylic moiety have been demonstrated in other biosynthetic enzymes, e.g., farnesyl diphosphate (FPP) synthases [21,22]. However, in FPP synthases, a nucleophilic substitution reaction takes place at a carbon center with a diphosphate acting as a leaving group instead of nucleotidyl diphosphate. In addition, mechanistically FPP synthases adopt a stereospecific SN1 reaction with an inversion of the configuration, leaving the actual mechanism behind the unique catalytic function of the retaining VldE enzyme unclear. Hopefully, structural studies would clarify whether or not a SNi mechanism is conserved within the retaining pseudo-glycosyltransferase, VldE.

Here, we report the three-dimensional structures of VldE in various liganded states using X-ray crystallographic techniques. The structure of VldE was solved by molecular replacement using the structure of OtsA as a search model. We have elucidated the structures of the unliganded VldE, in complex with guanosine 5’-
diphosphate (GDP), in complex with GDP and Trehalose (TRE), and in complex with GDP and VDO. Similar to OtsA, VldE is comprised of two Rossmann $\beta/\alpha/\beta$ domains which are oriented in a GT-B configuration (Figure 3). The active site, which is located at the interface of the Rossmann domains, and the selective interactions allowing for the binding of GDP but not uridine 5'-diphosphate (UDP) are described in our study (Figure 4A–B). The crystallographic investigation of VldE while binding the complete product complex, VDO and GDP, supports the proposed conservation of a $S_N$ catalytic mechanism analogous to the mechanism of OtsA. Both the “open” and “closed” conformations of the catalytic site are also described within this study.

Materials and Methods

Purification and Crystallization

VldE from *S. hygroscopicus* subsp. *limoneus* was expressed and purified with methods similar to those previously described [4]. (His)_6-tagged VldE was expressed in *E. coli* BL21 (DE3) pLysS and was purified using Ni-NTA affinity chromatography. Affinity
Crystal Structure of the VldE Product Complex

chromatography was performed using 40 mM Hepes buffer, pH 7.5 with 300 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, and 10% glycerol. Elution from the Ni-NTA resin was achieved using the same buffer but with 175 mM imidazole. Using dialysis, the affinity chromatography buffer was exchanged with VldE storage buffer consisting of 10 mM Tris-HCl, pH 7.5 with 5% glycerol, 0.1 mM dithiothreitol, and 1 mM MgCl₂. VldE was concentrated to 10 mg/mL, and crystals were grown using sitting drop vapor diffusion.

In the case of VldE and VldE-GDP crystals, the mother liquor consisted of 100 mM Tris-HCl, pH 8.0 with 30–35% polyethylene glycol 3,350. GDP, at a concentration of 3 mM, was added to the protein stocks. The Crystals consisted of reservoir solution enriched with 20% ethylene glycol 3,350. GDP, at a concentration of 3 mM, was added to the cryoprotectant solution. Crystals were flash-cooled in liquid nitrogen after soaking in the cryoprotectant solution for 20–40 minutes.

In the case of VldE-GDP-TRE crystals, the mother liquor consisted of 50 mM Tris-HCl, pH 8.0, 10–28% polyethylene glycol 3,350, 200–500 mM NaCl, and 20 mM MgCl₂. VldE was kept at a concentration of 10 mg/mL, and crystals were grown using sitting drop vapor diffusion with a 1:1(v/v) mixture of protein and mother liquor. Before the drops were set, 3% trehalose and 1 mM GDP were added to the protein stocks. The Crystals were grown over a period of four to eight weeks at a temperature of 293 K. Cryoprotectant solutions, consisting of reservoir solution enriched with 20% ethylene glycol, 3% trehalose, and 1 mM GDP were added to the crystal drops at a 1:1(v/v) ratio. Crystals were flash-cooled in liquid nitrogen after soaking in the cryoprotectant solution for 20–40 minutes.

Data Collection and Processing

All diffraction data except for the VldE-GDP dataset was collected at the Northeastern Collaborative Access Team (NE-CAT) beamline at the Advanced Photon Source (Argonne National Laboratory). Data was recorded at 100 K on an ADSC Q315 (315 mm x 315 mm) detector and processed by NE-CAT’s RAPD automated processing (https://rapd.necaps.anl.gov/rapd), which uses XDS [23] for integration and scaling. A 2.0 Å VldE dataset was collected at a wavelength of 0.9479 Å while oscillating the crystal 0.5 degrees for each frame. A 2.11 Å VldE-GDP dataset was collected at a wavelength of 0.9792 Å while oscillating the crystal 1.0 degrees for each frame. A 2.81 Å VldE-GDP-TRE dataset was collected at a wavelength 0.9792 Å while oscillating the crystal 1.0 degrees for each frame. The 2.15 Å VldE-GDP dataset was collected at NSLS Beamlıne X6A at a wavelength 1.0 Å while oscillating the crystal 1.0 degrees for each frame. X-ray data was collected on a ADSC Quantum CCD detector at 100 K and was integrated, merged, and scaled using HKL2000 [24]. Although the cell dimensions were similar, the unliganded VldE crystal belonged to the P2₁ space group, while the VldE-GDP and VldE-GDP-TRE crystals belonged to the P2₁ space group.
Figure 4. The Catalytic Site of VldE. Shown is a comparison of the VldE and OtsA catalytic sites in ribbon diagrams. Residues/molecules of interest are represented in stick models. The dotted lines mark hydrogen bonds and ionic interactions. The preferential binding of GDP by VldE is demonstrated by comparing (A) the protein-ligand interactions within the VldE active site (green) with (B) the protein-ligand interactions between OtsA and UDP within the OtsA active site (gray). The protein interactions with ribose and phosphate are conserved between VldE and OtsA. However, there are differing interactions with the nucleotide base groups. The large purine makes interactions with the residues Arg321, Asn323, Asn361, and Thr366. The ribose and phosphate moieties interact with Arg290, Lys295, Leu387, Ser388, and Glu391. Within OtsA, Leu344, Ile295, Pro297, and His338 only allow for the binding of the smaller pyrimidine. The ribose and phosphate moieties make interactions with residues Arg262, Lys267, Leu365, and Glu369. The mesh represents the $|F_o| - |F_c|$ electron density omit map of the GDP binding site. The map is contoured at 3.0σ levels. (C) Shown is a superimposition of the VldE cyclitol binding sites in the presence (yellow) and absence (green) of validoxylamine A 7'-phosphate. The mesh represents the $|F_o| - |F_c|$ electron density omit map of the VDO binding site. The map is contoured at 3.0σ levels. VDO makes interactions with residues the side-chains of residues Asp158, His182, Arg12, Asn325, Arg326, and Asp383. VDO also makes interactions with the backbones of residues 384–387. Binding of the acceptor cyclitol is recognized by conformation changes by the side-chains of residues Asp158, Tyr159, and Arg326. (D) Shown is a comparison by superimposition of VDO binding within the catalytic sites of VldE (yellow) and OtsA (gray). Note the strong conservation of residue and ligand positions.

doi:10.1371/journal.pone.0044934.g004
The unit cell dimensions of the VldE-N-GDP-N-TRE dataset were dissimilar and belonged to the C2 space group. Diffraction data statistics are summarized in Table 1.

Structure Determination and Refinement

To determine the structure of VldE, molecular replacement (MR) was performed. Generation of a search model from the OtsA structure (PDB accession code 1UQU [25]) and molecular replacement was carried out using MrBUMP [26] to orchestrate molecular replacement via the MOLREP [27] module in the CCP4 [28] program suite. The resulting MR model was used to build a partially sequenced model (~400 residues) using the AUTOBUILD [29–32] function of the PHENIX [33] software suite. Further automated model building was carried out using the ARP/wARP [34] module within the CCP4 [28] software suite. The model was finished by manual building within COOT [35]. The model was refined using restrained refinement by REFMAC5 [36] with TLS parameters defined by the TLSMD server [37,38]. Two monomers were modeled within the asymmetric unit, and non-crystallographic symmetry (NCS) restraints were used during refinement. Waters were incorporated into the models by referring to the |Fo| - |Fc| omit maps. Out of the possible 994 residues, 957 residues were modeled with 97.8% within the most favored regions of the Ramachandran plot. Although 0.5% are reported as outliers, they are all within well-defined electron densities. The final model, which lacked residues 1, 2, and 482–497, has a Rwork of 0.190 and an Rfree of 0.222. The structure refinement statistics are summarized in Table 1.

Table 1. Statistics of reflection data and structure refinements.

| Liganding  | VldE | VldE-GDP | VldE-GDP-VDO | VldE-GDP-TRE |
|------------|------|---------|-------------|-------------|
| Space Group| P2   | P2_1   | P2_1        | C2          |
| Unit Cell Dimension (Å) | 84.75, 48.43, | 47.82, 121.15, | 47.96, 120.71, | 320.82, 122.82, |
| Unit Cell β-angles (°) | 91.88 | 91.74 | 91.63 | 91.62 |
| Wilson Plot B Value (Å²) | 48.74 | 35.96 | 40.37 | 53.40 |
| Resolution Range (Å) | 48.92–1.95 | 37.52–2.15 | 47.94–2.11 | 49.15–2.81 |
| Reflections Observed | 245,918 | 368,911 | 205,530 | 220,052 |
| Unique Reflections | 69,317 | 51,924 | 55,049 | 88,243 |
| Reflections Rfree Set | 3,559 | 2,645 | 2,790 | 4,421 |
| Completeness (%) | 98.1 (91.8) | 99.9 (100.0) | 99.7 (99.0) | 99.3 (98.5) |
| Redundancy | 3.5 (3.3) | 7.1 (7.1) | 3.7 (3.7) | 2.5 (2.5) |
| | 13.0 (2.7) | 25.2 (3.1) | 16.5 (2.7) | 12.2 (2.7) |
| Rsym | 0.045 (0.421) | 0.078(0.522) | 0.049(0.433) | 0.058(0.304) |
| Rwork | 0.190 | 0.197 | 0.191 | 0.211 |
| Rfree | 0.222 | 0.209 | 0.222 | 0.258 |
| No. TLS Bodies | 6 | n/a | n/a | n/a |
| No. of Amino Acids | 957 | 948 | 950 | 2,738 |
| No. of Protein Atoms | 7,474 | 7,378 | 7,361 | 21,796 |
| No. of Hetero Atoms | 0 | 58 | 120 | 214 |
| No. of Waters | 492 | 405 | 488 | 202 |
| RMSD Bond Lengths (Å) | 0.022 | 0.012 | 0.017 | 0.016 |
| Angles (°) | 1.67 | 1.30 | 1.76 | 1.47 |
| Mean B Factor | 41.85 | 40.21 | 39.62 | 62.41 |
| Protein Atoms (Å²) | 42.63 | 40.19 | 39.59 | 62.61 |
| Hetero Atoms (Å²) | n/a | 34.34 | 37.63 | 62.57 |
| Water Atoms (Å²) | 29.38 | 40.93 | 40.50 | 40.82 |
| Ramachandran Outliers (%) | 0.5 | 0.4 | 0.4 | 0.4 |
| Ramachandran Favored (%) | 97.8 | 97.8 | 97.3 | 95.5 |
| Poor Rotamers (%) | 1.6 | 1.3 | 1.6 | 1.5 |

Rsym = Σ_{h}(|I_{h} - <I_{h}>)|/Σ_{h}I_{h}, where h = set of Miller indices, j = set of observations of reflection h, and <I_{h}> = the mean intensity. RMSD values are deviation from ideal values. Rwork = Σ_{h}(|F_{o,h} - |F_{c,h}|)|/Σ_{h}|F_{o,h}|, was calculated using 5% of the complete data set excluded from refinement. The numbers in parentheses represent values from the highest resolution shell (2.08–2.00 Å for VldE, 2.23–2.15 Å for VldE-GDP, 2.22–2.11 Å for VldE-GDP-VDO, and 2.96–2.81 Å for VldE-GDP-TRE).
monomers were modeled within the VldE-GDP-VDO the asymmetric unit. NCS restraints were used during all refinements. In these structures, 0.4% of residues are reported as Ramachandran outliers; however, they are all within well-defined electron densities, and outliers at residues Asp158 and Asn386 were common within all the monomers of all the models of VldE.

Diffraction and structure refinement statistics are summarized in Table 1. Coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers: VldE (PDB ID: 3VDM), VldE-GDP (PDB ID: 4F96), VldE-GDP-VDO (PDB ID: 4F97), and VldE-GDP-TRE (PDB ID: 4F9F).

Results

Overall Structure

As was described in a recent crystallographic study of ValL [40], a VldE homolog from S. hygroscopicus subsp. jingangensis, VldE is a homodimer. Each monomer consists of twin Rossman β/α/β domains in the GT-B configuration [41] (Figure 3A) with the terminal helix (α16) extending back across the C-terminal Rossman-like domain to reach the N-terminal Rossman-like domain with a turn at Trp458 as it crosses the interface of the two domains. Despite a modest 19% sequence identity (29% homology) (Figure 2), the overall fold of VldE is remarkably similar.
to OtsA (Figure 3B), a fellow member of the GT20 family. Unlike the previous study [40] however, the core of the N-terminal domain of our apo enzyme is found with the catalytic site in an “open” conformation instead of “closed”. In this “open” conformation, the core β-sheet of the N-terminal Rossman-like domain consists of only ten strands instead of twelve. Two strands described as part of the core sheet in the previous study now are observed in a unique β-hairpin motif (β2 and β3) extending away from the catalytic site. Simultaneously, there is a dramatic shift of residues 33–47 away from the catalytic center. Unfortunately, coordinates from the structural study of ValL were not publicly available at the time of this study, so a complete comparison could not be performed. However, the closed conformation of VldE will

### Table 2. Notable interactions between VldE and ligands.

| Protein Atom | Guanine | Ribose 5'-phosphate | Donor/Acceptor | Donor/Acceptor |
|--------------|---------|----------------------|----------------|----------------|
| Arg321*      | NH2     | O6 (3.3)             | O2B (2.8)      | O6P (3.7)      |
| Asn323*      | ND2     | N1 (3.3)             | O2B (2.8)      | O6P (3.7)      |
|              |         | N2 (3.4)             | O1B (2.9)      |                |
| Asp360       | O       | O6 (3.3)             | O2B (2.8)      | O6P (3.7)      |
| Asn361       | OD1     | N1 (2.6)             | O2B (2.8)      | O6P (3.7)      |
|              |         | N2 (2.9)             | O1B (2.9)      |                |
| Asp362       | N       | O6 (2.9)             | O2B (2.8)      | O6P (3.7)      |
|              |         | O6 (3.2)             | O1B (2.9)      |                |
| Thr368*      | OG1     | N7 (2.6)             | O2B (2.8)      | O6P (3.7)      |
|              |         | O6 (3.6)             | O1B (2.9)      |                |
| Arg290       | NE      | O2B (2.8)             | O1B (2.9)      | O6P (3.7)      |
| Lys295       | NZ      | O3A (3.1)             | O2B (2.8)      | O6P (3.7)      |
|              |         | O1B (3.0)             | O1B (2.9)      |                |
| Leu387       | N       | O1A (3.4)             | O2B (2.8)      | O6P (3.7)      |
|              |         | O2A (2.8)             | O1B (2.9)      |                |
| Ser388       | OG1     | N7 (2.6)             | O2B (2.8)      | O6P (3.7)      |
|              |         | O1A (3.1)             | O1B (2.9)      |                |
| Glu391       | OE1     | O2' (2.5)             | O1B (2.9)      | O6P (3.7)      |
|              |         | O2' (2.6)             | O1B (2.9)      |                |
| His182       | ND1     | OAO (2.8)             | O3P (3.3)      |                |
| Asp383       | OD1     | OAS (2.9)             | O3P (3.3)      |                |
| Gly384       | N       | OAS (3.0)             | O3P (2.6)      |                |
| Gln385       | N       | OAS (2.7)             | O3P (2.2)      |                |
|              |         | OAR (2.8)             | O4P (3.2)      |                |
| Asn386       | N       | OAS (3.4)             | O3P (3.4)      |                |
|              |         | OAR (2.7)             | O4P (3.2)      |                |
| Leu387       | N       | OAR (3.6)             | O4P (3.7)      |                |
| Asp158       | OD1     | OAO (3.3)             |                |                |
|              |         | OAO (3.3)             |                |                |
| Asn325       | ND2     | OAX (2.4)             |                |                |
|              |         | OAX (2.4)             |                |                |
| Arg326       | NH1     | OAY (2.7)             |                |                |
|              |         | OAY (2.7)             |                |                |
| Arg290       | NE      | OAY (3.6)             |                |                |
|              |         | OAY (3.6)             |                |                |

Values in parentheses are distances given in angstroms.
Donor/Acceptor is an indicator of relative position within the catalytic site.
Distances describing GDP interactions with VldE are taken from the VldE-GDP model.
*Indicates residues not conserved in OtsA that make guanine specific interactions.
doi:10.1371/journal.pone.0044934.t002
be described in later sections of this article using our own experimental data.

The GDP bound structure of VldE (VldE-GDP) described here is also in an “open” conformation and the binding of GDP did not have an effect on the global conformation of the apo enzyme (RMSD Cα = 0.350 Å). Surprisingly, the structure of VldE in complex with the entirety of its products (VldE-GDP-VDO), GDP and VDO was also in an open conformation and a comparison to the VldE-GDP model showed that the binding of VDO also did not have an effect on the global conformation of VldE (RMSD Cα = 0.262 Å).

Lastly, we were also elucidated the structure of VldE in complex with both GDP and TRE (VldE-GDP-TRE). Within the VldE-GDP-TRE structure, six monomers were modeled within the asymmetric unit (ASU). The six monomers of the ASU assumed three alternative conformers. The monomers of the first conformation contained both GDP and trehalose, and this first conformer was found to also have an “open” catalytic site with similar global conformation to the VldE-GDP-VDO complex (RMSD Cα = 0.705 Å). The last two conformers within the VldE-GDP-TRE structure had GDP but not trehalose bound, and these two conformers contained a “closed” catalytic site as described in the previous studies [40]. These alternative conformations are significantly different when compared the “open” VldE-GDP structure (RMSD Cα = 2.304 Å, RMSD Cβ = 2.395 Å) and are further discussed in later sections.

The Nucleotide Binding Site

Due to the conservation of key residues within their binding pockets, the binding of ligands within VldE are thought to be analogous to OtsA [19]. Here we provided experimental support for the analogous binding of GDP within VldE and UDP within OtsA. (Figure 4A) Within VldE, the ribose and phosphate moieties of GDP make interactions with the side-chains of Asp383, His182 and Arg290. (B) Shown is a superimposition of the catalytic sites of VldE·GDP·VDO model (yellow) and the VldE·GDP·TRE model (pink) in ribbon diagrams. Trehalose does not assume a binding pose comparable to VDO. This is most likely to do the absence of a phosphoryl group. Due to the absence of the phosphate moiety, Arg326 and Asn325 also swing out of the catalytic site.

doi:10.1371/journal.pone.0044934.g006

Figure 6. Trehalose within the Catalytic Site. Shown is a trehalose (TRE) within the VldE cyclitol binding site in ribbon diagrams. Residues/molecules of interest are represented in stick models. The dotted lines mark hydrogen bonds. (A) The mesh represents the |Fo|−|Fc| electron density omit map of trehalose within the VldE catalytic site (pink). The map is contoured at 3.0σ levels. Trehalose makes interactions with the backbone amides of residues Gly384, Glu385, Asn386, and Leu387 as well with the side-chains of Asp383, His182 and Arg290. (B) Shown is a superimposition of the catalytic sites of VldE·GDP·VDO model (yellow) and the VldE·GDP·TRE model (pink) in ribbon diagrams. Trehalose does not assume a binding pose comparable to VDO. This is most likely to do the absence of a phosphoryl group. Due to the absence of the phosphate moiety, Arg326 and Asn325 also swing out of the catalytic site.

doi:10.1371/journal.pone.0044934.g006
Figure 7. Conformational Changes. Shown are ribbon diagrams of the three conformers of VldE modeled using the VldE•GDP•TRE crystallographic data. Residues/molecules of interest are represented in stick models. Arrows indicate the direction of residue movement. Ligands bound within each conformer are shown to provide a point of reference for comparison. (A) The global conformation of each conformer (gray) is shown with the areas of conformational change highlighted by coloring (pink, blue, cyan). (B) Residues 11–50 for each conformer is shown. Note that this region of residues is capable of 10.9 Å shift towards the catalytic center and that strands of the β-hairpin motif (β2 and β3) move simultaneously with strands β6 and β5 to extend the core β-sheet from ten to twelve strands. (C) A view of helix α3 for each conformer is shown. Note that residues at the kink of the helix are capable of reorganizing.

doi:10.1371/journal.pone.0044934.g007
binding of a uracil moiety. Interactions between VldE and GDP are summarized in Table 2.

**Cyclitol Binding Sites**

The first crystallographic structure of VldE in complex with the entirety of its products, VDO and GDP, was elucidated (Figure 4C). The interactions between GDP and VldE are described within the previous section, but here the interactions between VDO and VldE are shown to be analogous to the interactions previously described by the OtsA-VDO complex [20].

The hydroxyls of the donated cyclitol make numerous interactions with the backbone amides of VldE at residues Gly384, Gln385, Asn386, and Leu387. The donated cyclitol also makes interactions with the side-chains of residues His182 and Asp383 (Figure 5B). The interactions by donated cyclitol are conserved within the catalytic site of OtsA (Figure 4D). Also conserved is the interaction of the \( \beta \)-phosphate with the formerly bound carbon of the donated cyclitol. As in OtsA, the phosphate of the leaving nucleotide is involved in the stabilization of the transition state during the concerted, front-faced nucleophilic attack of a SN\(_{i}\) reaction, and at a distance of 2.6 Å from the attacking nucleophile, the phosphate can act as a general base to deprotonate the amine [20].

The binding of the acceptor cyclitol within VldE is recognized by a shift in side-chain conformations within the catalytic site. Asp158 swings 62–83° towards the catalytic center in recognition of the hydroxyls of the acceptor cyclitol. Upon the binding of the phosphorylated acceptor group, the guanidine moiety of Arg326 enters into an interaction with the phosphate oxygens. This interaction allows Tyr159 to swing 110–112° to make an interaction with Arg326. The acceptor cyclitol also interacts with the side-chain of Asn325. These interactions are conserved within OtsA except at Asn325. At the same position within OtsA there is Ser299 which does not make an interaction with VDO (Figure 4D) [4].

Despite the conservation of the interactions between the catalytic site and VDO, there is no observed conformational closing of the catalytic site upon the binding of the acceptor cyclitol as was witnessed in structural studies of OtsA [19,20,25]. However, a closed conformation was described in the recently published structural studies of ValL [40]. In later sections, such a conformational shift will be described within our structural studies of VldE, but this shift was not triggered by the binding of the acceptor cyclitol.

**Table 3. DALI Server Results.**

| Enzyme Name                                                                 | RMSD |
|----------------------------------------------------------------------------|------|
| Trehalose-Phosphate Synthase (OtsA)                                        | 2.4  |
| N-Acetyl-\( \alpha \)-D-Glucosaminyl L-Malate Synthase (\( \beta \)d\( \beta \)shA) | 2.9  |
| 3-Phospho-1-D-Inositol-2-Acetamido-2-Deoxy-\( \alpha \)-D-Glucopyranoside Synthase (MshA) | 3.7  |
| Trehalose Synthase (TreT)                                                   | 3.1  |
| Phosphatidylinositol Mannosyltransferase (PimA)                             | 3.1  |
| Glycogen Synthase (GlgA)                                                    | 3.1  |

- Listed in order of DALI ranking.

**Figure 8. The \( S_ni \) mechanism of VldE.** Shown is a figure comparing the \( S_ni \) mechanism of (A) OtsA to the proposed \( S_ni \) mechanism of (B) VldE. The olefinic moiety of GDP-valienol plays a critical role in facilitating the coupling reaction through a mechanism similar to the formation of an oxonium ion-like transition state upon the detachment of the nucleotide phosphate within OtsA. Both transition states are formed by the coordination of the allylic carbon on the donated group, the bridging nucleophile of the acceptor group and the leaving oxygen of the donor diphosphate-nucleotide.

doi:10.1371/journal.pone.0044934.g008
We were also able to elicit the structure of VldE in complex with both GDP and trehalose (VldE-GDP TRE). (Figure 6A) Within this model, GDP maintains the interactions previously described elsewhere within this study. The binding pose of trehalose is similar to what was described by the recent structural study of VldE [40]. The sugar moiety of trehalose within donor cyclitol binding site makes interactions with the backbone amides of residues Gly304, Gln503, Asn506, and Leu507 as well with the side-chains of Asp503 and His182. Unsurprisingly, this preserves the interactions between the enzyme and the structurally similar donor cyclitol as was described in the VldE-GDP-VDO model. However, the sugar moiety of trehalose occupying the acceptor cyclitol binding site makes only a single hydrogen bond with Arg290 in our study. A superimposition of trehalose and VDO within the VldE catalytic site shows that trehalose does not assume a binding pose comparable to VDO (Figure 6B). This is most likely due to the absence of a phosphorylated ligand. Within the VldE-GDP-VDO model, Arg326 and Asn325 interact with the phosphorylated cyclitol and most likely help to ensure the proper position of validamine 7-phosphate for catalysis. Indicatively, Arg326 and Asn325 swing out of the catalytic site within our trehalose bound structure. Interactions between VldE and VDO and TRE are summarized in Table 2.

**Observed Changes in Conformation**

While solving the VldE-GDP-TRE structure, six monomers of VldE were placed within the asymmetric unit. Represented within the six monomers were three differing conformations of the N-terminal Rossmann-like domain (two monomers per conformation) (Figure 7A). However, no interesting conclusions regarding possible allosteric interactions in this enzyme could be derived from these observations alone.

The first conformer had GDP and trehalose bound and is described in the previous section. This conformer is quite similar to the other models with “open” catalytic sites expect for residues 325–328 which swing out of the catalytic site upon the binding of trehalose. The second and third conformers have only GDP within the catalytic site and both of these conformers share a dramatic shift in residues 11–50 which result in the “closing” of the catalytic site (Figure 7B). Resultantly, Gly33, Gly34, and Thr35 move 10.9 Å towards the catalytic center, and within 4 Å of the ligand atoms. Additionally, helix α2, which originally consisted of residues 36–41, coils further to include residues 42–47. This shift occurs concurrently with the rotation of the β-hairpin motif (β2 and β3) unique to VldE. Additionally, the last two of ten strands (β6 and β5) comprising the core β-sheet of the N-terminal Rossmann-like β/α/β domain move towards the compacting catalytic site. The movements of the unique β-hairpin motif and the two strands of the core β-sheet narrow the distance between them and allow the necessary interactions to unite them into a single twelve-stranded core β-sheet. The “closed” conformation described here is similar to what was described in the structural study of ValL [40].

A conformational change which resulted in the closing of the catalytic site was described within the structural studies of OtsA. Residues were observed to make a >10 Å movement towards the catalytic center upon binding of the accepting sugar in OtsA [19,25]. The conformational shift was thought to be due to the release of the phosphorylated acceptor glucose 6-phosphate by Arg9. However, with the lack of an acceptor group within the catalytic site, the conserved arginine at position 12 of VldE cannot be responsible for the closing of the catalytic site. Therefore, the closing of the VldE catalytic site is more likely to work at least partially through a more dynamic, equilibrium based mechanism.

An additional conformation change was also observed within the third conformer found within the VldE-GDP-TRE crystal (Figure 7C). Within the α3, there is a kink at residues 96–102. Within the third conformer, the kinked residues shift to form a more organized secondary structure. Again, the observed conformational change is not concerted with the binding of ligand and further supports the notion that VldE moves through equilibrium based conformations. Conformational change at this position was observed in the previous structural studies, and similarly the change seems to be focused around Gln96 [40].

**Discussion**

**Mechanistic Considerations**

The net retention of the substrate stereochemistry is thought to occur through either a double displacement (S_n2 X_2) or internal return mechanism (S_i) [16]. Recent evidence substantiates claims that the conservation of the anomeric center configuration within OtsA is due to an internal return mechanism [18,20]. If the reaction of VldE were to alternatively go through a double displacement nucleophilic substitution mechanism, a residue must be immediately available for a covalent intermediate. Comparatively, the hydrolysis of glycosides is known to occur through a nucleophilic substitution involving a covalent intermediate [42,43]. The most likely candidate residue within VldE would be a nucleophile His182. As can be seen in Figure 4D, this residue is also conserved within the catalytic site of OtsA (His154). At a 5.1 Å from the stereochemical center of the substrate, His182 is not observed to be immediately available for nucleophilic substitution in our structural studies, and as of yet there has been no significant, direct evidence to support a mechanism by which the non-glycosidic coupling reaction of VldE occurs through a covalent intermediate.

Our structural evidence demonstrates a strict conservation of nucleotide and (pseudo)sugar orientation within VldE and OtsA and suggests that the glycosyltransferase reaction of OtsA and the pseudo-glycosyltransferase reaction of VldE do occur through similar, concerted but asynchronous internal return mechanisms. Therefore, the olefinic moiety of GDP-valentol must play a critical role in facilitating the coupling reaction through a mechanism similar to the formation of an oxonium ion-like transition state upon the detachment of the nucleotide phosphate within OtsA as was previously suggested [4]. Through such a mechanism, the transition state is formed by the simultaneous binding of the allylic carbon on donated cyclitol, the bridging amine of the acceptor cyclitol-phosphate and the leaving oxygen of the donor diphasate-nucleotide. As in OtsA [20], the leaving phosphate is in a position to deprotonate the incoming nucleophile of the acceptor cyclitol (Figure 8A–B).

Supportively, a distance matrix search using DALI [44] revealed a strong similarity between VldE and OtsA, N-Acetyl-α-D-Glucosaminyl L-Malate Synthase (BbBshA, EC 2.4.1.x), 3-Phospho-1-D-Inositol-2-Acetamido-2-Deoxy-α-D-Glucopyranoside Synthase (MsaA, EC 2.4.1.250), Trehalose Synthase (TreT, EC 2.4.1.245), Phosphodiinositol Mannosyltransferase (PinA, EC 2.4.1.57), and Glycogen Synthase (GlgE, EC 2.4.1.11) (Table 3). All are retaining glycosyltransferases that have been associated with internal return mechanisms [20,45–49] (Table 3).

**Significance**

The biosynthetic pathways of natural products exhibiting antibiotic-like properties have recently become of great interest. As seen in the discovery of the antibacterial erythromycin, the
antihelmintic avermectins, and the antitumor indolocarbazoles, the genetic manipulation of biosynthetic gene clusters alongside the use of alternative biosynthetic precursors, and combinatorial proteins for chemoenzymatic synthesis can result in the generation of an entire array of biologically active compounds [50–53]. The generation of a library of the biosynthesis of validamycin A has only recently been discovered, this study and others are underway to elucidate the individual processes within this biosynthetic pathway of this C27-N-aminocyclitol [2].

The C27-N-aminocyclitols (e.g., acarbose, validamycin, cetoniacyanate, and salbostatin) belong to a class of natural products which include the aminoglycosides (e.g., streptomycin, hygromycin, butirosin, and neomycin), and the five membered aminocyclitols or cyclopeptitols (e.g., pactamycin, trehalozin, and allosamidin). The aminoglycosydrases are a diverse group of natural products which were amongst the first clinical antibiotics. The C27-N-aminocyclitols are an even more diverse group of natural products with an entire range of biological activities [10]. A better understanding of the biosynthesis of C27-N-aminocyclitols at the molecular level could allow for the generation of novel, natural product analogs which could include an array of structurally altered antibiotics. The line of inquiry provided within this article hopes to add to that understanding.

Acknowledgments

The authors thank Dr. Henry Bellamy at The Center for Advanced Microstructures and Devices, Louisiana State University, Baton Rouge, LA, for his exhaustive discussions on the topics of data collection and processing strategies. The authors also thank the staffs at The Northeastern Collaborative Access Team (NE-CAT) beamline at the Advanced Photon Source (Argonne National Laboratory), Argonne, IL, and the National Synchrotron Light Source (NSLS) Beaman X6A at the Brookhaven National Laboratory, Upton, NY for their time and efforts. Lastly, the authors thank Matthew Kode for his help during his visit to NSLS, and Robert B. Crochet for proofreading the manuscript.

Author Contributions

Conceived and designed the experiments: MCC YSY SA DN KHA TM YHL. Performed the experiments: MCC YSY DN YHL. Analyzed the data: MCC YSY DN TM YHL. Contributed reagents/materials/analysis tools: MCC YSY SA DN KHA TM YHL. Wrote the paper: MCC TM YHL.

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