Published in final edited form as:
Curr Opin Struct Biol. 2010 October ; 20(5): 543–550. doi:10.1016/j.sbi.2010.08.002.

Biosynthetic Enzymes of Unusual Microbial Sugars

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Abstract

The biological importance of proteins and nucleic acids in the natu ral world is undeniable, and research efforts on these macromolecules have often overshadowed those directed at carbohydrates. It is now known, however, that carbohydrates not only play roles in energy storage and plant cell wall structure, but are also intimately involved in such processes as fertilization, the immune response, and cell adhesion. Indeed, recent years have seen an explosion in research efforts directed at uncovering and understanding new sugar moieties. The di- and trideoxysugars, which are synthesized by a variety of bacteria, fungi, and plants, represent an especially intriguing class of carbohydrates. They are found, for example, on the lipopolysaccharides of some Gram-negative bacteria or on antibacterial agents such as erythromycin. Many of them are formed from simple monosaccharides such as glucose-6-phosphate or fructose-6-phosphate via a myriad of enzymatic reactions including acetylations, aminations, dehydrations, epimerizations, reductions, and *C*-, *N*-, and *O*-methylations. In this review we focus on the recent structural investigations of the bacterial *N*-acetyltransferases and the PLP-dependent aminotransferases that function on nucleotide-linked sugar substrates.

Introduction

Carbohydrates are essential elements in nearly every p hysiological process and represent the most abundant biomolecules in living systems. Indeed, it has been estimated that ~50% of the Earth's biomass is composed of carbohydrates. Apart from their role in providing metabolic energy, carbohydrates are involved in a wide range of biological processes including the immune response, cell-cell interactions, fertilization, viral infection, and drug efficacy, among others. The diversity of the complex carbohydrates observed in nature is derived through both the identity of the individual monosaccharides themselves and the manner in which these sugar moieties are linked together to form polymeric molecules. A simple disaccharide composed of two glucosyl units alone can, for example, lead to eleven possible structures! Add to this the enormous variety of monosaccharides found throughout

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the biological kingdom , and one is left with a dazzling array of macromolecules with unique chemical properties and three-dimensional structures.

The di- and trideoxysugars, which are the topic of this review, represent an especially important and intriguing class of carbohydrates that are synthesized by plants, fungi, and bacteria [1]. They are found, for example, on the lipopolysaccharides of some Gram-negative bacteria, on the *S*-layers of some Gram-positive and Gram-negative bacteria, on extracellular polysaccharides, and on antibiotic, antifungal, anthelminitic, and antitumor agents. While research papers concerning unusual sugars began to appear in the literature as early as 1929, until recently these carbohydrates and the enzymes required for their biosynthesis were somewhat overlooked. This was due in part to the fact that most di- and trideoxysugars were not and are still not commercially available, thus making it difficult to biochemically characterize the enzymes involved in their production.

Many of the unusual sugars produced by microbes are formed from simple monosaccharides such as glucose-6-phosphate or fructose-6-phosphate via a myriad of enzymatic reactions including dehydrations and reductions. Most, but certainly not all, of the deoxysugars of biological relevance are constructed around the 2,6-dideoxyhexoses or the 2,3(4),6-trideoxyhexoses, which are further modified by acetylation, amination, epimerization, and *C*-, *N*-, and *O*-methylation reactions. More than one hundred different di- and trideoxysugars have been isolated from prokaryotic sources thus far [1], some of which are shown in Figure 1.

The pathways for the biosynthesis of most di- and trideoxysugars begin with the attachment of α-D-glucose-1-phosphate (or α-D-mannose-1-phosphate) to an NMP moiety via a nucleotidyltransferase [1]. The second step in unusual sugar biosynthesis typically involves removal of the C-6’ hydroxyl group and oxidation of the C-4’ hydroxyl moiety thereby leading to NDP-4-keto-6-deoxyglucose. This reaction is catalyzed by an NDP-glucose 4,6-dehydratase. Importantly, NDP-4-keto-6-deoxyglucose represents the branching point for the subsequent enzymatic reactions that lead to the production of such nucleotide-linked sugars as GDP-colitose, dTDP-desosamine, and CDP-tyvelose (Figure 1).

At first glance, the diversity of the di- and trideoxysugars observed in the natural world seems overwhelming. Yet, once NDP-4-keto-6-deoxyglucose has been formed, there are seven common enzymatic reaction types that ultimately lead to the production of these unusual sugars: acetylations, aminations, dehydrations, epimerizations, isomerizations, ketoreductions, and methylations. In some of the more exotic sugars, such as D-kijanose or D-sulphoquinovose, additional reactions are required such as amine oxidation or sulfation, respectively [2]. For this review we will focus on the recent structural and functional analyses of enzymes that catalyze either *N*-acetylations or PLP-dependent aminations (or dehydrations) using nucleotide-linked sugars as substrates.

N-acetyltransferases

N-acetyltransferases catalyze the transfer of acetyl groups from acetyl-CoA to primary amine acceptors. To date, both the GNAT and the LβH superfamilies of *N*-acetyltransferases have been intensively studied. The term GNAT is an abbreviation for “GCN5-related *N*-

acetyltransferase" whereas the designation "LβH" refers to the left-landed β-helix motif that characterizes members of the superfamily.

The first X-ray structure determined for an LβH superfamily member was that of UDP-*N*-acetylglucosamine acyltransferase or LpxA [3]. This enzyme plays a key role in Lipid A biosynthesis by catalyzing the transfer of (*R*)-3-hydroxymyristic acid from an acyl carrier protein to the C-3' hydroxyl of UDP-*N*-acetylglucosamine. As such, LpxA is technically an *O*-acyltransferase rather than an *N*-acetyltransferase. The overall three-dimensional architecture of LpxA was truly remarkable in its abundance of exceedingly rare left-handed crossover connections and its repeated isoleucine rich hexapeptide motif.

In recent years, the three-dimensional structures of *N*-acetyltransferases specifically functioning on nucleotide-linked sugars and belonging to the LβH superfamily have been reported [4-10]. Of particular interest is the *N*-acetyltransferase referred to as PglD from *Campylobacter jejuni* [8••]. This enzyme is involved in the production of 2,4-diacetamido-2,4,6-trideoxy-D-glucose or QuiNAc4NAc (Figure 1). *C. jejuni* is a highly unusual Gram-negative bacterium in that it contains *N*-glycosylated proteins similar to those observed in eukaryotes [11]. QuiNAc4NAc serves as the linker between the asparagine residue of a given glycosylated protein and the attached *N*-glycan moiety.

A ribbon representation of PglD in complex with CoA is presented in Figure 2a. The enzyme functions as a trimer with each subunit folding into two separate domains (Figure 2b). The CoA ligand binds to the enzyme in a curved conformation, and the three active sites of the trimer are situated between subunits (Figure 2a).

On the basis of the PglD/ CoA complex structure and model building of a "tetrahedral" intermediate species into the active site, it was proposed that His 125, through its interactions with Glu 124 and Glu 126, functions as the general base required for catalysis. Following this initial investigation, binary structures of PglD in complex with acetyl-CoA or its UDP-4'-amino sugar substrate were reported [9••]. A close-up view of the PglD active site with bound cofactor and substrate is displayed in Figure 2c. Residues from both subunits contribute to the anchoring of the acetyl-CoA and UDP-linked sugar into the active site. In light of these two binary complexes of PglD with either acetyl-CoA or a nucleotide-linked sugar, a more detailed catalytic mechanism was put forth with His 125 again serving as the active site base [9••]. In a subsequent biochemical analysis of PglD, however, an H125A mutant protein was shown to be active [12•], and thus the role of this histidine in PglD catalysis should still be considered open to debate.

Recently, the three-dimensional architecture of a second *N*-acetyltransferase belonging to the LβH superfamily and functioning on a nucleotide-linked sugar was reported [10••]. This enzyme, referred to as QdtC, catalyzes the last step in the biosynthesis of 3-acetamido-3,6-dideoxy-α-D-glucose or Qui*p*3NAc (Figure 1), a sugar found in the *O*-antigens of various Gram-negative bacteria and in the *S*-layer glycoprotein glycans of some Gram-positive bacteria. For this X-ray crystallographic analysis, three structures of the wild -type enzyme (from *Thermoanaerobacterium thermosaccharolyticum* E207-71) complexed with acetyl-CoA, with CoA and dTDP-3-amino-3,6-dideoxy-α-D-glucose, or with CoA and dTDP-3-

amino-3,6-dideoxy-α-D-galactose were determined. Like PglD, QdtC is a trimer with each subunit participating in extensive interactions with the other monomers. The structure of an individual subunit of QdtC is dominated by 32 β-strands that form the canonical LβH motif. QdtC lacks, however, the N -terminal domain observed in PglD.

The manners in which the nucleotide-linked sugars are accommodated within the active sites of PglD and QdtC are stunningly different (Figure 3). Whereas primary structural alignments of these two enzymes suggested that His 123 in QdtC corresponds to His 125 in PglD, in fact, His 123 is ~8 Å from the sugar amino group! The PglD and QdtC structures clearly provide an important lesson in the dangers of making biochemical assumptions based solely on amino acid sequence alignments.

A catalytic mechanism for QdtC has been proposed on the basis of detailed structural, kinetic, and site-directed mutagenesis experiments [10••]. According to the proposed mechanism, catalysis by QdtC proceeds without a general base provided by the protein. Rather the sulfur of acetyl-CoA ultimately serves as the catalytic base by accepting a proton from the sugar amino group. Very recently the structure of WlbB from *Bordetella petrii* has been reported [13••]. This *N*-acetyltransferase is involved in the production of 2,3-diacetamido-2,3-dideoxy-D-mannuronic acid, a rare sugar found in the outer membranes of pathogenic bacteria such as *Pseudomonas aeruginosa* and *Bordetella pertussis*. Like that observed for QdtC, the active site of WlbB is devoid of potential catalytic bases, and most likely its reaction mechanism proceeds in a similar manner to that proposed for QdtC.

PLP-Dependent Aminotransferases and Dehydratases

For the *N*-acetyltransferases described above to carry out their reactions, a free amino group on the sugar is required. Addition of amino groups to the sugar rings occurs through the action of aminotransferases that require pyridoxal 5'-phosphate or PLP for activity. The first sugar modifying aminotransferase structure to be determined was that of ArnB from *Salmonella typhimurium*. It catalyzes the amination of the 4'-keto group of a UDP-linked sugar substrate to yield UDP-4-amino-4-deoxy-L-arabinose (Figure 1) [14]. As revealed by this initial structural analysis, ArnB belongs to the aspartate aminotransferase or Type I family of PLP-dependent proteins. Catalysis by these enzymes is known to proceed via a ping-pong mechanism in which the enzyme oscillates between PLP and PMP (pyridoxamine 5'-phosphate)-bound forms. Typically, the PLP cofactor forms a Schiff base with the α-amino group of a conserved active site lysine. This is referred to as the internal aldimine. The α-amino group of an incoming amino acid, which is typically glutamate, attacks the C4' atom of PLP, displacing the lysine and yielding the external aldimine. A series of proton extractions and donations by active site residues results in the collapse of the external aldimine to yield α-ketoglutarate and PMP. Formation of PMP represents the "ping" half of the reaction. The "pong" half of the mechanism is the reaction of PMP with ketones such as keto sugars to form transaminated products.

A ribbon representation of the overall fold of the ArnB dimer is displayed in Figure 4. Each subunit contains two domains with the N -terminal region responsible for PLP-binding. The two active sites of the enzyme are located in clefts at the subunit:subunit interfaces. Since the initial report of the ArnB structure, additional papers have appeared in the literature

describing the three-dimensional architectures of other aminotransferases that function on nucleotide-linked sugars including PseC from *Helicobacter pylori* [15], DesV from *Streptomyces venezuelae* [16], DesI from *S. venezuelae* [17], GDP-perosamine synthase from *Caulobacter crescentus* [18••,19•], and QdtB from *Thermoanaerobacterium thermosaccharolyticum* [20•].

The structural analysis of PseC was of particular importance because it represented the first glimpse of an aminotransferase with a bound nucleotide-linked sugar, specifically in the external aldimine form [15]. PseC is involved in the biosynthesis of pseudaminic acid and catalyzes amination of the sugar C-4' position to yield UDP-4-amino-4,6-dideoxy-*N*-acetyl-β-L-altrosamine (Figure 1). Note that in the reaction catalyzed by PseC, the amino group is transferr ed to the axial position. Following this investigation of PseC, the structure of DesI was reported. DesI is one of six enzymes required for the biosynthesis of dTDP-desosamine in *S. venezuelae* [21]. In the case of DesI, the amino group is transferred to the equatorial position to produce dTDP-4-amino-4,6-dideoxyglucose (Figure 1) [17]. The major difference in ligand binding between PseC and DesI is the nearly 180^{o} rotation of the hexose in the active site. This difference accounts for the axial versus equatorial amino transfer in PseC and DesI, respectively.

Another fascinating PLP-dependent enzyme whose structure was solved within the last several years is GDP-4-keto-6-deoxy-D-mannose 3-dehydratase or ColD [22-24]. This enzyme is involved in the production of colitose (Figure 1), a 3,6-dideoxysugar found in the *O*-antigens of some Gram-negative and marine bacteria. Four enzymes are required for colitose biosynthesis [22,25,26], the third step of which is catalyzed by ColD. Instead of an amination, however, ColD catalyzes a remarkable reaction, namely the removal of the hydroxyl group at C-3' (Figure 5). Unlike most PLP-dependent enzymes, the cofactor in ColD is not covalently attached to the protein via a lysine residue. In place of the lysine is His 188. A possible catalytic mechanism for ColD, based on all presently available biochemical and structural data, has recently been proposed [22,27].

As noted in Figure 5, ColD catalyzes a dehydration at C-3'. Another enzyme, GDP-perosamine synthase uses the same substrate as ColD, but catalyzes an amination at C-4'. Perosamine (4-amino-4,6-dideoxy-D-mannose) is found in the *O*-antigen of *Vibrio cholerae* O1, the causative agent of cholera in humans. An *N*-acetylated version of it has also been isolated from a variety of Gram-negative bacteria, including *Escherichia coli* O157:H7, the infamous contaminant of packaged spinach that resulted in widespread infections in the United States in 2006. ColD and GDP-perosamine synthase are decidedly similar in structure [18••,19•]. There are several variations present within their active sites, however, that determine whether they function as an aminotransferase or a dehydratase. Among these is the replacement of Asn 185 in GDP-perosamine synthase with Ser 187 in ColD. Also, GDP-perosamine synthase contains the typical lysine residue that forms an internal aldimine with the PLP-cofactor. Recently it was shown that changing Ser 187 to an asparagine and His 188 to a lysine residue resulted in the conversion of ColD from a C-3' dehydratase to a C-4' aminotransferase [28••].

Following the X-ray crystallographic analysis of ColD, the structure of another C-3' dehydratase was reported [29••], namely CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-dehydratase or E_1 from *Yersinia pseudotuberculosis*. This enzyme converts CDP-4-keto-6-deoxy-D-glucose to CDP-4-keto-3,6-dideoxy-D-glucose, which then serves as a precursor for the formation of such unusual sugars abequose, ascarylose, paratose, and tyvelose that are found attached to the lipopolysaccharides of Gram negative bacteria (Figure 1). E_1, like ColD, has an active site histidine (His 220) rather than a lysine. Unlike ColD, however, E_1 contains a [2Fe-2S] cluster and uses the NADH-dependent accessory protein E_3 to complete its dehydration reaction. Furthermore, E_1 does not require glutamate to convert the PLP cofactor into PMP, but instead directly releases the sugar product as a ketone, leaving the cofactor in the PMP form, ready for the next catalytic cycle. Interestingly, in the case of E1, four site-directed mutations (D194H/ Y217H/ H220K/ F345H) were required to convert it from a PMP-dependent dehydratase to a PLP-dependent aminotransferase [29••].

Concluding remarks

The di- and trideoxysugar moieties found throughout nature serve a variety of biological roles. When attached to aglycone scaffolds, they have been shown to enhance the efficacy of the natural product. By understanding the structures and functions of the enzymes required for the biosynthesis of these sugars, it should be possible to redesign them to produce novel carbohydrates, which may have important ramifications for the development of new therapeutics. But di- and trideoxysugars are not confined solely to natural products. They are also found, for example, in the *O*-antigens of Gram-negative bacteria, and it has been suggested that they play roles in microbial virulence. Consequently, many of the enzymes required to produce these sugars have and will continue to serve as drug targets. Even from this limited review it is clear that bacteria, fu ngi, and plants have evolved an amazing arsenal of enzymes for the biosynthesis of unusual di- and trideoxysugars. Undoubtedly, the next several years will be particularly exciting as more details are unraveled concerning the enzymes involved in the production of these unusual and fascinating microbial-derived sugars.

Acknowledgments

Research in our laboratory was supported in part by a grant from the National Institutes of Health (DK47814 to H. M. H.). We gratefully acknowledge the helpful discussions of Professors W. W. Cleland and Ivan Rayment.

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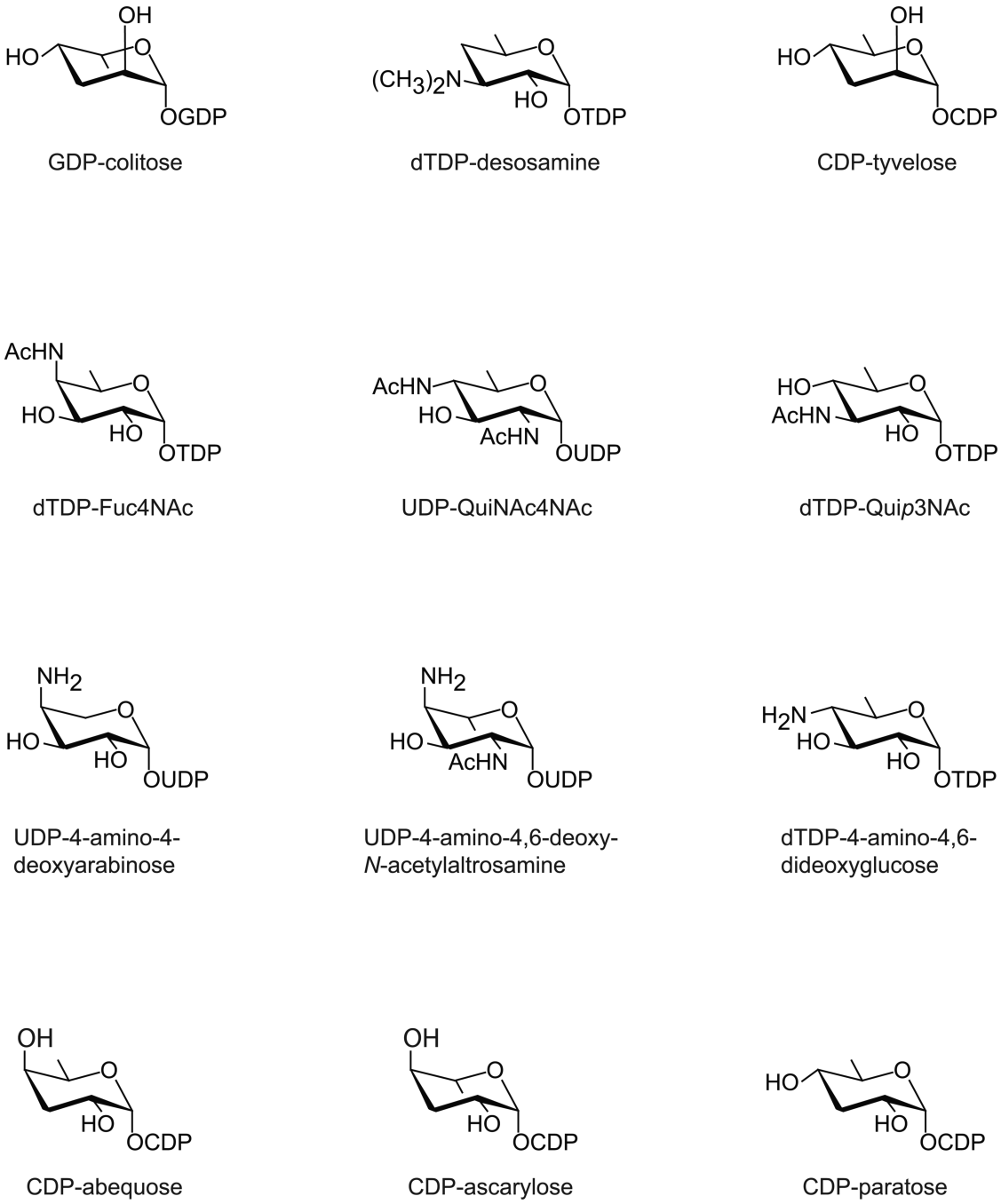

Figure 1.
Examples of di- and trideoxysugars produced by microbial sources.

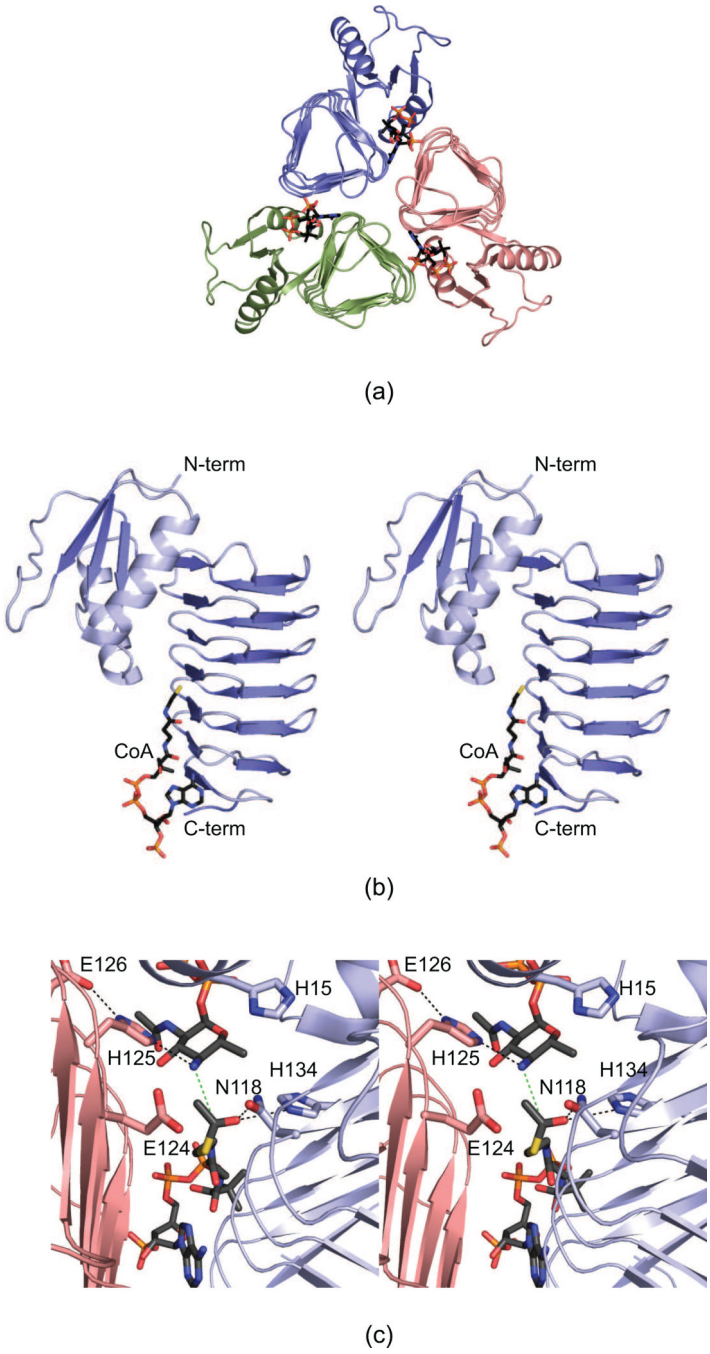

Figure 2.

The structure of PglD. A ribbon representation of the PglD trimer is depicted in (a) with the CoA ligands drawn as sticks (PDB code 2VHE [8]). A stereo view of an individual subunit is presented in (b). Shown in (c) are the positions of acetyl-CoA and the UDP-4'-amino sugar substrate when bound in the PglD active site (PDB codes 3BSY and 3BSS [9]). The active site is formed between two subunits as colored in salmon and light blue. The black dashed lines represent possible hydrogen bonding interactions, whereas the green dashed

line indicates the direction of attack at the carbonyl carbon of acetyl-CoA by the sugar amino group.

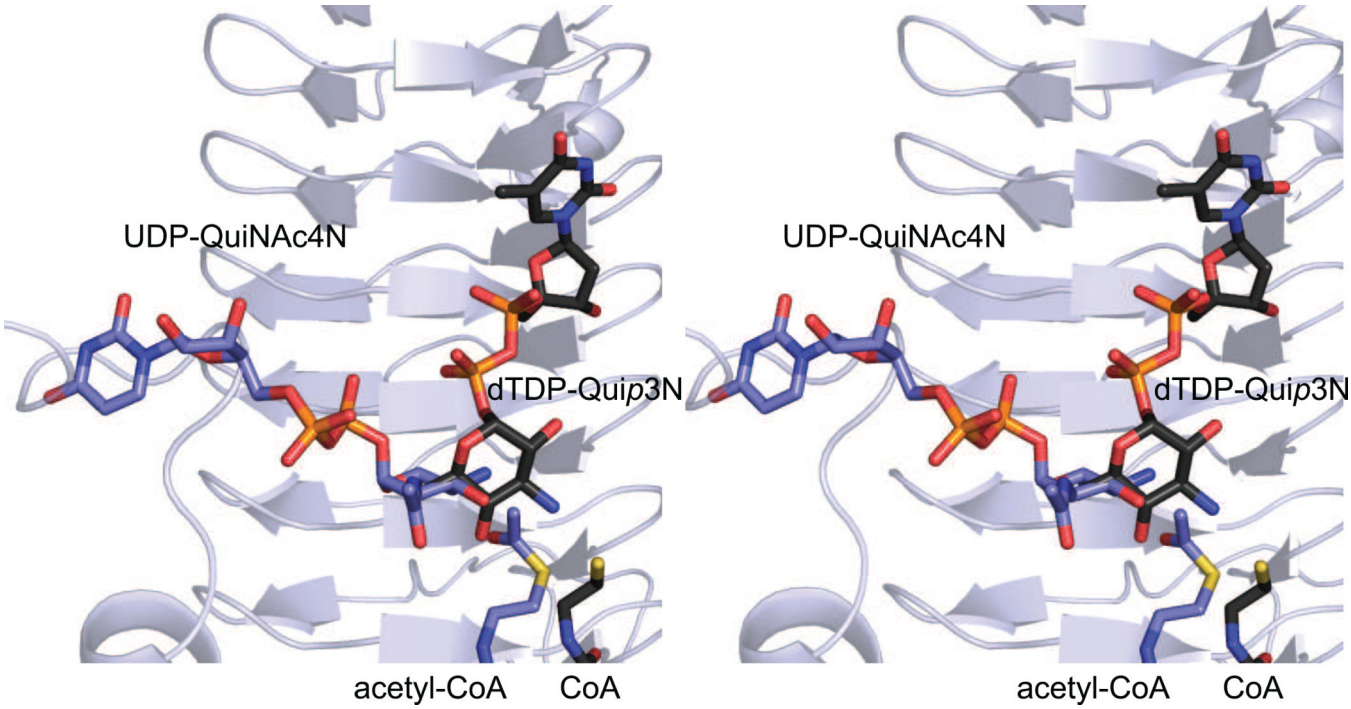

Figure 3.

Differences in NDP-sugar binding between PglD and QdtC. The ribbon representation shown is that for QdtC. The carbon atoms for the acetyl-CoA and UDP-QuiNAc4N ligands, when bound to PglD, are highlighted in blue whereas those for the CoA and dTDP-Qui*p*3N ligands, when anchored to QdtC, are displayed in black.

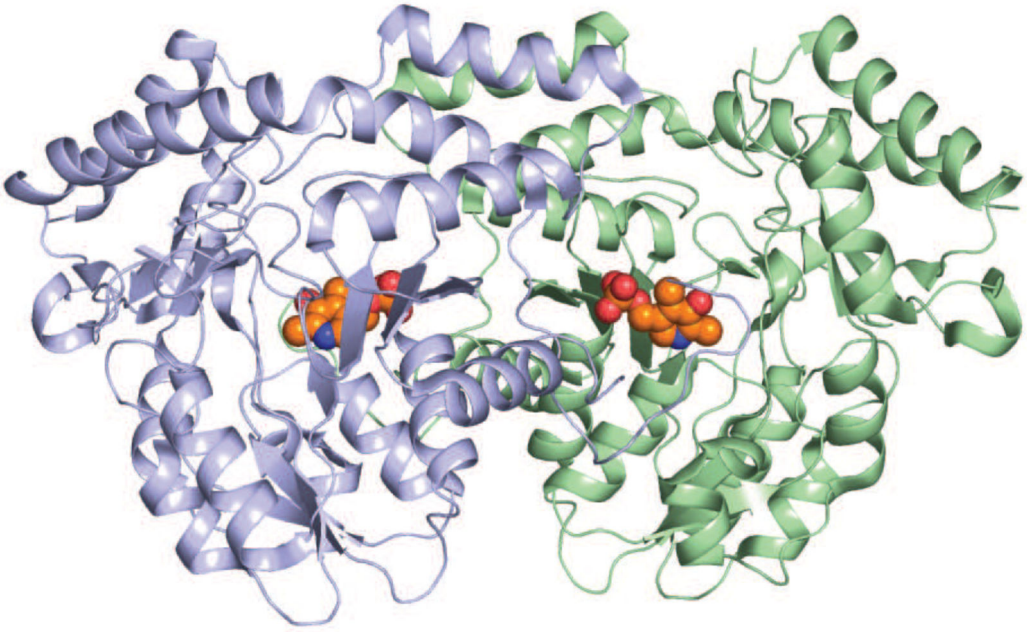

Figure 4.

The structure of the ArnB dimer. The two subunits are highlighted in light blue and light green and the PLP cofactors are depicted in space-filling representations.

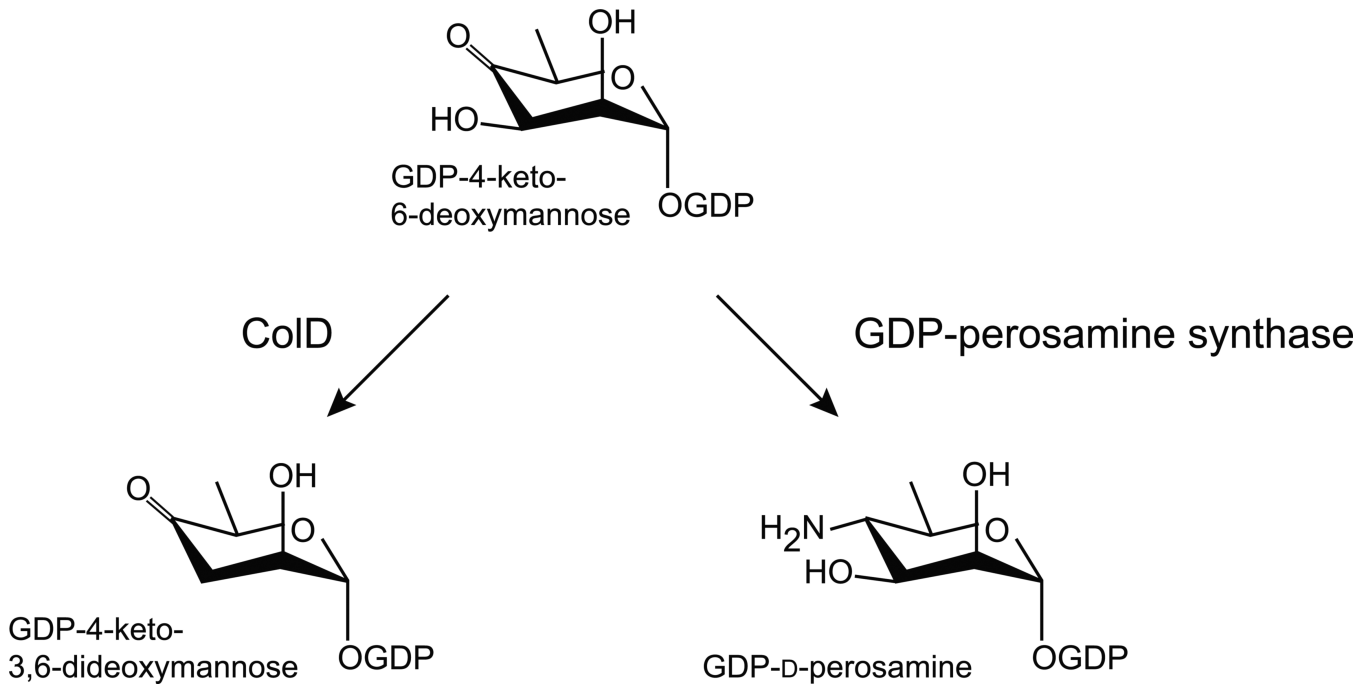

Figure 5.
Reactions catalyzed by ColD and GDP-perosamine synthase.