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Current Topics

Structural and Mechanistic Basis of Bacterial Sugar Nucleotide-Modifying Enzymes†

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ABSTRACT: Recently, carbohydrates have come to the fore because of their central role in many biological processes. One area of current interest concerns the enzymatic modification of sugar nucleotides, in relation to both secondary metabolite glycosylation and the formation of complex cell surface-associated glycoconjugates. Bacteria, in particular, have proven to be a rich field in which to study these transformations, because they are often unique to specific classes of organisms. This has led to the realization that such microbial biosynthetic pathways might be exploited in the generation of novel antibiotics, or indeed serve as targets for such compounds. This work illustrates the interplay between protein structure determination, chemistry, and molecular biology in providing insight into the mechanism of such biochemical transformations.

Enzymes involved in sugar nucleotide modification represent an area of increasing research activity. In microorganisms, they play a key role in the formation of building blocks for both secondary metabolite glycosylation (*1*) and for the synthesis of complex cell surface glycoconjugates, such as lipopolysaccharide (*2*) and components of microbial cell walls (*3*). Such processes may provide invaluable tools for enzymatic synthesis of novel antibiotics (*1*), or conversely serve as new targets for such compounds (*4*, *5*). While the mechanism of sugar nucleotide-modifying enzymes (*6*) and deoxysugar nucleotide biosynthesis have been reviewed recently (*6*, *7*), this work focuses on examples where a combination of protein structure determination, chemistry, and molecular biology has been used to provide details about the mechanism of such biochemical transformations.

Oxidation and/or Reduction of a Hydroxyl Function to a Keto Function

Oxidation of a hydroxyl group to a ketone is a common strategy in the biosynthesis of sugars. It is used to activate the protons α to the ketone group, for amination and for direct epimerization. Some enzymes both generate the keto function and carry out further reactions themselves. dTDP-D-glucose 4,6-dehydratase (EC 4.2.1.46) (*8*) and GDP-D-mannose 4,6-dehydratase (*9*) dehydrate across the pyranose C-5 and C-6 positions. GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase/4-reductase (GMER) epimerizes the C-3 and C-5 positions (α to the C-4 keto function) (*10*) before reducing the keto group to an alcohol. UDP-galactose epimerase (*11*, *12*) and ADP-L-glycero-D-mannoheptose 6-epimerase (*13*) oxidize and reduce the carbohydrate. All the enzymes share the short chain dehydrogenase superfamily fold and have

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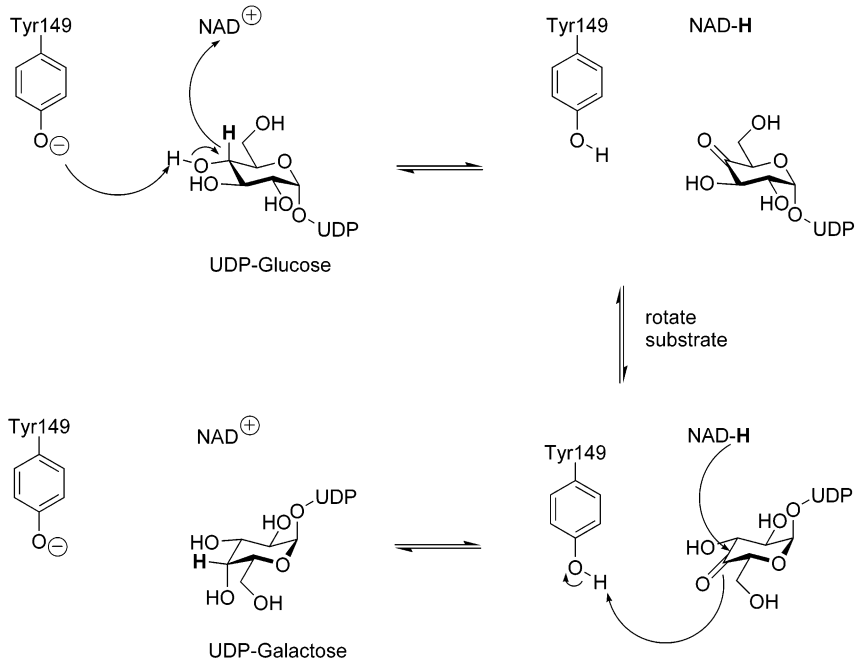

FIGURE 1: Reaction catalyzed by UDP-galactose 4-epimerase.

the highly conserved TyrXXXLys couple (where X represents any residue) and a conserved Ser residue (also found to be Thr) (*14*). Clearly, the common requirement for hydride transfer chemistry dominates these proteins.

The most intensively studied protein of this class is UDP-galactose 4-epimerase, which oxidizes C-4 (hydride abstraction) and then reduces the resulting ketone from the opposite face of the sugar, effecting freely reversible conversion between a *gluco*- and *galacto*-configured pyranose ring. From a combination of site-directed mutagenesis, spectroscopy, and structural biology, much of the detail of hydride transfer has recently been deduced. The Tyr149 residue has its pK_a adjusted by the conserved Lys153 such that it can abstract or donate a proton to the carbohydrate (*15*−*17*). Binding of sugar nucleotide induces a protein conformational change (*18*) which potentiates the nicotinamide, facilitating hydride abstraction from the sugar. The precise detail of how active site residues effect catalysis has only recently become clear. The Ser124 residue had been thought to relay the proton from the sugar to Tyr149 (*19*). This arose because in crystal structures of the *Escherichia coli* UDP-galactose 4-epimerase with a substrate, the Tyr residue was too far from the hydroxyl group to bring about direct deprotonation (*17*, *19*, *20*). However, the structures of substrate complexes of SQD1 (involved in the biosynthesis of UDP-sulfoquinovose) (*21*), human UDP-galactose 4-epimerase (*22*), and dTDP-D-glucose 4,6-dehydratase (*23*) have shown that in fact the Tyr residue can act directly as a base (Figure 1). This suggests that the position of the Tyr residue in substrate complex structures with the *E. coli* enzyme was an artifact. The observation that tyrosine acts as the base in the short chain dehydrogenase superfamily directly raises questions concerning the need for a conserved Ser/Thr residue. This amino acid is often found to be involved in short hydrogen bonds (approximately 2.5 Å in length) with the substrate. Thoden et al. have recently described this as a low-barrier hydrogen bond (LBHB) (*24*), as found in human UDP-galactose 4-epimerase (*22*). An alternative proposal, based on studies on RmlB (*23*) and dTDP-6-deoxy-L-*lyxo*-4-hexulose reductase (RmlD) (*25*), suggests that the short chain dehydrogenase mechanism proceeds through a LBHB between the catalytic tyrosine and the substrate. To form a LBHB, the pK_a values of the participating groups need to be matched; otherwise, the proton cannot be shared. The pK_a of the active site tyrosine in SDR enzymes is less than 7, and that of the substrate is either −7 or 16, depending on whether it is a carbonyl group or a hydroxyl group. Consequently, on first inspection, transferring a proton from or to the substrate with tyrosine acting as an acid or base, respectively, is problematic. However, it is the pK_a value of the transition state that will determine the efficiency of LBHB generation and therefore catalysis. This pK_a value can be expected to lie between the two extremes of a carbonyl and a hydroxyl group. Any hydrogen bond to the intermediate will modify this pK_a value of the transition state; the serine/threonine residue has been proposed to fine-tune the pK_a of the substrate to match that of the tyrosinate, thus facilitating catalysis.

Dehydration

The dTDP-D-glucose 4,6-dehydratase (RmlB) mechanism, originally proposed in 1963, involves a three-step oxidation−dehydration−reduction process (*26*). Very elegant labeling and biochemical studies subsequently proved that these individual chemical steps occur sequentially during turnover; these studies have been reviewed elsewhere (*27*, *28*). Essentially, the first step in the process involves C-4 oxidation of the hydroxyl group to a ketone (discussed above), activating the C-5 proton for abstraction. The C-6 hydroxyl group is protonated and leaves in a concerted elimination to form a glucosene intermediate, the existence of which has now been shown by mass spectrometry (*29*). The glucosene is subsequently reduced by addition of hydride at C-6 and protonation at C-5. The dehydratase mechanism is shared by GDP-D-mannose 4,6-dehydratase (GMD), the first carbohydrate dehydratase to be structurally characterized

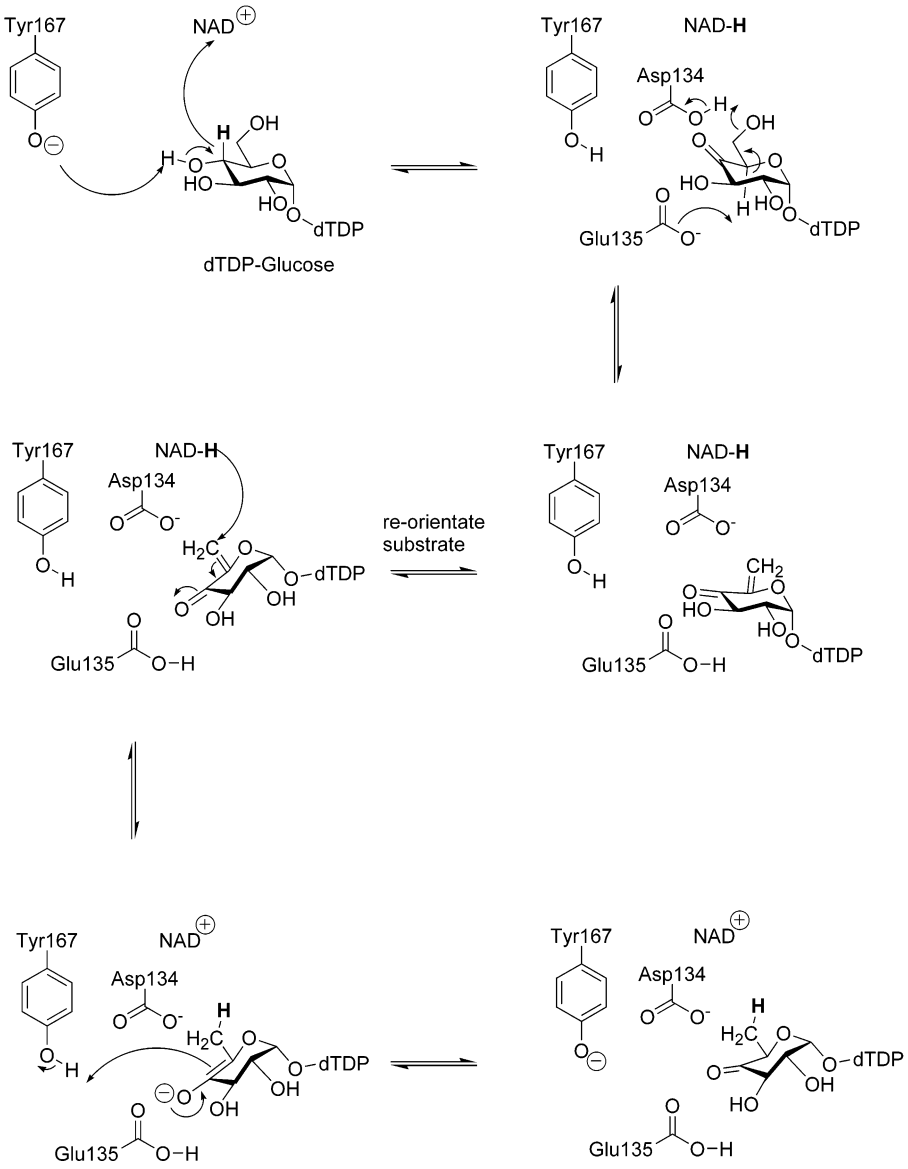

FIGURE 2: Reaction catalyzed by dTDP-glucose 4,6-dehydratase.

(*30*). This structure and site-directed mutagenesis data on GMD (*30*, *31*) and RmlB (*23*, *32*, *33*) identified a key conserved glutamic acid (residue 135 in the *Salmonella typhimurium* sequence) as the base which abstracts the C-5 proton. The GMD structure did not have a substrate bound, and the nature of the acidic residue that protonates the C-6 hydroxyl group was not identified. This residue was identified by two effectively simultaneous studies. dTDP 6-fluorodeoxyglucose initially undergoes the same reaction with RmlB as the normal substrate, the only difference being that, unlike hydroxide, fluoride is a good leaving group and does not require protonation. Using this substrate in an elegant series of experiments showed that a conserved Asp residue (adjacent to the conserved Glu) was required for processing of the native substrate but not its fluorinated analogue (*33*). The obvious conclusion was that Asp134 served as the required general acid. The structure of both *S. typhimurium* and *Streptococcus suis* RmlB with substrate and analogue (dTDP-xylose) confirmed the role of the conserved Glu135 and showed that the conserved Asp134 residue is hydrogen bonded to O-6 of the substrate (*23*). Once again, the only interpretation of the data is that Asp134 supplies a proton to O-6. The structure showed that the Glu and Asp side chains, although not in contact, could access conformers that were able to directly interact, accounting for the altered pK_a of the Asp residue. This study focused attention on a problem that had been neglected, namely, hydride transfer to C-6. From a survey of a database of structures, it had been reported that hydride transfer follows a fairly well-defined trajectory (*34*). This is, of course, reasonable as judged from cursory consideration of the theoretical constraints of orbital alignment. The RmlB complex structures showed that while C-4 was in an optimal position for hydride abstraction, C-6 was not in a position to accept hydride (*23*) in the final step of the reaction. Elimination of water from C-6 transforms a covalent bond into a noncovalent bond, creating severe steric strain. It was proposed that, since the water remains bound to the protein, sugar rotation around the glycoside linkage relieves the nonbonded contact (*23*) (Figure 2). The rotation creates no steric clash but moves C-6 to an appropriate location for accepting hydride. In this position, the source of the proton could be either the Tyr167 (which was protonated during hydride abstraction) or the conserved Glu135 that is protonated during the water elimination (*23*).

Epimerization α to a Keto Function

RmlC catalyzes the conversion of dTDP-6-deoxy-D-*xylo*-4-hexulose into dTDP-6-deoxy-L-*lyxo*-hexulose (epimerization of C-3 and C-5 of the keto sugar). RmlC is thought to be the rate-limiting enzyme in normal dTDP-L-rhamnose biosynthesis (*35*, *36*). Unlike the well-known galactose/glucose epimerase (*18*), RmlC has no cofactor and does not operate by a crypto-redox process. It represents a growing list of enzymes that invert the chirality of carbon atoms α to a keto function (*37*), although molecular details of its mechanism have remained obscure. Although it is known from early experiments with D_2O that acid−base catalysis is important (*38*), the key residues and the nature of reaction intermediates are unknown. Structures of the RmlC enzyme from *Salmonella enterica* (*39*), *Methanobacterium thermoautotrophicum* (*40*), and *St. suis* (*41*) have been reported. These enzymes belong to the cupin superfamily (*42*), which includes a diverse range of protein functions within a *β*-sandwich-type fold. Study of the *St. suis* enzyme in complex with dTDP-glucose and dTDP-xylose provided experimental evidence for the location of the active site. This study identified the principal catalytic residues, on the basis of the structure, but noted that some rearrangement would be necessary for catalysis (*41*). The *St. suis* study proved to be particularly important because, unlike all other sequences, the streptococcal enzyme has only one, not two, conserved His-Asp diads (His76-Asp180) (*41*). In previous mechanistic proposals, two diads were proposed to act as bases for C-3 and C-5 epimerization (*39*, *40*). Clearly, this had to be revised; the *St. suis* structures showed that the His63-Asp118 diad is almost certainly a dual-purpose base, being involved in both C-3 and C-5 epimerization reactions (*41*). The study suggested that Tyr140 was responsible for donating a proton to C-5, and water for donating a proton to C-3 (*41*) (Figure 3).

The enzyme GMER has already been mentioned in the context of the oxidation−reduction chemistry as a member of the short chain dehydrogenase superfamily, but it also epimerizes the C-3 and C-5 positions of the GDP-4-keto-6-deoxy-D-mannose (*10*). The two epimerized stereocenters lie α to a keto group, directly analogous to the RmlC reaction discussed earlier. In the GDP-4-keto-6-deoxy-D-mannose binding model proposed by Somers et al. (*43*), the His179 side chain of GMER is in a suitable position to fulfill the role of a general acid or base during catalysis. On the basis of structural studies and mutagenesis (*44*), a mechanism for GMER has been proposed. His179 is thought to act as a base, abstracting a proton from C-3, while Tyr136 transiently protonates the C-4 oxygen and thus stabilizes the enediol/enolate intermediate. This is followed by a reprotonation from the opposite face of the sugar ring, either by a water molecule or by a GMER residue that has not yet been identified. Finally, the C-3 epimerization would be completed by the deprotonation of the C-4 keto group. The same residues could then proceed to epimerize at the C-5 position following a realignment of the sugar inside the catalytic site to put the C-5 proton in an optimal position for abstraction. However, as judged from the superimposition of the substrate complexes of RmlB (which is homologous to GMER), Cys109 in GMER is in the same position as RmlB Glu135, the residue that abstracts the proton from C-5 in the RmlB reaction. It therefore seems likely that the Cys109 residue is involved in the GMER epimerization process, which is supported by mutagenesis studies (*44*).

Cleavage of the Pyranose Ring

Hexoses can exist in both the five-membered (furanose) and six-membered ring forms (pyranose). In solution, galactose freely interconverts between galactofuranose (Gal*f*) and galactopyranose (Gal*p)* by a ring opening reaction that goes through the open chain form in which O-1 is an aldehyde rather than a hydroxyl group. The five-membered ring form, Gal*f*, is a constituent of a polymer linking the peptidoglycan and mycolic acid layer of the cell wall of *Mycobacterium tuberculosis* (*45*). The sugar is also found in the O-antigens of several Gram-negative bacteria, including *Klebsiella pneumoniae* (*46*), a common noscomial pathogen, and *E. coli* (*47*), as well as in cell surface glycoconjugates of numerous other microorganisms (*3*, *48*). The synthesis of galactofuranose is essential for the viability of *M. tuberculosis*; however, it is not found in humans, and its biosynthesis is therefore a potential therapeutic target (*49*). Gal*f* is derived from UDP-Gal*f*, which is in turn synthesized from UDP-galactopyranose (UDP-Gal*p*) (*50*). There is no known naturally occurring nucleotidyl transfer reaction that utilizes the Gal*f* sugar. The interconversion of UDP-Gal*p* and UDP-Gal*f* is catalyzed by the flavoprotein UDP-galactopyranose mutase (mutase). The uncatalyzed reaction has no detectable rate, since unlike Gal*p*, UDP-Gal*p* is unable to open rings.

Mutase from *K. pneumonia* is reported to require the presence of NADH or NADPH for its activity, despite the fact that there is no net transfer of electrons (*50*−*52*). Further studies on the enzyme demonstrated that the transfer of hydride from the R face of NADH is rate-limiting under aerobic conditions (*51*). Subsequent reports showed that reducing conditions (e.g., reaction with dithionite) and anaerobiosis activate the enzyme, while oxidative conditions [e.g., reaction with $K_3Fe(CN)_6$] inactivate it (*52*). Other studies suggest that the oxidized enzyme retains some activity (*54*); flavoproteins can undergo photoreduction, which could lead to such inconsistencies. Positional isotope exchange experiments demonstrated that there is cleavage of the galactose C-1−O-1 bond during the mutase reaction (*54*). Fluorodeoxy analogues of UDP-galactose (substituted at the 2 and 3 positions) are substrates for the enzyme, although as expected their k_{cat} values are massively reduced (71000- and 1000-fold, respectively, compared to that of UDP-Gal*p*). Neither compound eliminated HF on treatment with mutase (*55*), ruling out a mechanism that involves oxidation at C-2 or C-3 of the galactose ring. A subsequent study on the *E. coli* UDP-galactopyranose mutase confirmed these results, although covalent intermediates were apparently formed between the enzyme and substrate (*53*). An elegant mechanism in which O-4 of galactose acts as a nucleophile to displace the UDP group, forming a bicyclic sugar and UDP, was proposed (*54*, *55*). This mechanism does not require transfer of electrons from reduced flavin, which might act to promote the reaction by stabilizing the oxocarbonium ion-like transition state. The crypto-redox behavior of flavoenzymes is well-known (*58*). A recent study has shown that substrate binding to mutase stabilizes the blue (neutral) semiquinone form of the flavin and the fully reduced flavin

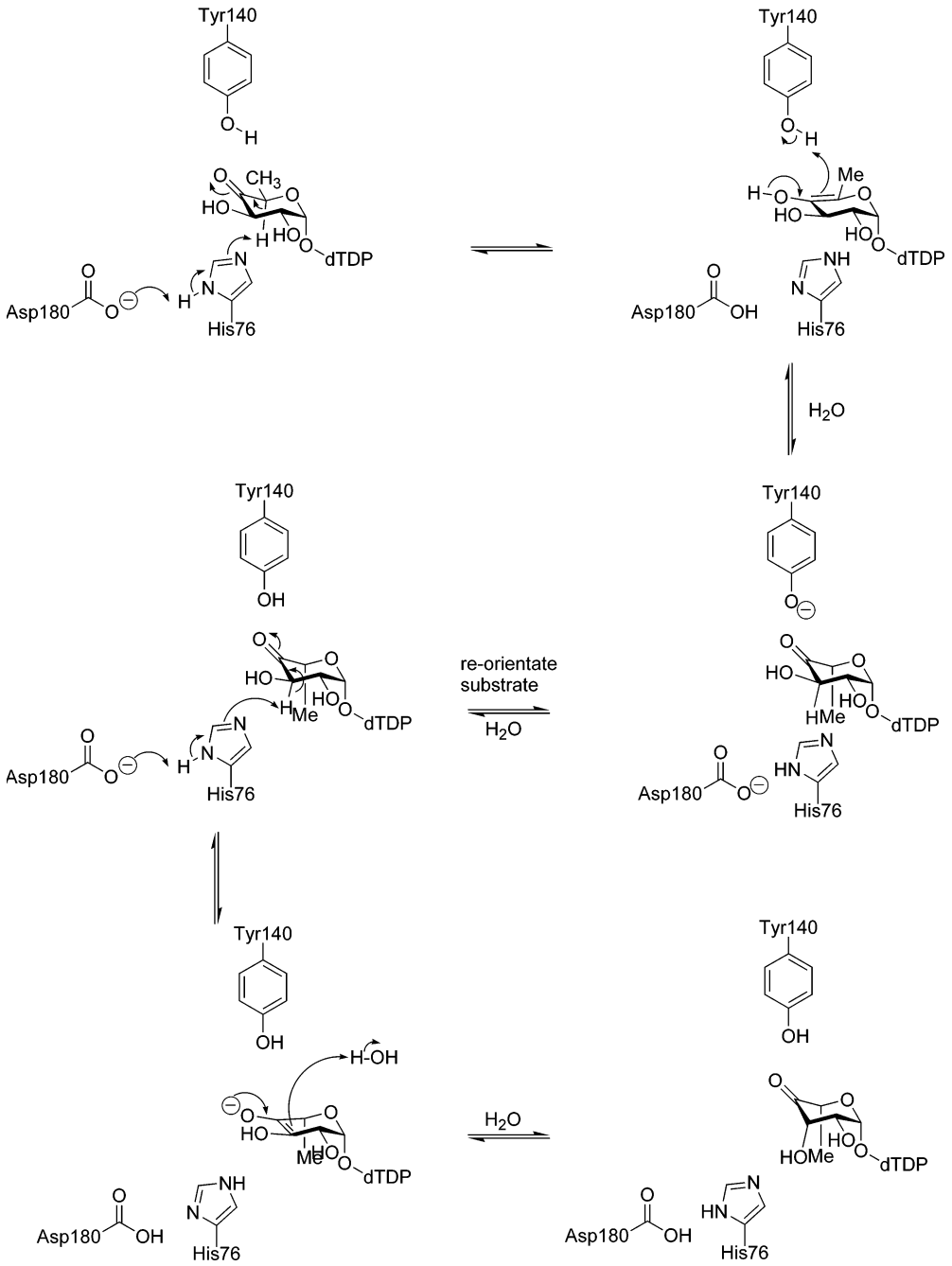

FIGURE 3: Reaction catalyzed by dTDP-6-deoxy-α-D-*xylo*-4-hexulose 3,5-epimerase.

is found to be the anionic hydroquinone (*57*). The anionic hydroquinone is poised to transfer a single electron hydroquinone to semiquinone in a rapid manner and uncouples electron transfer by the isoalloxazine ring from proton transfer. This study proposed a radical-based mechanism, but the question of whether the enzyme proceeds by transient electron transfer remains (Figure 4).

The structure of the *Klebsiella* mutase revealed a three-domain enzyme (*52*). The largest domain (N-terminal) binds the FAD and is reminiscent of glutathione reductase. A second hinge domain connects the third α-helical domain to the N-terminal domain. Between the α-helical domain and the N-terminal domain is a large cleft in which the isoalloxazine ring is exposed to the solvent (*52*). Modeling studies have shown that the substrate could be bound such that the sugar ring is adjacent to the flavin (*52*). Unfortunately, in the absence of substrate, structures available to date do not settle the debate about the mechanism of the enzyme.

trans-Amination of a Keto Sugar

Gram-negative bacteria have developed an assortment of defense mechanisms to combat the effect of antimicrobial peptides produced by the infected host. One such mechanism involves the covalent modification of the microbe's lipid A with 4-amino-4-deoxy-L-arabinose (*58*), which reduces the charge on the LPS, thus decreasing its potential to interact with cationic antimicrobial peptides (*59*). ArnB from *S. typhimurium* catalyzes amino group transfer from glutamate to the C-4″ position of uridine 5′-*β*-L-*threo*-pentopyranosyl-4″-ulose diphosphate, giving uridine 5′-*β*-L-4-amino-4-deoxyarabinopyranose (L-Ara4N) diphosphate. Orthologs of ArnB from three organisms known to modify lipid A with

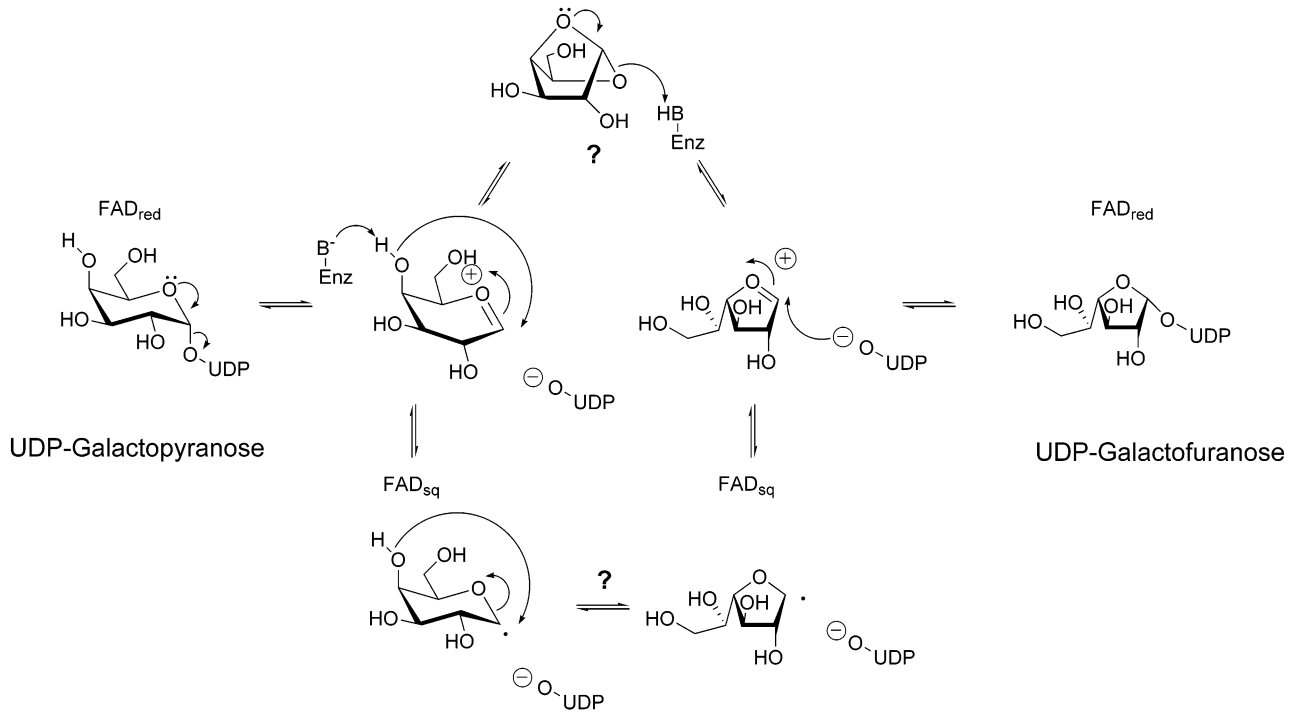

FIGURE 4: Reaction catalyzed by UDP-galactose mutase.

L-Ara4N (*E. coli* K-12, *Yersinia pestis*, and *Pseudomonas aeruginosa*) are highly conserved (*60*).

ArnB is the only sugar nucleotide *trans*-aminase to be structurally characterized to date (*60*), although the RfbE *trans*-aminase from *Vibrio chlolerae* O1 has also received attention in relation to the enzymatic synthesis of GDP-perosamine (*61*). ArnB possesses a type 1 aminotransferase fold, typified by aspartate aminotransferase (*62*); its closest structural homologue (rmsd of 2.3 Å for 351 equivalent α-carbons; 28% identical) is 3-amino-5-hydroxybenzoic acid synthase from *Amycolatopsis mediterranei* (*63*). The ArnB protein is homodimeric [RfbE is tetrameric (*61*)] with an extensive dimer interface that buries more than 2500 Å^2 of solvent accessible surface per monomer. The large N-terminal cofactor binding domain consists of a central seven-stranded, mixed polarity β-sheet surrounded by eight α-helices. The smaller C-terminal domain consists of a three-stranded β-sheet and five α-helices (*60*).

The active sites of type 1 aminotransferases are composed of residues from both halves of the dimer, with the pyridoxal phosphate cofactor interacting with several conserved residues. In ArnB, the side chain of Trp89 makes direct contact with the pyridine ring of the cofactor and is oriented ~60° from parallel ring stacking, apparently dictating the approach of the substrate to the C-4′ position of the cofactor. The peptide bond between His329 and Phe330 adopts a *cis* conformation, which is unusual for a non-prolyl amide bond. When this arrangement exists, it is generally found in a functionally important region of a peptide chain. The role of the non-prolyl *cis* amide bond in ArnB is currently unclear, although the His-Phe combination is strictly conserved among bacteria known to modify lipid A with L-Ara4N (*60*).

PLP-dependent *trans*-aminases catalyze the transfer of an amino group between an amine and a ketone, in this case glutamate and a keto sugar nucleotide, giving an amino sugar nucleotide and α-ketoglutarate. Transfer occurs through two half-reactions, where the aminated form of the cofactor, pyridoxamine 5′-phosphate (PMP), results from the first half-reaction. The absorbance spectrum of ArnB suggests that the cofactor is largely in the aminated PMP form in the purified protein. This was confirmed from the X-ray structure of the protein−cofactor complex, which showed electron density projecting from C-4′ of the cofactor; Lys188 was found in a retracted position, inconsistent with an internal aldimine structure. In the absence of a keto sugar nucleotide substrate for accepting amine, ArnB exists in a stable noncovalent E·PMP intermediate state. Addition of α-ketoglutarate, the product of the first half-reaction, to E·PMP ArnB results in generation of an internal aldimine between Lys188 and the pyridoxal 5′-phosphate (PLP) form of the cofactor (E-PLP) as is evident from spectroscopic changes (increased absorption at 430 nm and decreased absorption at 340 nm) and from crystallographic analysis (*60*) (Figure 5).

Although ArnB turnover in the presence of its keto sugar nucleotide substrate has not yet been investigated, all available information is consistent with an archetypal type 1 aminotransferase mechanism (*62*). A key issue that remains to be resolved is why ArnB gives rise to an axial amine whereas RfbE, for instance, produces an equatorial amine. It is to be expected that structural analysis of reaction intermediates involved in both processes will provide insight into the partitioning of the product into one stereoisomer or the other. It is interesting to note that imine intermediates analogous to those employed by ArnB have also been implicated in C−O bond cleavage during the biosynthesis of deoxysugar nucleotides (*64*).

Epimerization Adjacent to the Anomeric Center

UDP-*N*-acetylglucosamine 2-epimerase catalyzes the reversible interconversion of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylmannosamine (UDP-ManNAc). Early work plausibly led to the proposal that this enzyme

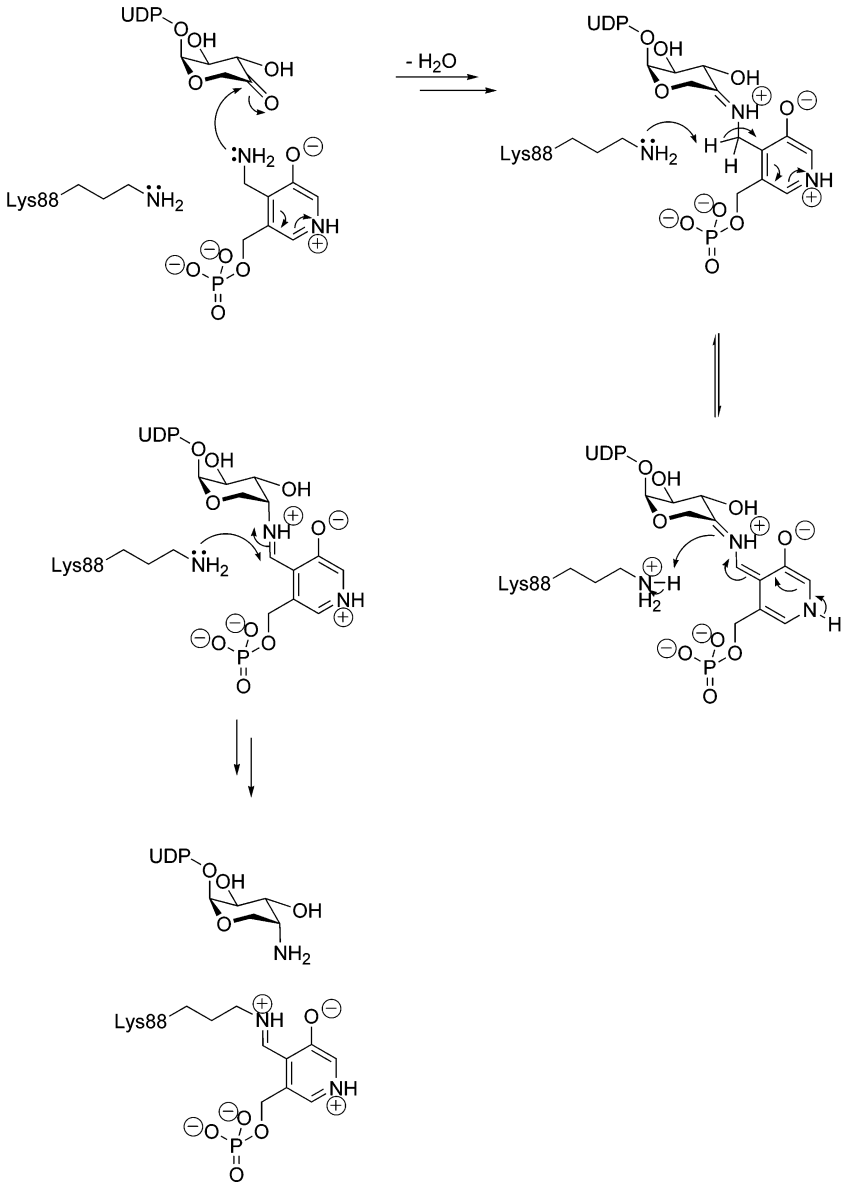

FIGURE 5: Second half-reaction catalyzed by UDP-5-β-L-pentopyranosyl-4-ulose 4-*trans*-aminase.

catalyzes C-3 oxidation of UDP-GlcNAc by a tightly bound NAD^+ cofactor, and so acidifies the proton at C-2 to effect epimerization. Subsequent reduction of the C-3 ketone then regenerates the NAD^+ cofactor and in so doing produces UDP-ManNAc (*65*). However, later work showed that the enzyme does not require exogenous cofactors (*66*), which was confirmed in more detailed mechanistic studies by Tanner and co-workers on the *E. coli* epimerase (RffE) (*67, 68*). These studies demonstrated a mechanism based on an elimination or re-addition that takes place through the cleavage of the anomeric C−O bond, with 2-acetamidoglucal and UDP as intermediates.

The structure of an RffE−UDP complex was determined to 2.5 Å by X-ray crystallography (*69*). Although the enzyme was crystallized in the presence of UDP-GlcNAc, in the final structure only the UDP moiety was clearly visible in both dimer subunits. This might be due either to UDP-GlcNAc hydrolysis, liberating UDP during the crystallization process, or to turnover of substrate with loss of the glycal intermediate. The structure shows the protein is dimeric, with notable conformational differences between the two subunits. Such differences might indicate a role for conformational change in substrate binding, or perhaps in the allosteric regulation of the enzyme [RffE is allosterically regulated by its substrate, UDP-GlcNAc (*66*)].

In the UDP complex, one subunit is in a "closed" conformation while the other one is "open". In the closed conformation, His213 is hydrogen bonded to the oxygen of the β-phosphate of UDP, whereas this interaction is absent in the open conformation. It seems likely that His213 acts as a general acid catalyst, assisting departure of UDP during the elimination step (*71*) (Figure 6). In the closed conformation of RffE, further stabilization of the bound UDP results from π-stacking of the uracil ring between the phenyl group of Phe276 and the guanidine group of Arg10 (*69*). While the Phe residue is conserved in some putative UDP-GlcNAc 2′-epimerases, such as *Staphylococcus aureus* MnaA and Cap5P, it is altered in *Sta. aureus* Cap5G (tryptophan), *Bordetella pertussis* WlbD, and *P. aeruginosa* WbpI (methionine). The consequences of these differences are unclear at present. Of the other residues identified as being important in either UDP binding (Arg10, Ser290, and Glu296) or the

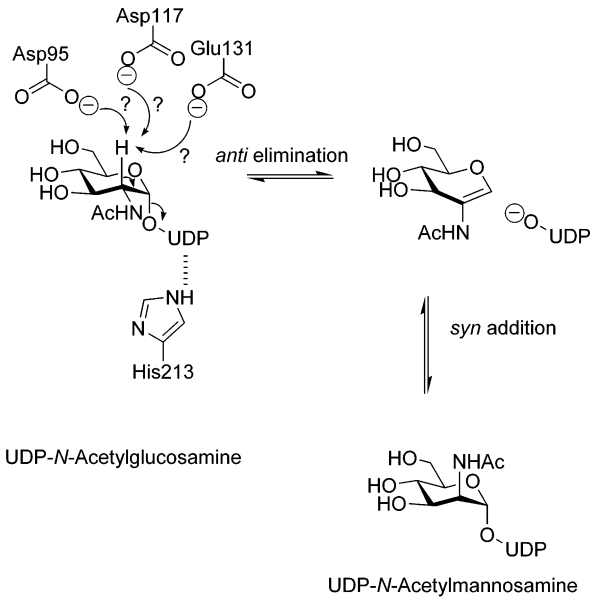

FIGURE 6: Reaction catalyzed by UDP-*N*-acetylglucosamine 2-epimerase.

possible sugar recognition site (Lys15, Asp95, Glu117, Glu131, Arg135, and His213), only Lys15 is not absolutely conserved in all putative 2′-epimerase (in three of 33 sequences, Lys is replaced with Arg). Preliminary inspection of the related *B. pertussis* WlbD structure (*70*) reveals that appropriate amino acid residues are correctly placed to carry out RffE-like chemistry. Superposition of 120 α-carbon atoms from WlbD residues conserved in RffE give an rmsd of only 1.03 Å, but there are differences in the structure that might have implications for substrate specificity.

In the case of RffE (*69*) and the distantly related mammalian UDP-GlcNAc epimerase/ManNAc kinase (*71*), the ability to catalyze both *syn* and *anti* elimination reactions that involve removal of nonacidic protons still must be examined. The RffE structure is similar to those of enzymes that, based on poor sequence identity, would initially appear to be unrelated. However, the ability to effect acid−base catalysis seems to have led UDP-GlcNAc 2′-epimerase, glycogen phosphorylase, and a DNA-modifying β-glucosyltransferase to share the structural features that are necessary to bring about such chemistry (*6*, *69*). Protein fold conservation in the glycotransferase family of enzymes, in particular, is striking (*72*). How generic structural motifs and catalytic machinery are able to conduct such disparate chemistry remains to be seen.

Transfer of Enolpyruvate to a Sugar Alcohol

UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) catalyzes the first committed step in the biosynthesis of bacterial cell wall peptidoglycan and as such is a potential drug target (*4*); in fact, MurA is known to be inhibited by the antibiotic fosfomycin. Structures of the free MurA enzyme (*73*, *74*), complexed with the substrate and covalently modified with fosfomycin, are available (*75*). The MurA reaction involves the transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 3′-hydroxyl group of UDP-*N*-acetylglucosamine (UDP-GlcNAc). The enzyme shares a unique three-dimensional topology and overall reaction mechanism with 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS). The latter enzyme is essential for the biosynthesis of aromatic amino acids in plants and bacteria, representing a prime target for new herbicides and antibiotics (*75*).

Both MurA and EPSP synthase follow an induced-fit mechanism (*76*). In the substrate-free form, the unliganded protein exhibits an open conformation. Once the substrates bind, the protein forms a much more tightly packed so-called closed form. In the closed form, the substrates are properly positioned for catalysis. Using limited tryptic digestion of MurA, it was shown that formation of the closed conformation of MurA was accompanied by a marked increase in stability toward proteolytic degradation. Once the substrate binds, the active site forms in the interdomain cleft, involving movements of the two domains of the protein and a reorientation of the Pro112−Pro121 loop, reducing the solvent accessibility of Cys115. This key residue has been proposed to act as a general acid and base in the MurA reaction and also serves as the primary site of action of the antibiotic fosfomycin (*77*). NMR experiments have shown that the Cys115 nucleophile may form a covalent *O*-phosphothioketal species with PEP, although this species has been shown to be nonessential for catalysis and may be off-pathway. The three-dimensional structure of MurA complexed with UDP-GlcNAc shows Asp305 close to the 3′-hydroxyl group of UDP-GlcNAc, suggesting that it may act as an acid−base catalyst (*78*). In addition to Asp305, Asn23 also interacts with the substrate 3′-hydroxyl group, although its role was initially unclear. Using site-directed mutagenesis, Asp305 mutants were shown to possess reduced affinity for UDP-GlcNAc, leading to Cys115's lower propensity to form the *O*-phosphothioketal with PEP and the thioether with the fosfomycin. These findings emphasize the dual role of Asp305 as a general base and an essential binding partner

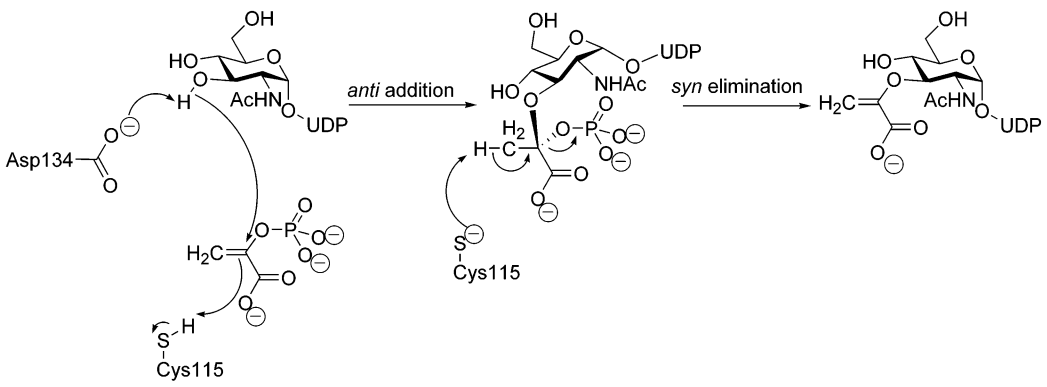

FIGURE 7: Reaction catalyzed by UDP-*N*-acetylglucosamine enolpyruvyltransferase.

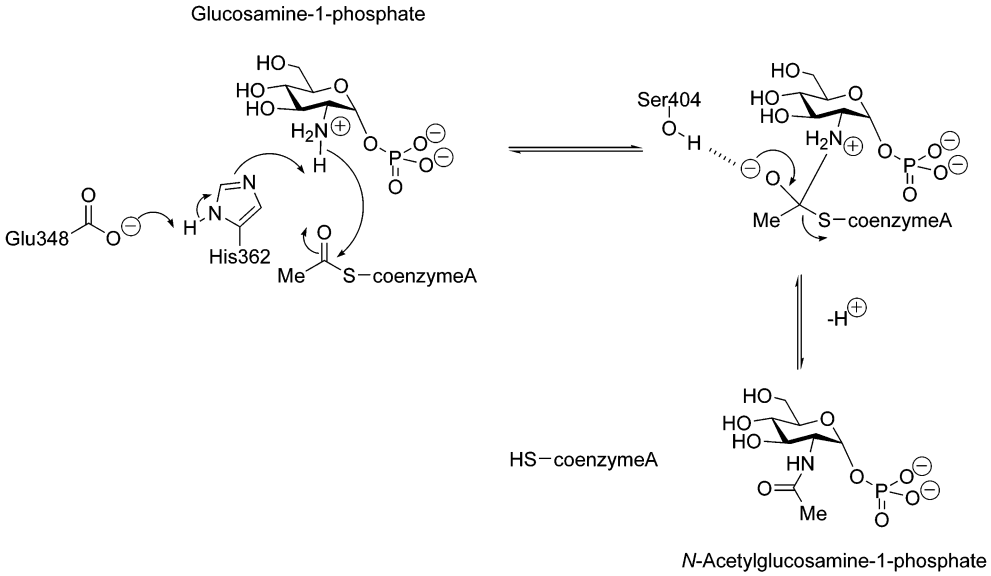

FIGURE 8: First half-reaction catalyzed by *N*-acetylglucosamine-1-phosphate uridyltransferase.

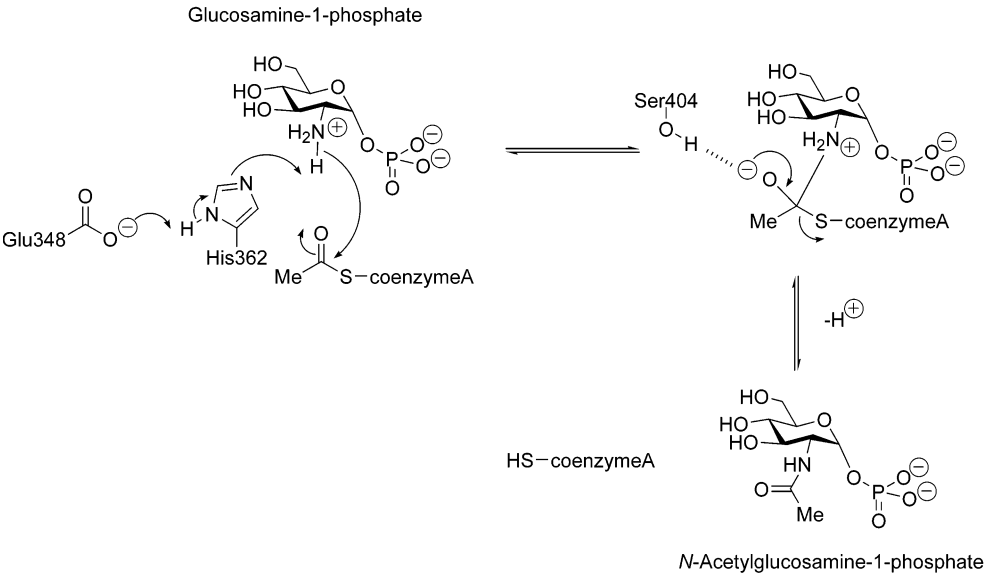

FIGURE 9: Reaction catalyzed by UDP-glucose dehydrogenase.

for UDP-GlcNAc (Figure 7). Similarly, Asn23 mutants exhibited a much lower catalytic activity, although binding of UDP-GlcNAc was not significantly affected, indicating that this residue is mainly involved in transition state stabilization.

Overall, MurA catalyzes pyruvyl transfer by means of a noncovalently bound tetrahedral phospholactoyl-UDP-*N*-acetylglucosamine intermediate, the structure and stereochemistry of which have been proven by crystallographic studies on the trifluoromethyl analogue of PEP with the MurA Cys115Ala mutant. These studies essentially provide a snapshot along the reaction pathway (*79*).

Acyl Transfer to Carbohydrates

Acyl transfer is often carried out differently in bacteria and eukaryotes, with some bacterial enzymes being defined by their left-handed β-helical fold. This was first seen in the structure of UDP-*N*-acetylglucosamine acyltransferase, LpxA, which transfers (*R*)-3-hydroxymyristate from its acyl carrier protein to the 3-OH of UDP-*N*-acetylglucosamine (*80*). This class of β-helical proteins typically shares a similar amino acid sequence in the form of an imperfect tandem-repeated hexapeptide sequence based on [LIV]-[GAED]-X_2-[STAV]-X. The most detailed structural studies that have been reported are for the bifunctional *N*-acetylglucosamine-1-phosphate uridyltransferase (GlmU), which converts glucosamine 1-phosphate and acetylcoenzyme A to *N*-acetylglucosamine 1-phosphate, and subsequently catalyzes UTP-dependent conversion of the sugar 1-phosphate to the sugar nucleotide (*81*). The protein contains two domains, one possessing a typical dinucleotide-binding Rossman fold for nucleotidyl transfer and the other a left-handed β-helical structure for effecting acyl transfer (*81*). The left-handed β-helical folds form a trimer with a 3-fold rotational axis parallel to the β-helix (perpendicular to the individual β-strands). Further studies mapped out the substrate binding site at the nucleotidyl transfer domain (*82*−*84*) and reported the full structure of the acyltransferase domain but did not

identify the key residues for catalysis. From sequence analysis and modeling studies, the active site was located at each of the interfaces between the monomers. The structure of GlmU from *Streptococcus pneumoniae* (*85*) with acetylcoenzyme A bound, together with structures of the related tetrahydrodipicolinate *N*-succinyltransferase (*86*) and xenobiotic acetyltransferase (*87*), provided structural insights into the acyl transfer mechanism. Conserved residues His362 and Glu348 interact to increase the basicity of His362, which in turn serves as a general base for deprotonation and activation of the amino group of the sugar 1-phosphate substrate (*85*−*87*) (Figure 8). The amine is then able to attack the acetate carbonyl group of acetylcoenzyme A, forming a tetrahedral intermediate that is thought to be stabilized by Ser404 (*85, 86*), and which subsequently collapses to complete acetyl group transfer to the sugar. The key point about the acyl transfer mechanism is that it involves a direct acyl group transfer from coenzyme A to substrate; structural data rule out formation of a covalent protein intermediate.

Oxidation of a Primary Alcohol

UDP-glucose dehydrogenase (UDP-D-glucose:NAD-6-oxidoreductase) catalyzes a two-fold (four-electron) NAD^+-linked oxidation of UDP-glucose to UDP-glucuronic acid. The structure of the enzyme has now been determined from *Streptococcus pyogenes* (*88*). The first step in the reaction is the transfer of the *pro-R* hydride from C-6 to NAD^+ and deprotonation of O-6 (*89*), very similar to the C-4 oxidation discussed earlier but in this case generating an aldehyde. The identity of the base required to deprotonate O-6 is still ambiguous; Lys204 seems to be the only likely candidate, reminiscent of HMG-CoA reductase (*90*). However, a water molecule observed in the structure bound to a conserved Asp residue may function as a proton relay in the abstraction of the proton. Protein chemistry studies have shown that the aldehyde once formed undergoes nucleophilic attack by the sulfur of Cys260 to form the first tetrahedral intermediate (*91*, *92*). The structure suggests that the intermediate is stabilized by Asn208 and the general base Lys204, or water. This first intermediate collapses by transfer of a second hydride to a fresh molecule of NAD^+ to form a covalent thioester (Figure 9). Support for such a thioester intermediate comes from work with a Cys260Ser mutant, which forms a covalently bound ester intermediate that turns over slowly and hence accumulates (*93*). The thioester is finally hydrolyzed to liberate the free carboxylic acid and regenerate the protein thiol.

Summary

The combined use of protein structure determination, chemistry, and molecular biology has led to significant developments in our understanding of bacterial sugar nucleotide processing. Further challenges are presented, for instance, by recent observations suggesting that the *P. aeruginosa* WbjB protein (and likewise *Sta. aureus* Cap5E) catalyzes not only 4,6-dehydration of UDP-*N*-acetylglucosamine but also 3- and 5-epimerization (*94*). Clearly, there is much to be done before we are in a position to predict with confidence the substrate specificity of, or even the chemical transformation catalyzed by, sugar nucleotide-modifying enzymes.

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