Novel Enzymatic Mechanisms in Carbohydrate Metabolism

Xuemei He, Gautam Agnihotri, and Hung-wen Liu*

Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455, Division of Medicinal Chemistry, College of Pharmacy, and Department of Chemistry and Biochemistry, University of Texas, Austin, Texas 78712

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I. Introduction

Carbohydrates form the most abundant group of natural products and are found in all classes of living organisms.[1−8] They serve as a direct link between the energy of the sun and the metabolic energy that is required to sustain life. In organisms capable of photosynthesis, solar energy is harvested to drive reactions in which glucose is synthesized from carbon dioxide and water. The energy stored in this "carbon fixation" process then gradually moves upward into the food chain. The living organisms that partake the products of photosynthesis obtain useful energy by oxidizing the carbohydrates back into carbon dioxide and water through the processes of glycolysis and respiration. The carbohydrates that are most frequently used as metabolic vehicles are glucose, fructose, sucrose, lactose, and starch.

In addition to their pivotal role in metabolism, carbohydrates also play an important structural role in many organisms. Some examples of the latter type include cellulose, chitin, lipopolysaccharide, and the bacterial murein, all of which are derived from repeating sugar units which may have additional cross-linking components for rigidity. Furthermore, many bioactive secondary metabolites such as cardioglycosides, macrolide antibiotics, and aminoglycoside antibiotics rely on these sugar components for solubility and activity. In addition, carbohydrates are used as convenient precursors for the biosynthesis of other important building blocks such as aromatic amino acids. Carbohydrates also have many applications in industrial processes. For example, the food industry uses sucrose as a sweetening agent, a preservative, and a raw material for fermentation. Starch is used as a raw material for the manufacture of many goods. Cotton is still one of the most popular fabrics and an important raw material for the textile industry. Paper and other derivatives of cellulose are important for the manufacture of packaging materials and plastics.

* To whom correspondence should be addressed. Phone: 512-232-7811. Fax: 512-471-2746. E-mail: h.w.liu@mail.utexas.edu.

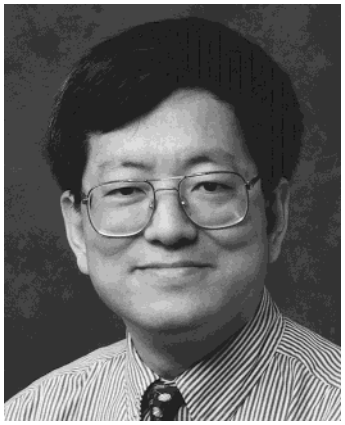
More recently, carbohydrates have been the focus of growing attention among biological molecules due to an increased recognition of their vital roles in many physiological processes.[9] The diversity of structures that is made possible by carbohydrate building blocks is greater than that of oligonucleotides or oligopeptides.[10] As an example, the number of all linear and branched isomers of a hexasaccharide is calculated to be over 1×10^{12}. Even a simple disaccharide composed of two glucose units can be represented by 11 different structures. In addition, nature has further enhanced the structural variations in carbohydrates by creating modified/unusual sugars such as deoxysugars, aminosugars, and branched-chain sugars.[11] Such modifications can greatly influence the hydrophobicity and the overall topology of the glycosylated macromolecules.

Both normal and unusual sugars play important roles in cellular adhesion and cell−cell recognition, fertilization, protein folding, neurobiology, xenotransplantation, and target recognition in the immune response. For example, glycosylation is a major posttranslational modification of membrane and secreted proteins in eukaryotic cells. Alterations in the structures of glycans are associated with a variety of diseases, including metastatic cancer.[4] Similarly, glycosphingolipids, which contain oligosaccharides, are present in the plasma membrane, Golgi bodies, endosomes, and neuronal and synaptic membranes.[12−14] Changes in the cell-surface expression profile of these compounds are associated with processes such as development, differentiation, organ regeneration, and oncogenic transformation. Another example of the biological importance of carbohydrates is the posttranslational attachment of glycosylated phosphatidylinositol (GPI) anchors to a wide variety of proteins found on the exterior surface of the eukaryotic plasma membrane. GPI anchors have also been implicated as a sorting and targeting signal that marks the modified proteins for transport to the cell surface. There is evidence for the involvement of GPI anchors in the activation of tyrosine kinases of the Src-kinase family. These molecules may also serve as second messengers as well. Therefore, it is clear that carbohydrates are not only critical for the storage and production of energy, but also intricately involved in many recognition and signaling events. Given their biological significance, it is not surprising that a large number of enzymes participate in the metabolism of carbohydrates.

In this review, we have attempted to highlight the reaction mechanisms of enzymes that are involved in the biosynthesis and/or transformations of monosaccharides, most of which are derived from common sugars. Alterations in the structure and properties of common sugars can be achieved by a variety of conversions. For example, C−O bond-cleavage reactions result in the formation of a wide range of mono-, di-, tri-, and even tetradeoxygenated sugars. Many intermediates in the deoxysugar biosynthetic pathways are also substrates of enzymes that catalyze C−N bond-formation reactions to generate aminosugars, C−C bond-formation reactions to produce branched-chain sugars, and epimerization reactions

Xuemei He grew up in the city of Guangzhou, P. R. China. She earned her Bachelor of Science degree in Organic Chemistry from Zhongshan University in 1990. She then spent one year as an Assistant Chemical Engineer in the Zhongshan Fine Chemical Group Companies, where she received three awards from the provincial and municipal governments for her technical innovations. In fall 1991, she entered the graduate program at the University of Minnesota, where she carried out her doctoral work on the biosynthesis of deoxysugars found in a variety of Gram-negative bacteria and antibiotics under the direction of Professor Hung-wen Liu. After finishing her Ph.D. degree in 1997, she continued her postdoctoral study with Professor Liu. She is currently a Research Associate at the University of Texas at Austin. Her research interests include the mechanistic studies of enzyme-catalyzed reactions and the molecular basis of biological processes, especially those involved in the development of cancers. Her spare time is occupied by lovely son Jonathan, and she enjoys writing, reading, and gardening. She wishes to dedicate this review to her parents, Professor Jinchang He and Ms. Zhi Huang.

Gautam Agnihotri was born in New Delhi, India, in 1970. After finishing high school, he spent two years at the National Defence Academy, Khadakvasla. Thereafter, he graduated with his Bachelor of Science degree from Rani Durgavati University, Jabalpur, in 1992. After obtaining his Master of Science degree in Organic Chemistry from the Indian Institute of Technology (Bombay) in 1994, he joined the graduate program at the Department of Chemistry, University of Minnesota, Minneapolis. A desire to work at the interface of chemistry and biology prompted him to join the research group of Professor Hung-wen Liu, under whose guidance he obtained his Ph.D. degree in Biological Chemistry in 2000. His research interests are exploring the chemical basis of biological events through the application of tools such as molecular biology, protein chemistry, and enzymology. He is currently working as a Postdoctoral Scientist at Glaxo-Smith-Kline Pharmaceuticals. His hobbies include reading and traveling.



Hung-wen (Ben) Liu was born in Taipei, Taiwan, in 1952. He graduated with his Bachelor of Science degree in Chemistry from Tunghai University, Taichung, in 1974. After two years of military service, he began his graduate study at Columbia University, where he carried out research under Professor Koji Nakanishi. His work on the additivity relation in exciton-split circular dichroism curves and its application to the structural studies of oligosaccharides earned him his Ph.D. degree in 1981. He then joined the laboratory of Professor Christopher Walsh at Massachusetts Institute of Technology as a postdoctoral fellow, where he was introduced to the field of mechanistic enzymology. In 1984, he joined the faculty of Chemistry at the University of Minnesota, Minneapolis, where he was promoted to the rank of Full Professor in 1994 and to Distinguished McKnight University Professor in 1999. In 2000, he moved to the University of Texas at Austin, where he is now the George H. Hitchings Regents Chair in Drug Design and Professor of Medicinal Chemistry and Biochemistry. His research lies at the crossroads of organic and biological chemistry, with particular emphasis on enzymatic reaction mechanisms, protein function regulation, inhibitor design and synthesis, natural product biosynthesis, and metabolic pathway engineering. The goal of his work is to establish a more thorough understanding of the mechanisms of various biological processes and to rationally design methods to control or mimic their actions. In his spare time, he enjoys reading, traveling, photography, and playing with model train sets.

to afford sugars with stereoinverted centers. Representatives of enzyme catalyzing the above reactions along with those involved in C−O bond formation, carbonyl (1,2) rearrangements, and the formation/rearrangement of sugar skeletons will be discussed in this review. Some of these enzymes such as CDP−6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-dehydrase (E_1), MurA, and UDP−galactose 4-epimerase have been extensively studied, whereas investigations on the mechanism of others, such as UDP−glucosamine 2-epimerase and YerE, have only recently been initiated. Nevertheless, the common theme that unites these enzymes is their novelty and/or significance, whether from biochemical, mechanistic, pharmaceutical, or industrial perspectives.

Needless to say, many omissions have had to be made in order to keep this review within manageable dimensions. For example, enzymes involved in the attachment or removal of glycosyl units, such as glycosyltransferases[15] and glycosidases,[15b,16] are not included nor are the better known enzymes such as the ribonucleotide reductases[17] and aldolases.[18] In addition, many enzymes whose biochemical and mechanistic information is still limited are also excluded, such as the ADP−ribosyl transferases.[19]

As demonstrated by the examples described in this review, biochemical studies of enzymes involved in carbohydrate metabolism have aided in establishing their reaction mechanisms, many of which are novel and complicated. Such knowledge has facilitated the design of specific agents that can be used to control and/or mimic the action of these enzymes. Moreover, understanding of the biosynthetic pathways of unusual sugars in microorganisms has allowed the genetic manipulation of the corresponding biosynthetic machinery to produce rationally designed

"unnatural glycoconjugates".[9,20,21] It is believed that this combinatorial biosynthetic approach offers the promise for the development of new chemical entities that will ultimately serve in the battle against the many old and new threats to human health.

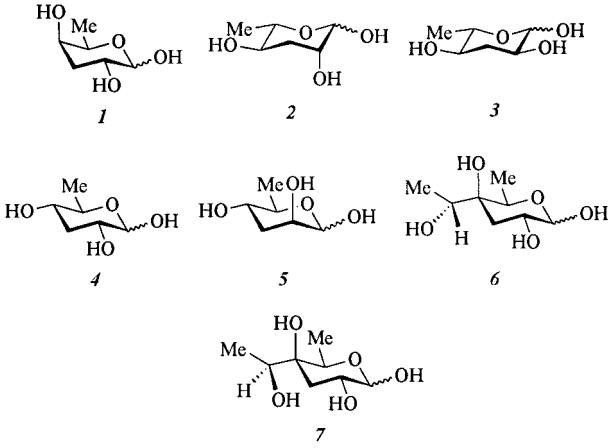
II. C–O Bond-Cleavage Reactions

Since carbohydrates possess a carbon skeleton that is decorated with hydroxyl groups, a variety of mechanisms have evolved for the deoxygenation of these molecules to generate modified sugar structures. The deoxysugars resulting from such reactions do not directly participate in the common energy-producing pathways of the cell. In fact, they are harnessed for their ability to mediate highly specific recognition events and to impart structural stability. The best known example of a C−O bond-cleavage reaction is the formation of 2-deoxyribose from ribose.[17,22] The stability of DNA as compared to RNA led nature to choose DNA as the carrier of the genetic code. However, there are numerous other structures where the presence of deoxysugars is also important. For example, these sugars are found as components of many glycoproteins, glycolipids, and a great variety of secondary metabolites. In each case, the presence of these sugars is crucial for recognition, binding, and/or activity.[11,23]

Several elaborate enzymatic mechanisms have been discovered in the study of the C−O bond-cleavage reactions of carbohydrates.[9,24−26] In this section, we will discuss these reactions as they apply to hexoses. Interestingly, deoxygenation at the C-2 or C-6 position of a hexose substrate proceeds through an anion-induced dehydration mechanism, whereas the removal of the hydroxyl group at C-3 in the formation of 3-deoxyhexoses requires the participation of coenzyme B_6 and involves the formation of radical intermediates. However, in each case, a 4-hexulose acts as the precursor.

A. Biosynthetic Pathways for Deoxyhexose Formation in *Yersinia pseudotuberculosis*

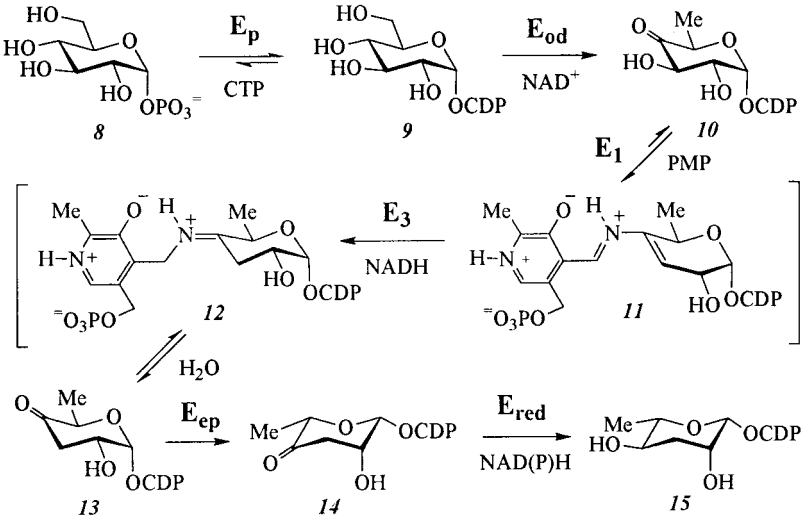
The 3,6-dideoxysugars are found almost exclusively in the lipopolysaccharide (LPS) of Gram-negative bacteria.[27] These sugars are well-known antigenic determinants that contribute to the serological specificity of many immunonologically active polysaccharides. Therefore, they play an important role in the expression of the pathogenicity and virulence of bacteria. Among the biosynthesis of the seven known 3,6-dideoxyhexoses, abequose (**1**), ascarylose (**2**), colitose (**3**), paratose (**4**), tyvelose (**5**), and yersiniose A and B (**6** and **7**), the formation of ascarylose by *Yersinia pseudotuberculosis* is the most thoroughly studied.[9,11,24−26] As an introduction, the pathway for



the formation of CDP−ascarylose (**15**) in *Yersinia pseudotuberculosis*[28] will be briefly described. This will not only serve as a primer for the in-depth mechanistic discussions of the enzymes involved in the C−O bond-cleavage reactions, but will also be relevant for enzymes catalyzing the formation of C−N and C−C bonds that are discussed in the subsequent parts of this review.

As shown in Scheme 1, the first step of the biosynthesis of CDP−L-ascarylose (**15**) is the generation of CDP−D-glucose (**9**) by the coupling of α-D-glucose-1-phosphate (**8**) and cytidine triphosphate (CTP), which is catalyzed by α-D-glucose-1-phosphate cytidylyltransferase (E_p).[29] The second step features the first C−O bond-cleavage event via an intramolecular oxidation−reduction reaction that is catalyzed by CDP−D-glucose 4,6-dehydratase (E_{od}), which is the

Scheme 1

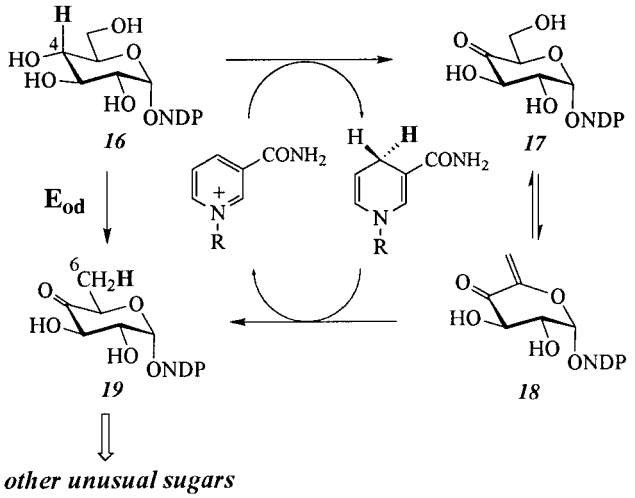


first enzyme that will be discussed in this section. This transformation is followed by the removal of the C-3 hydroxyl of **10** to generate **13** through the combined actions of two enzymes, CDP−6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-dehydrase (E_1) and its partner, CDP−6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-dehydrase reductase or E_1 reductase (E_3), which will be discussed in the second part of this section. Compound **13** acts as a substrate for CDP−3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose 5-epimerase (E_{ep}),[30] which inverts its C-5 configuration. The final stereospecific reduction of the 4-keto group of the product **14** by CDP−3,6-dideoxy-D-*glycero*-L-*glycero*-4-hexulose 4-reductase (E_{red})[30] concludes the overall pathway to give CDP−L-ascarylose (**15**). Since intermediates from this pathway are also involved in the biosynthesis of many unusual sugars such as other deoxysugars, aminosugars, and branched-chain sugars (see sections III.A.2, III.C, and IV.D), this pathway serves as the prototype for the formation of a wide variety of such sugars.

B. E_{od}: C–O Bond Cleavage at the C-6 Position

Most naturally occurring deoxyhexoses are deoxygenated at the C-6 position.[7] In fact, except in the case of some cardiac glycosides, monodeoxygenation of a hexose at positions other than C-6 is rare. It has been demonstrated that C-6 deoxygenation is the first committed step in almost all deoxyhexose biosynthetic pathways leading to the formation of di-, tri-, and tetradeoxysugars. An NAD^+-dependent nucleotidyldiphosphohexose (NDP−hexose) oxidoreductase (E_{od}) has been shown to catalyze this irreversible intramolecular oxidation−reduction.[31−35] Through the action of this enzyme, NDP−hexoses such as **16** are converted to the corresponding NDP−4-keto-6-deoxyhexoses (**19**, Scheme 2), which serve as the precursor for the biosynthesis of most deoxysugars, aminosugars, and branched-chain sugars.[9,24−26]

Scheme 2



1. Biochemical Properties of E_{od}

NDP−glucose 4,6-dehydratase (E_{od}) has been purified from a number of bacterial, plant, and mammalian sources with varying specificity for the nucleotide unit as well as the hexose.[36] The enzyme TDP−D-glucose 4,6-dehydratase isolated from *E. coli* is the best-studied member of its family.[37,38] This dehydratase is a homodimeric protein with a molecular mass of 40 kDa per monomer. Each pair of subunits of this enzyme contains one molecule of the cofactor NAD^+,[39] which is released from its binding site when the dissociation of the subunits is induced by the sulfhydryl-directed reagent HMB (*p*-hydroxymercuribenzoate).[40] Therefore, it has been suggested that the cofactor NAD^+ may play a role in subunit-association and regulation of the enzyme activity.

2. Catalytic Mechanism of E_{od}

The catalytic pathway of E_{od} has been established on the basis of the pioneering work of Glaser, Gabriel, and others.[31−35] The incubation of the enzyme with substrate that was isotopically labeled at C-4 produced TDP−4-keto-6-deoxyglucose with the exclusive retention of the isotope label at C-6.[41,42] On the contrary, incubation of the substrate with enzyme reconstituted with [4-^{3}H]NAD^+ did not produce ^{3}H-labeled TDP−4-keto-6-deoxyglucose.[43] These data clearly indicated that the hydrogen transfer from C-4 of substrate (**16**) to C-6 of product (**19**) is intramolecular in nature. Substantial kinetic isotope effects were also observed when TDP−[4-^{2}H]glucose was used in the incubation, indicating that the transfer of hydride from the substrate to the cofactor or its rebound to intermediate **18** may be rate-limiting.[32] The proton at C-5 of the substrate is exchangeable with solvent. In fact, enzymatic reaction carried out in ^3H_2O or ^2H_2O led to the formation of products with isotope incorporation at C-5.[41,44] On the basis of these observations, a mechanism composed of three discrete steps has been proposed for the E_{od} catalysis,[31−35] which is depicted in Scheme 2: substrate TDP−D-glucose (**16**) is first oxidized to TDP−4-ketoglucose (**17**) with a concomitant formation of NADH; subsequent elimination of water across C-5 and C-6, possibly facilitated by an active-site general base, leads to the formation of a 4-keto-$\Delta^{5,6}$-glucoseen intermediate **18**; a reduction using the hydride from NADH produces TDP−4-keto-6-deoxyglucose (**19**).

It is worth mentioning that the formation of NADH results in a conformational change in the enzyme which may impart it a higher affinity for the sugar nucleotide and prevent premature release of the reaction intermediate.[40,43] Indeed, incubation of a mixture of TDP−glucose and TDP−[U-$^2H_7$]-glucose gave rise to only nonlabeled and perdeuterated TDP−4-keto-6-deoxyglucose, excluding the likelihood of multisite hydrogen relay pathways or the possible liberation of reaction intermediates from the active site of the enzyme.[41]

As illustrated in Scheme 2, the enzyme-bound cofactor NAD^+ plays an important role in the transient oxidation of substrate. This cofactor accepts a hydride from C-4 of substrate **16** and later passes this reducing equivalent to C-6 of intermediate **18**. Consequently, NAD^+ is regenerated at the end of each catalytic cycle. Clearly, this enzyme is distinct from other nicotinamide-dependent enzymes that utilize $NAD(P)^+$ merely as a cosubstrate. In fact, E_{od} belongs

to a select group of enzymes which use the pyridine nucleotides as de facto catalytic prosthetic groups.[35]

3. Stereochemical Studies on the Catalysis of E_{od}

The stereochemical course of the E_{od} catalysis has been studied in detail. As illustrated in Scheme 3, Wang and Gabriel were able to show that the hydride transfer in the catalytic mechanism of the enzyme from *E. coli* occurs from the "*si* face" with respect to the nicotinamide ring by using TDP−[4-^{3}H]-6-deoxy-D-glucose (**20**) as the substrate under single turnover conditions.[45] The stereospecificity of a related enzyme, CDP−D-glucose 4,6-dehydratase from *Yersinia pseudotuberculosis*, has also been studied, albeit by an alternative approach.[46] It has been found that besides catalyzing its normal reaction, this cytidine-specific enzyme can convert its product CDP−4-keto-6-deoxyglucose (**10**) into CDP−6-deoxyglucose (**21**) using a reducing equivalent derived from NADH. Incubation of the enzyme with **10** in the presence of deuterium-labeled NADH revealed that the hydrogen being transferred to the 4-keto group of **10** derives from the *pro-S* hydrogen of NADH.[46]

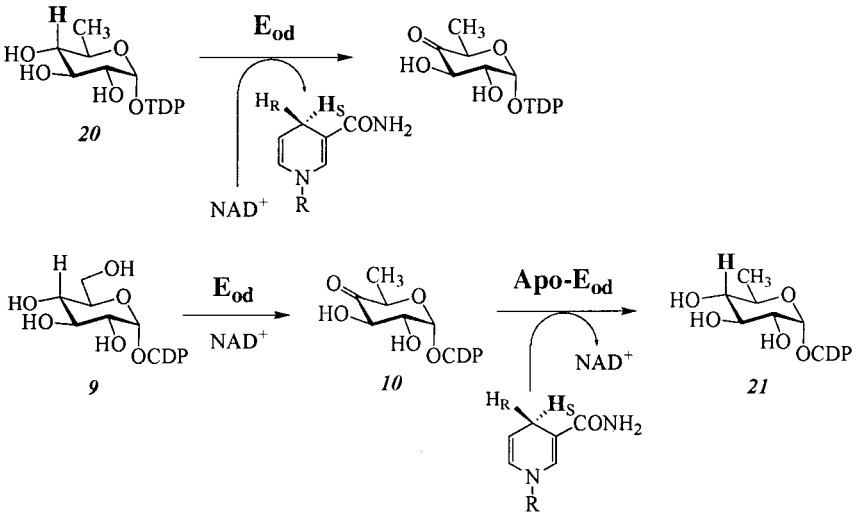
Interestingly, there are other NAD(P)$^+$-dependent enzymes whose mechanisms involve the regeneration of the cofactor at the end of each catalytic cycle, such as UDP−D-galactose 4-epimerase and L-*myo*-inositol-1-phosphate synthase.[35] A comparison of the catalytic features of these enzymes reveals that they are similar to E_{od} with respect to the binding orientation of the substrate/coenzyme since they display the same stereochemical preference for hydride transfer as E_{od}.[47,48] Thus, in all of these cases, the substrate appears to bind near the *si* face of the nicotinamide ring and the hydrogen from substrate transiently resides at the *pro*-S position of the cofactor in these enzymes. It has been speculated that they may have evolved from a single ancestor in a divergent fashion with the preservation of the parent catalytic core.[46]

The stereochemical investigations of other steps in the E_{od} reaction were performed by chiral methyl analysis studies.[49−52] Stereospecifically labeled (6*S*)- and (6*R*)-CDP−[4-^{2}H,6-^{3}H]-D-glucose were synthesized and incubated with the *Y. pseudotuberculosis* enzyme.[51,52] With the assumption that the nicotinamide cofactor in E_{od} mediates a suprafacial hydride transfer from C-4 to C-6 of the sugar skeleton, it was determined that the elimination of water from C5/C6 of **17** is a syn process. In addition, the reduction of the 4-keto-$\Delta^{5,6}$-glucoseen intermediate **18** was established to proceed via an anti addition of a hydride and a proton across the double bond. Overall, the hydroxyl group of the substrate at C-6 is displaced by a hydride derived from C-4 with a net inversion of configuration. A similar stereochemical course was also established for TDP−D-glucose 4,6-dehydratase as well as for GDP-D-mannose 4,6-dehydratase.

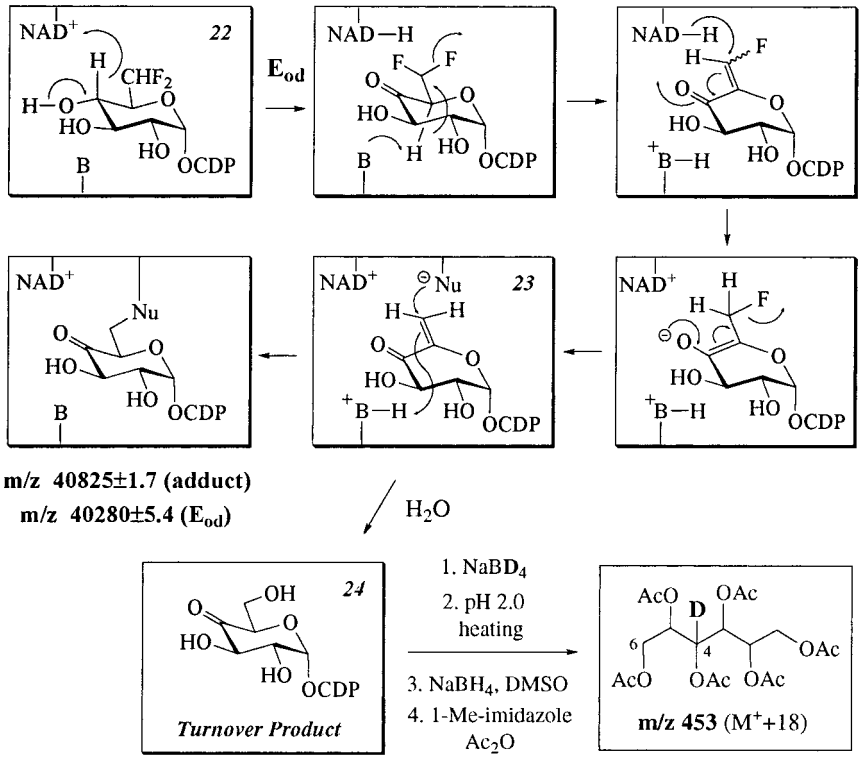
4. Cofactor Binding Studies on E_{od}

More detailed characterization of E_{od} has been performed on the *Y. pseudotuberculosis* enzyme, which has been overproduced in *E. coli*.[28] When this enzyme was purified from *Y. pseudotuberculosis*, it contained only 1 equiv of NAD$^+$ per dimer and required exogenous NAD$^+$ to be fully active.[52,53] Such a half-site saturation stoichiometry between cofactor and the functional enzyme has raised concerns about the affinity of E_{od} for its cofactor.[36] Sequence alignment of E_{od} with other ADP-binding proteins revealed the presence of an extended binding fold (^{16}**G**HT**G**FK^{22}**G**) instead of the commonly found motif **G**X**G**XX**G**, known as the Rossman consensus.[54] The presence of His17 in the β-turn region and Lys21 in a position typically occupied by a small hydrophobic residue may potentially introduce steric and electronic interactions unfavorable to enzyme−cofactor interactions. To test this hypothesis, mutant enzymes with an ADP-interacting region that bears a greater resemblance to the Rossman fold have been constructed.[36] With the elimination of adverse interference, all of the mutants exhibited improved affinity for NAD$^+$, which supports the hypothesis that the affinity of E_{od} for its cofactor NAD$^+$ is correlated to its primary sequence. However, enhanced cofactor binding ability does not alleviate the dependence of these mutant proteins on the addition of exogenous NAD$^+$ for full activity. Thus, the apparently low NAD$^+$ content of E_{od} may not be solely attributed to

Scheme 3



Scheme 4



its weak cofactor affinity as previously surmised. In fact, by titrating E_{od} against NAD^+, it has been determined that the enzyme contains two cofactor binding sites per dimer, which display a large anti-cooperativity ($K_1 = 40$ nM, $K_2 = 540$ nM). In addition, a closer examination of the purified enzyme revealed the presence of tightly bound reduced cofactor NADH ($K_1 = 0.21$ nM, $K_2 = 7.46$ nM). It is likely that the active sites of E_{od} are occupied by nicotinamide cofactor in either form and the enzyme becomes fully active only when all its active sites are reconstituted by NAD^+.[36] Similar results have also been observed for UDP−galactose 4-epimerase, which will be discussed in section IV.A of this review.

5. CDP–6-Deoxy-6,6-difluoro-α-D-glucose—A Mechanism-Based Inhibitor

The C-4 oxidation is a significant event in the catalytic mechanism of E_{od}. The resulting electron-withdrawing 4-keto group not only increases the acidity of the proton at C-5 to facilitate the subsequent C5/C6 dehydration, but also serves as an activating group for the downstream reactions in the biosynthetic pathway. Therefore, a thorough understanding of this E_{od}-catalyzed reaction is indispensable for designing methods to control and/or regulate deoxysugar biosynthesis. To this end, a substrate analogue of E_{od}, CDP−6-deoxy-6,6-difluoro-α-D-glucose (**22**), was synthesized and incubated with the enzyme. It was determined that this compound is a mechanism-based inactivator for E_{od} ($K_I = 0.94$ mM and $k_{inact} = 2.4 \times 10^{-2}$ min^{-1}).[55] ESI-MS analysis suggested that the enzyme inactivation is the result of the covalent trapping of an active-site nucleophile by the 4-keto-$\Delta^{5,6}$-glucoseen intermediate **23** which was formed when **22** was processed by E_{od} (Scheme 4). The fact that a small amount of the hydration product **24** was collected and characterized lent further credence to the proposed mechanism of inactivation. Since the X-ray crystal structure of E_{od} is not yet available, further study of the modified protein will provide valuable information about the residues associated with the active site of the enzyme.

C. E_1/E_3: C–O Bond Cleavage at the C-3 Position

The most intriguing step in the biosynthesis of ascarylose (**2**) is the C-3 deoxygenation of the E_{od} product, CDP−6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**10**), to form CDP−3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**13**). This C−O bond-cleavage event involves a complicated mechanism catalyzed by two enzymes: CDP−6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-dehydratase (E_1) and its reductase (E_3) (Scheme 1).[9,11,24−26] The enzymatic system that catalyzes this complex transformation, including the basic properties of the enzymes involved, cofactor requirements, and the sequence of events in the reaction process was first established through the pioneering research of Strominger and co-workers.[56−58] The genes encoding E_1 and E_3 were later identified and subcloned by the efforts of Liu and co-workers.[28,59] Extensive mechanistic studies on the recombinant proteins available from heterologous expression of the corresponding genes in *E. coli* have helped unlock the mysteries of this intriguing C−O bond-cleavage reaction.

1. Biochemical Properties of CDP–6-Deoxy-L-threo-D-glycero-4-hexulose 3-Dehydrase (E_1) and CDP–6-Deoxy-L-threo-D-glycero-4-hexulose 3-Dehydrase Reductase (E_3)

E_1 is a dark red-brown protein that exists as a homodimer in its native state with a subunit molec-

ular mass of approximately 49 kDa.[57,60] When the wild-type enzyme was originally purified from *Yersinia pseudotuberculosis*, it was only pale yellow in color. One of the chromophores in this enzyme was subsequently identified as pyridoxamine 5′-phosphate (PMP).[56,57] It was later found that the fully reconstituted enzyme contains 1 equiv of PMP per subunit, as determined by radiometric[60] and fluorometric[61] quantitations. As opposed to other coenzyme B_6-dependent enzymes in which the pyridoxal 5′-phosphate (PLP) coenzyme is associated with the protein through the formation of a Schiff base with a lysine residue, the PMP cofactor in E_1 is associated with the active site only through noncovalent ionic interactions.[56] Initial preparations of E_1 suffered from low enzyme activity due to the dissociation of PMP from the active site. Besides cofactor PMP, analytical assays of E_1 also revealed the presence of stoichiometric amounts of iron and sulfur.[62] Subsequent EPR analysis confirmed that a [2Fe−2S] cluster is associated with E_1 ($g = 2.007, 1.950, 1.930$).[62,63] Since E_1 is obtained as a mixture of the holo- and apoenzyme after purification by column chromatography, exogenous PMP, Fe^{2+}, and sulfur are usually required to fully reconstitute its activity.

E_3 is a monomeric red-brown protein with a molecular mass of 36 kDa. The purified enzyme contains 1 mol of FAD per mol of enzyme.[64] In addition, E_3 also harbors a plant-type ferredoxin [2Fe−2S] center, as revealed by UV−vis and EPR spectrometric analysis and iron and sulfur quantitation.[64] on the basis of sequence homology, E_3 is closely related to other iron−sulfur proteins in the flavodoxin−$NADP^+$ reductase family.[65] This enzyme can transfer reducing equivalents from NADH to many acceptors, including O_2, FMN, FAD, and 2,6-dichlorophenolindophenol (DCPIP), with varying degrees of efficacy.[59] While the FAD can operate independently of the [2Fe−2S] cluster in transferring electrons from NADH to alternative oxidants, the presence of the iron−sulfur cluster is necessary for the reducing equivalents to be channeled into the active site of E_1.[64] The stereospecificity of hydride transfer from NADH has been determined to be *pro-R* specific.[66] The activity of E_3 can be abolished by sulfhydryl-directed reagents such as 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *N*-ethyl maleimide (NEM).[67] The cysteine residues that are targeted by these reagents are Cys75 and Cys296.[65] Subsequent investigations have revealed that these two cysteines may interact with the [2Fe−2S] cluster and the NADH binding site of E_3, respectively.

Sequence alignments have shown that E_1 shares good homology with other coenzyme B_6-dependent enzymes, with two important distinctions.[68] First, a highly conserved lysine that forms a Schiff base with PLP in most B_6-dependent enzymes is missing in E_1. Instead, a histidine residue is present in its place at position 220 in E_1. Apart from E_1, only a few other sequences are known to have this unique replacement.[69] In addition, E_1 also contains a unique iron−sulfur cluster binding motif. It is believed that the presence of this histidine residue and the iron−sulfur cluster in E_1 represents a different evolutionary path undertaken by this enzyme, which ultimately converted E_1 to a PMP-dependent dehydrase instead of a normal PLP-dependent transaminase.

2. Role of Pyridoxamine 5′-Phosphate in the Catalytic Mechanism of E_1

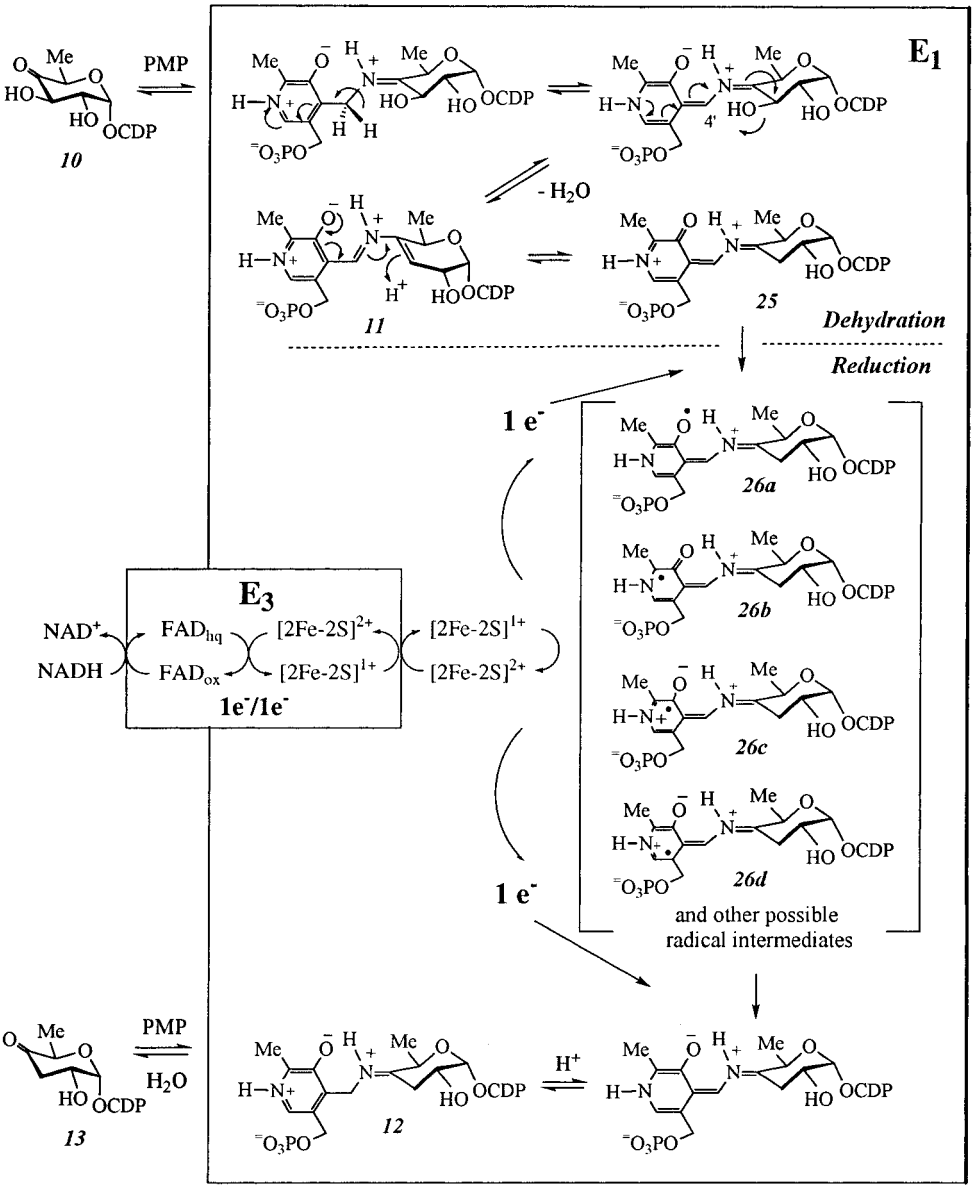
E_1 is able to bind its substrate CDP−4-keto-6-deoxyglucose (**10**) only in the presence of the coenzyme PMP. The enzyme−substrate complex shows increased absorbance in the range of 370−410 nm, indicating the formation of a conjugated imine that is seen in the catalytic mechanisms of most coenzyme B_6-dependent enzymes.[56] As shown in Scheme 5, the catalytic mechanism has been proposed to be initiated by the formation of a Schiff base between the amino group of PMP and the C-4 keto group of substrate **10**. Since no release of ^3H was detected from the incubation of CDP−[U-^{14}C-3-^{3}H]-6-deoxy-L-*threo*-D-*glycero*-4-hexulose with the enzyme,[56,57] a mechanism involving C-3 deprotonation can be ruled out. Instead, it is the abstraction of the *pro-S* 4′-hydrogen of PMP that triggers the expulsion of the C-3 hydroxyl leading to the formation of the conjugated $\Delta^{3,4}$-glucoseen intermediate **11**.[70] Through sequence alignment with other PMP/PLP-dependent proteins and site-directed mutagenesis studies, His220 has been identified as the active site base responsible for the proton abstraction that results in the C−O bond cleavage at C-3.[61] Interestingly, the H220N mutant of E_1 is not totally inactive. This may be attributed to the partial rescue of activity by the presence of another histidine at position 221, presumably through a small perturbation in the active site. As expected, the H220N/H221N double mutant is no longer catalytically active.

The C−O bond cleavage at C-3 is a reversible reaction, as indicated by the incorporation of ^{18}O-label at C-3 of the recovered substrate when unlabeled substrate was incubated with E_1 in the presence of $[^{18}O]H_2O$.[60] The dehydration and rehydration reactions exist in an equilibrium, which is driven to completion by the reduction of the $\Delta^{3,4}$-glucoseen intermediate **11** by electrons originating from E_3. Besides E_3/NADH, other reductants such as dithionite, diaphorase, and methane monooxygenase reductase can also be used to reduce the $\Delta^{3,4}$-glucoseen intermediate, albeit with a much lower efficacy.[70,71] A comparison of the substrate and product configurations at C-3 revealed that the hydroxyl group in the substrate is directly displaced by a hydrogen atom derived from solvent.[72] Considering the *pro-S* specific abstraction of the C-4′ proton on PMP and the net retention of configuration at C-3 of the deoxygenated sugar product, the dehydration reaction catalyzed by E_1 is probably a suprafacial process that occurs on the solvent-accessible *si* face of the PMP−substrate complex.

3. Roles of FAD and [2Fe–2S] Clusters in the Catalytic Mechanisms of E_1 and E_3

As mentioned earlier, the E_{od} product **10** and the $\Delta^{3,4}$-glucoseen intermediate **11** exist in an equilibrium at the active site of E_1. A two-electron reduction of the $\Delta^{3,4}$-glucoseen intermediate is necessary to drive

Scheme 5



the equilibrium to completion and regenerate the PMP coenzyme. It was subsequently determined that the reducing equivalents are relayed from the E_3-bound NADH to the active site of E_1 via a chain of redox-active cofactors, including FAD and the two iron−sulfur centers.[63,66,73] The presence of these iron−sulfur clusters, which are obligatory one-electron-transfer cofactors, dictates that a radical mechanism is operative in the E_1−E_3-catalyzed dehydration.[63,71]

As illustrated in Scheme 5, the initial steps in the catalytic mechanism of E_1 are based on anionic chemistry. However, E_3, which shares high sequence homology to the ferredoxin−$NADP^+$ reductase (FNR) family of reductases, uses the bound flavin coenzyme as a two-electron/one-electron switch in mediating the transfer of electrons from NADH to the [2Fe−2S] centers.[66,73] As shown in Scheme 5, E_3 first binds NADH, resulting in the formation of a charge-transfer complex between FAD and NADH. This complex may be stabilized by Cys296, a residue that was labeled in previous chemical-modification studies.[65,67] The first chemical step in the reductive half-cycle is the transfer of a hydride from NADH to the isoalloxazine ring of the FAD cofactor in E_3, reducing it to the hydroquinone form. From this point onward, the chemistry switches from anionic to one-electron based as the FAD hydroquinone performs two successive one-electron relays through the E_3-bound [2Fe−2S] cluster and the E_1-bound [2Fe−2S] cluster to the PMP−glucoseen intermediate in E_1.[63] As a result of these electron-transfer reactions, intermediate **11** is reduced to the Schiff base **12**. The hydrolysis of this Schiff base releases the C-3 deoxygenated product **13**, marking the end of one catalytic cycle.

The iron−sulfur clusters in the E_1−E_3 system act in a very modular fashion, since their activities can be separated from other chemical events in the catalysis. For example, the FAD cofactor in E_3 retains its ability to mediate electron transfer from NADH to electron acceptors, even if the iron−sulfur center of the enzyme is depleted.[66] Similarly, removal of the iron−sulfur center of E_1 does not hamper its ability to catalyze the PMP-mediated reversible dehydration of its substrate.[62] Whereas E_3 displays a typical

sequence motif that is seen in plant-type ferredoxins, E_1 and its homologues do not display any of the common sequence motifs seen in other [2Fe−2S] proteins. The C−X_n−C−X−C−X_7−C motif, which is conserved in E_1 and its homologues, may represent a special coordination motif that is unique for nucleotide−sugar 3-dehydrases. This motif may be important for modulating the properties of the iron−sulfur cluster to allow better redox communication with its receptor, the PMP−glucoseen intermediate **11**.

4. Interactions Between E_1 and E_3 and Redox Properties of the E_1–E_3 System

Since the $\Delta^{3,4}$-glucoseen intermediate **11** is anchored at the active site of E_1, the transfer of electrons from E_3 to this intermediate must involve the formation of an intermolecular complex between E_1 and E_3. Accordingly, when a mixture of these two proteins was injected in a FPLC gel-filtration column, a new peak which corresponds to the protein−protein complex was observed.[36] When E_1 was titrated against E_3, no sharp inflection points were observed in a plot of the reaction rate vs amount of E_1,[36] which implied that the interaction between E_1 and E_3 is relatively weak compared to those of other redox pairs such as adrenodoxin/adrenodoxin reductase. In fact, the affinity of the E_1:E_3 pair has been estimated to be approximately 288 nM, which is weaker than the low nanomolar affinities observed in other systems. However, the formation of an in vivo complex between E_1 and E_3 was clearly substantiated by using a yeast two-hybrid system[74] in which the interaction between E_1 and E_3 brings together the LexA and VP16 domains, allowing the reconstitution of a functional transcription complex that activates the expression of the *lac*Z reporter gene.[36]

Studies on the redox potentials of the electron-transfer cofactors in E_1 and E_3 also revealed the importance of intramolecular and intermolecular cofactor communications in this mechanism.[73] For example, the removal of the iron−sulfur cluster from E_3 has a significant impact on the redox behavior of the flavin coenzyme, changing its midpoint potential from −212 to −227 mV. Interestingly, it was found that the proposed electron-transfer pathway (E_3−FAD → E_3−[2Fe−2S] → E_1−[2Fe−2S]) may not be energetically favorable since the midpoint potentials for these three cofactors were determined to be −212, −257, and −209 mV, respectively.[73] It is possible that an excess amount of NADH may be required to overcome the thermodynamic barrier against the transfer of electrons from E_3−FAD to E_3−[2Fe−2S]. Therefore, the energy gap between these two cofactors may serve a regulatory function, suppressing the E_1/E_3 reaction until an excess supply of NADH is present. The redox titrations also revealed that the barrier for the electron transfer from E_3−FAD to E_3−[2Fe−2S] was considerably reduced at higher pH values.[73] This was corroborated by kinetic studies on the reductive half-reaction of E_3 using stopped-flow spectroscopy, which revealed that the FAD_{sq}/FAD_{hq} couple has a higher reduction potential than the [2Fe−2S] cluster at pH 7, but this trend is reversed at pH 10.[66] Therefore, it appears that the electron transfer in the E_1/E_3 system may also be regulated by pH.

5. Formation of Radical Intermediates During the Catalysis of E_1 and E_3

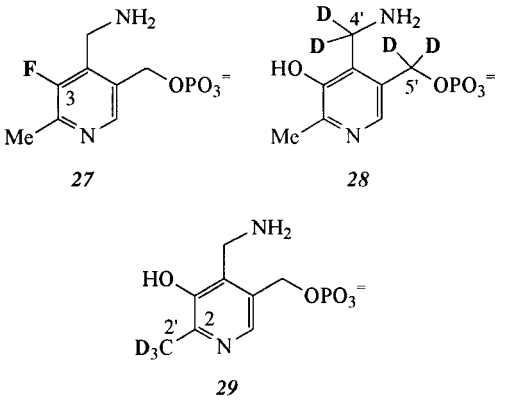
As mentioned earlier, iron−sulfur clusters, which are one-electron carriers, are intimately involved in the catalytic mechanism of E_1 and E_3. Therefore, it is expected that transient free radicals are formed in the catalysis of the E_1−E_3 reaction. In fact, two radicals have been observed by EPR spectroscopy during turnover.[63,71] Of these, the first radical was unambiguously identified as the flavin semiquinone radical. The second radical displayed a featureless EPR singlet centered at $g = 2.003$ and was assigned as a non-flavin organic radical.[63] Since the latter radical was observed only in the presence of substrate, it was more likely to be substrate-based than enzyme-based. However, because the $\Delta^{3,4}$-glucoseen part of **11** has at least one hydrogen at each of its carbons, the apparent lack of hyperfine coupling associated with the radical signal suggested that the unpaired electron spin may reside on the PMP part of the PMP−deoxysugar complex **26**.[63] The detailed kinetics, especially those of the early stages of the E_1/E_3 reaction, were monitored using stopped-flow spectroscopy.[63,66] To determine the individual microscopic rate constants, the absorbance data obtained at different wavelengths using a diode-array spectrophotometer was fit to a series of sequential exponential equations. The spectroscopic data indicated the formation of at least six intermediates in the catalytic mechanism, as judged by the minimum number of equations that were needed to obtain a good fit.[63] Rapid freeze−quench EPR studies revealed that the kinetics of formation and decay of the transient organic radical corresponded most closely to those of Intermediate V in the stopped-flow studies. This intermediate accumulates to its maximum concentration roughly 150 ms after mixing. The spectrum of Intermediate V displayed an absorbance maximum at 456 nm with a shoulder at 425 nm. This suggested that Intermediate V may exist as a sugar−PMP quinonoid species typically observed in PLP-dependent β-elimination reactions.[63] The additional longer wavelength absorption bands may be due to the flavin semiquinone and the reduced [2Fe−2S] clusters.

6. Studies on the Localization of the Unpaired Electron Spin in the E_1/E_3 Mechanism

The general mechanistic paradigm associated with PMP/PLP-catalyzed reactions involves the formation of anionic intermediates. However, many lines of evidence outlined in the previous section implicated an unprecedented role for PMP in the radical-based E_1/E_3 reaction. Therefore, extensive attempts were made to further characterize the radical intermediate, especially the localization of the spin density on the PMP ring. It was first assumed that the organic radical is a phenoxyl-type radical (**26a**) with its spin density primarily residing on the phenolic oxygen atom of the PMP ring.[71] This hypothesis was consistent with the observation of a featureless singlet

in the EPR spectrum. As depicted in Scheme 5, a tautomerization mechanism, which involves the rearrangement of the PMP-$\Delta^{3,4}$-glucoseen intermediate **11** to the PMP−quinoid species **25**, was proposed to account for the formation of the phenoxyl radical.

To test this assumption, 3-deoxy-3-fluoropyridoxamine-5′-phosphate ([3-F]PMP, **27**) was prepared as a mechanistic probe.[75] This coenzyme B_6-analogue should be isosteric and isoelectronic to PMP; however, it would not be able to undergo the proposed tautomerization. As expected, the deoxygenation of the substrate was inhibited when **10** was incubated with E_1 reconstituted with [3-F]PMP and E_3. Surprisingly, the [3-F]PMP-reconstituted enzyme also lost its ability to catalyze the reversible formation of the $\Delta^{3,4}$-glucoseen intermediate **11**.[75] It is likely that the inhibition of its formation is a reflection of the inability of [3-F]PMP to act as a competent PMP analogue rather than of the constraints imposed on the tautomerization process. Therefore, whether the tautomerization to form a PMP−quinoid species **25** is part of the mechanism remains as yet unproven.



To determine the localization of the radical intermediate on the PMP skeleton, several isotopically labeled PMP-analogues, including [4′,5′-$^2H_4$]PMP (**28**) and [2-C^2H_3]PMP (**29**), were synthesized.[76] Apo-E_1 was reconstituted with these analogues and then subjected to EPR analysis. When [4′,5′-$^2H_4$]PMP-reconstituted E_1 was used, the radical signal was narrowed by approximately 3 G as compared to that of the reference spectrum. Such a deuterium-induced sharpening effect on the EPR signal is indicative of the replacement of strongly hyperfine-coupled ^1H by ^2H and provides strong evidence for the direct involvement of PMP in harboring the unpaired electron spin.[76]

Pulsed electron nuclear double-resonance (pulsed ENDOR) spectroscopy was also used to further probe the structure of this PMP-based radical.[76] The Mims ENDOR spectra of the radical obtained by incubating the substrate with E_1 reconstituted with PMP and its labeled analogues allowed the assignment of transitions specifically caused by the presence of deuterium. It was determined that moderately large ^2H hyperfine couplings exist between the unpaired electron spin and each of the two sets of deuterium labels (4′/5′-$^2H_4$ and 2-$^2H_3$) residing on at least two separate positions within the PMP cofactor. These results clearly established that the unpaired electron spin which has been characterized kinetically[63] is indeed localized on the PMP.[76] Therefore, this study provides the most conclusive evidence for the formation of an unprecedented PMP-based radical in the mechanism of the E_1/E_3 reaction. The participation of PMP in deoxygenation is unique, but the direct involvement of PMP in the electron-transfer reduction via a radical mechanism truly places E_1 in a class by itself.

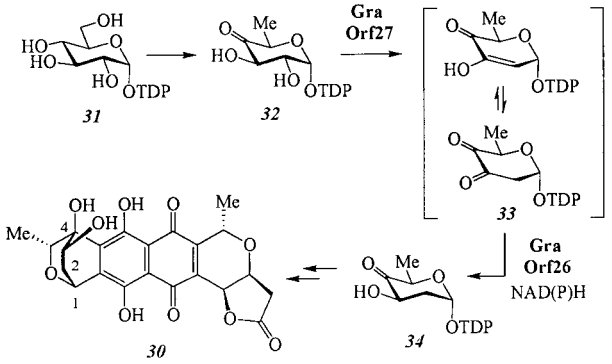
D. TylX3/TylC1: C–O Bond Cleavage at the C-2 Position

While the mechanisms of C-6 and C-3 deoxygenations of hexose substrates have been studied in great detail, the corresponding C−O bond cleavage at the C-2 position has been explored only recently. It should be pointed out that the C−O bond cleavage at the C-2 position of a pentose substrate catalyzed by ribonucleotide reductase is a very well-studied deoxygenation reaction. Since the ribose substrate for ribonucleotide reductase does not possess a convenient activating group, this enzyme has to resort to a complex mechanism using a protein-based radical to initiate the reaction.[17,22] The C-2 deoxygenation of hexoses, on the other hand, relies on the presence of a C-4 keto group in the substrate to promote the elimination of the C-2 hydroxyl located at its β position. A second enzyme that functions as a reductase is also required to produce the C-2 deoxygenated product.[77,78] Since unusual sugars that lack a hydroxyl at C-2 are relatively abundant in secondary metabolites, especially in macrolide and aminoglycoside antibiotics, studies on this reaction have been facilitated by the identification of the corresponding genes from the biosynthetic pathways of granaticin,[77] oleandomycin,[77] and tylosin,[78] all of which contain a 2,6-dideoxyhexose in their structures.

1. Identification and Biochemical Characterization of Enzymes Involved in C-2 Deoxygenation

The first evidence that shed light on the mechanism of C-2 deoxygenation was obtained recently by Floss and co-workers in their studies on the formation of the 2,6-dideoxy-D-hexose moiety in the antibiotic granaticin (**30**) that is produced by *Streptomyces violaceoruber* Tü22.[69d,77] As shown in Scheme 6, using the crude extracts of the transformed *E. coli* cells which harbor the plasmids bearing *gra* orf 27 and *gra* orf 26 genes, Draeger et al. characterized the

Scheme 6



products of these genes as a dehydratase and a reductase, respectively. These two enzymes work in concert to convert TDP−4-keto-6-deoxy-D-glucose (**32**) into TDP−4-keto-2,6-dideoxy-D-glucose (**34**) via the intermediate TDP−3,4-diketo-2,6-dideoxyglucose (**33**).[77]

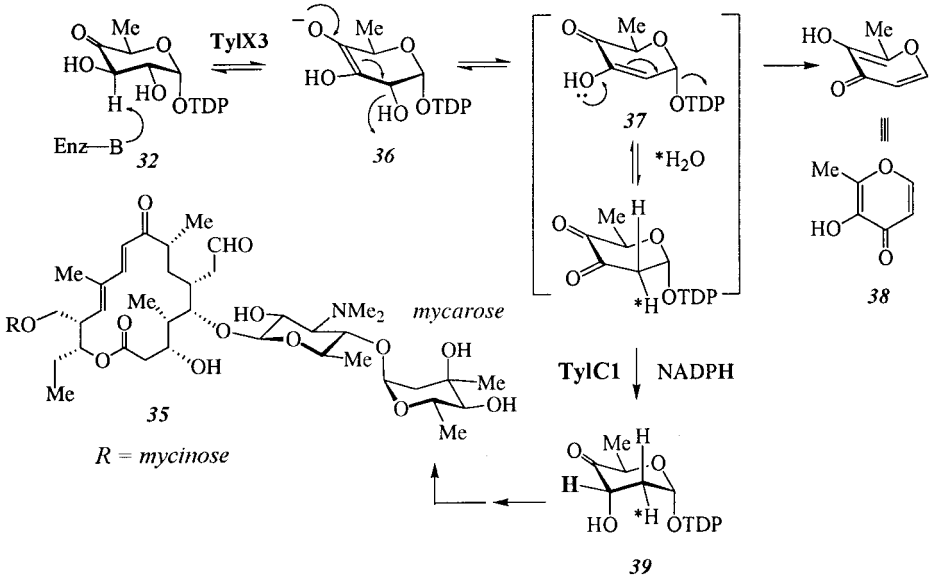
More biochemical studies on this mechanism were performed by Liu and co-workers in their studies on the biosynthetic pathway of mycarose, a 2,6-dideoxy C-3 methyl-branched sugar found in several antibiotics such as tylosin (**35**)[80] and erythromycin.[81] The proteins encoded by the genes *tylX3* and *tylC1* in the *tyl* cluster have been suggested to participate in the biosynthesis of mycarose.[79−81] Both *tylX3* and *tylC1* have been subcloned and overexpressed in *Escherichia coli* BL21 (DE3).[78] The recombinant TylX3 exists as a homodimer with a subunit molecular mass of 55 kDa, while TylC1 is a single polypeptide of mass 36 kDa. The UV−vis spectra of these two enzymes, which are transparent above 300 nm, indicate that they are devoid of common redox cofactors such as an iron−sulfur center or a FAD. Instead, the activity of TylX3 has been found to correlate directly to its Zn^{2+} content, an indication of this metal ion's important role in the enzyme catalysis.[78] Analysis of the deduced sequence of *tyl*C1 has revealed the presence of an extended Rossman fold (^{277}**G**VS**G**AVI**G**^{284}).[54] In fact, the activity of TylC1 is dependent on the reduced form of the nicotinamide cofactor and demonstrates a preference of NADPH over NADH.

2. Mechanism of C-2 Deoxygenation

When substrate TDP−4-keto-6-deoxy-D-glucose (**32**) was treated with TylX3 alone, compounds TDP and maltol (**38**) could be isolated by HPLC and were structurally identified by ^{1}H NMR.[78] Similar outcomes were also noted in the Gra Orf27-catalyzed reaction by Draeger et al., who suggested that maltol comes from the degradation of the expected dehydration product **33**.[77] Addition of TylC1 and NADPH to this incubation mixture resulted in the formation of TDP−2,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**39**) as the sole product. The stereochemical course of the TylX3/TylC1 catalysis was studied by carrying out the incubations in buffer prepared with D_2O. It was observed that deuterium is incorporated at the C-2 equatorial position of the product while the hydroxyl group at C-3 is in the axial orientation in **39** as determined by ^{1}H NMR.[78] These results clearly established that the reduction catalyzed by TylC1 must occur at C-3 and the substitution of 2-OH with a solvent hydrogen must proceed with a retention of configuration. Recent experiments have shown that TylC1 stereospecifically transfers the *pro-R* C′-4 hydrogen of NADPH. A mechanism for C−O bond cleavage at the C-2 position of a 4-ketohexose can be envisaged on the basis of these data (Scheme 7). The enzymatic transformation is initiated by a deprotonation at C-3, followed by the expulsion of 2-OH from enolate **36**. This process is analogous to the first half-reaction of the C-6 deoxygenation event for the generation of a 4-keto-6-deoxyhexulose from a 4-keto precursor (see section II.B) and the conversion of ribulose-5-phosphate into 3,4-dihydroxybutanone-4-phosphate in the biosynthesis of riboflavin.[82] The resulting α,β-unsaturated TDP−2,3-dienol-4-keto-6-deoxyglucose (**37**) could undergo an elimination of TDP and a tautomerization to form the more stable maltol (**38**).[77,78] However, in the presence of TylC1/NADPH, this unstable intermediate can be reduced to give the desired product, TDP−4-keto-2,6-dideoxyhexose (**39**).[78]

As mentioned earlier, the catalysis of TylX3 appears to rely on the presence of Zn^{2+} ions. This cation could activate a water molecule, which serves as the general base responsible for the C-3 deprotonation. Alternatively, it could polarize the C-4 keto functionality of substrate **32** to facilitate the formation of the enediolate intermediate **36**. Finally, this ion could act like an active-site Lewis acid assisting the departure of 2-OH. It should be noted that intermediate **37** is highly unstable. Its ready decomposition to TDP and maltol **38** in the absence of TylC1 suggests that TylX3 and TylC1 might have to interact closely in

Scheme 7



vivo in order to capture this reactive species efficiently. However, attempts to detect such an interaction using either the yeast two-hybrid system[74] or gel filtration chromatography were not fruitful.

Similar functions have also been assigned to the *orf10* and *orf11* encoded enzymes responsible for synthesis of the L-oleandrose moiety in antibiotics oleandomycin produced by *S. antibioticus* Tü99.[77] The deduced sequences of *tylX3*, *gra orf27*, and *Tü99 orf10* are all highly homologous to other genes that are believed to participate in the C-2 deoxygenation step of the 2,6-dideoxyhexoses biosynthesis, such as *eryBVI* from the erythromycin pathway,[81] *lanS* from the landomycin pathway,[83] *orf23* in the vancomycin pathway,[84] *snoH* in the nogalamycin pathway,[85] and *dnmT* from the doxorubicin pathway.[86] It is possible that these genes may constitute a small family of Zn^{2+}-dependent dehydratases that are structurally and mechanistically distinct from enzymes catalyzing the C-3 deoxygenation in the biosynthesis of CDP−ascarylose (see section II.C). Although TylC1, Gra Orf26, and Tü99 Orf11 are all NADPH-dependent keto-reductases with the same substrate specificity, their sequences do not demonstrate any significant similarity. It is worth mentioning that final products from the action of TylC1 or Gra Orf26/Tü99 Orf11, **39** and **34**, respectively, are different in their configurations at C-3,[77,78] which may reflect the structural variation of the enzyme active sites. While Gra Orf26 and Tü99 Orf11 are yet to be classified, TylC1 meets most of the criteria for a long-chain alcohol dehydrogenase:[87] a polypeptide of 329 amino acid residues, a Rossman-fold near its *C*-terminus, and stereoselectivity of the *pro-R* C′-4 hydrogen of NADPH. It would be interesting to see if TylC1, like other members of the long-chain dehydrogenase family, also utilizes Zn^{2+} in its catalytic mechanism.

III. C–X Bond-Formation Reactions

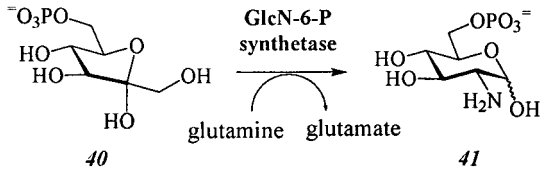
The formations of C−N, C−O, and C−C bonds are the most fundamental reactions in living organisms. In this part of the review, we will discuss the mechanisms of several enzymes involved in the transformations of common hexoses into amino- or branched-chain sugars.

A. C–N Bond-Formation Reactions

Aminosugars occur in a wide variety of natural products such as glycoproteins, glycolipids, and numerous secondary metabolites. The amino groups in these sugars may be found in the free, methylated, or acetylated states. The replacement of a hydroxyl group with an amino group can significantly change the properties of the parent sugar by influencing factors such as the hydrogen-bonding capacity, charge, and overall hydrophobicity of the molecule. Therefore, it is not surprising that aminosugars have been found to play important physiological roles in many glycoconjugates such as aminoglycoside antibiotics and the lipopolysaccharide of Gram-negative bacteria. In this section, two enzymes that catalyze formation of C−N bonds in carbohydrates are discussed. The first enzyme, glucosamine-6-phosphate synthase, catalyzes the C−N bond formation in the biosynthesis of glucosamine, a common aminosugar distributed widely in nature. This reaction involves a nucleophilic addition of the amide-hydrolysis-derived ammonia to a ketosugar precursor. The second enzyme, TylB, is involved in the biosynthesis of mycaminose, an aminosugar that is found in the macrolide antibiotic tylosin. TylB catalyzes the formation of the C−N bond through a PLP-dependent transamination reaction.

1. Glucosamine-6-phosphate Synthetase: C–N Bond Formation via Transamidation

Glucosamine-6-phosphate synthetase, also known as 1-glucosamine D-fructose-6-phosphate amidotransferase, catalyzes the conversion of D-fructose-6-phosphate (**40**) into D-glucosamine-6-phosphate (**41**).

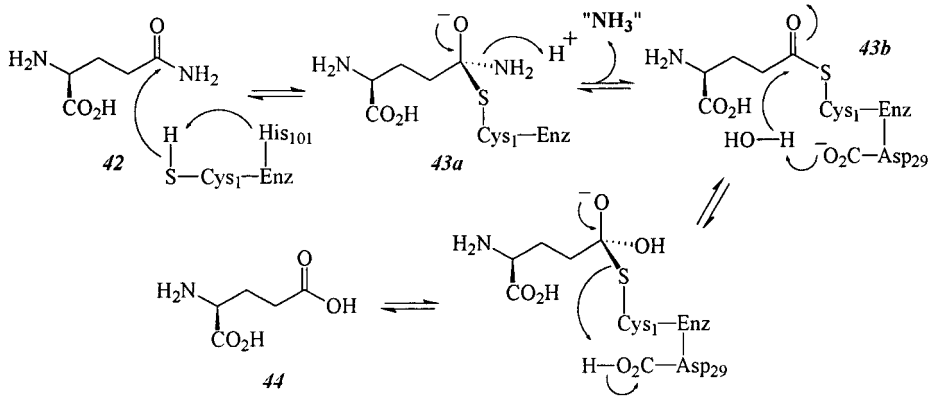


40 *41*

The nitrogen source in this C−N bond-formation reaction is ammonia, which is derived from the cosubstrate L-glutamine (**42**). The product D-glucosamine-6-phosphate (**41**) is further transformed by downstream enzymes into UDP−*N*-acetylglucosamine, which is the central structural entity of many important biopolymers, such as peptidoglycan and chitin. Because glucosamine-6-phosphate is a key precursor for the biosynthesis of many essential biopolymers, glucosamine-6-phosphate synthetase has been considered as an attractive target for antibacterial or antifungal agents. In fact, studies on the active site and the catalytic mechanism of this enzyme have helped in the design of glutamine analogues and their peptide conjugates as potential drug leads with in vivo antimicrobial activities.[88,89]

a. Glucosamine-6-phosphate Synthetase as a Member of the Amidotransferase Family. The activity of glucosamine-6-phosphate synthetase was first reported by Ghosh et al.[90] Most of the early studies were performed on the partially purified enzymes from rat liver,[91−95] bacteria,[96,97] fungi,[98] and Baker's yeast.[99,100] Recently, *glmS*, the corresponding gene that encodes this enzyme in *E. coli*, was discovered and cloned.[101−103] Overexpression of *glmS* has allowed the recombinant *E. coli* glucosamine-6-phosphate synthetase (GlmS) to be purified to homogeneity. This enzyme is a homodimeric protein with a subunit molecular mass of 70.8 kDa.[103] Studies on the inhibition of this enzyme with the glutamine analogue 6-diazo-5-oxo-L-norleucine (DON) resulted in an alkylation at the *N*-terminus of the polypeptide.[90] However, the stoichiometry of labeling in the absence of fructose-6-phosphate was only 0.5 mol of DON/mol of subunit. Therefore, this enzyme exhibits half-of-the-sites reactivity, indicating that the binding of one molecule of DON to one subunit induces a conformational change that prevents the binding of a second molecule of DON to the other subunit.[103] Interestingly, the first amino acid residue at the

Scheme 8



N-terminus of the mature protein is cysteine instead of methionine, as predicted by the gene sequence.[103] Such a modification appears to be a common feature shared by other enzymes of its class.[104−106]

Glucosamine-6-phosphate synthetase from *E. coli* exhibits high sequence homology to other amidotransferases that utilize the amide functional group of glutamine as the nitrogen source for the synthesis of amino acids, nucleotides, and coenzymes.[105] Detailed sequence alignments have revealed that this synthetase belongs to the PurF subfamily of glutamine-dependent amidotransferases, which is named after the *purF*-encoded glutamine phosphoribosylpyrophosphate amidotransferase.[107] Three conserved residues in this subfamily, Cys^{1}-His^{101}-Asp^{29} (purF numbering), have been implicated to participate in a catalytic triad similar to that found in serine proteases.[107,108] Indeed, derivatization of the *N*-terminal cysteine of glucosamine-6-phosphate synthetase by reagents such as iodoacetamide[103] or the N^{3}-fumaroyl-L-2,3-diaminopropionate derivatives is effective in abolishing the amidohydrolase activity of this enzyme.[109,110] Similarly, substitution of Cys-1 with alanine results in a totally inactive mutant protein.[111]

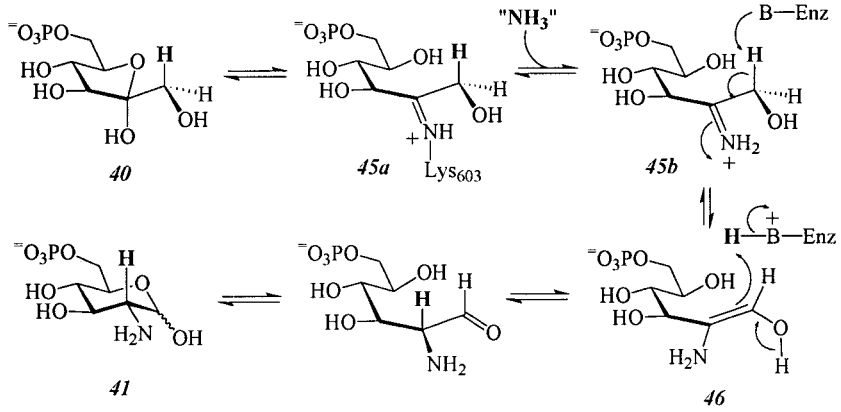
It has been proposed that the overall catalytic mechanism of this enzyme consists of two transformations: (1) the hydrolysis of glutamine to generate L-glutamate and ammonia and (2) the isomerization of fructose-6-phosphate to an aldose (analogous to the Heyns rearrangement), followed by the amination of the resulting aldose with ammonia to form glucosamine-6-phosphate.[112,113] Kinetic measurements have revealed that the overall reaction proceeds by an ordered Bi−Bi mechanism in which the binding of D-fructose-6-phosphate (**40**) occurs prior to that of the second substrate, L-glutamine (**42**). Limited proteolysis of glucosamine-6-phosphate synthetase has shown that the protein is organized into two domains, each bearing a distinct catalytic activity. It has been established that the glutamine amidohydrolase/glutaminase activity is associated with the *N*-terminal domain (residues 1−240) while the *C*-terminal domain (residues 241−608) is responsible for the synthetase activity, catalyzing the isomerization and amination of D-fructose-6-phosphate.[114]

b. Glutaminase Activity of Glucosamine-6-phosphate Synthetase. Mechanistic studies of the glutaminase activity associated with glucosamine-6-phosphate synthetase have led to a catalytic model in which Cys-1 acts as a nucleophile to attack the amide carbonyl of the substrate glutamine (**42**) to initiate the reaction, resulting in the production of ammonia and a γ-glutamyl thioester adduct **43b**. The likely presence of a tetrahedral intermediate **43a** during the course of glutaminase reaction is supported by the potent inhibition of this enzyme by glutamate γ-semialdehyde ($K_i = 3 \times 10^{-8}$ M), which is a minor component in equilibrium with pyrroline-5-carboxylate.[115] The roles of the other two residues in the putative catalytic triad have also been postulated. The histidine residue may be used to enhance the nucleophilicity of Cys-1, while the aspartate residue may be responsible for the regeneration of the free thiol of Cys-1 following the release of NH_3 and L-glutarate **44** (Scheme 8).[108] Chemical modification studies of the enzyme from Baker's yeast have shown that a similar catalytic triad might exist in that enzyme.[100] Moreover, an arginine residue which is not part of the putative catalytic triad was also implicated to play an important role based on these modification experiments.[100]

The structure of the glutaminase domain of the *E. coli* enzyme cocrystallized with the byproduct L-glutamate at 1.8 Å resolution has provided important insights into the mechanism of this enzyme.[116,117] One of the most intriguing observations from this study is that no protease-like catalytic triad could be identified in the crystal structure. Instead, the *N*-terminal cysteine residue appears to play the role of a general base in the hydrolysis of glutamine.[117] Residue Arg26, which was probably targeted in the chemical modification experiments on the yeast enzyme, is suggested to be crucial for the communication between the glutaminase domain and the synthetase domain. However, structural studies of this enzyme have not clarified the actual functions of the histidine and aspartate residues, which were deemed important in the chemical modification studies. The inability to assign functions to these residues based on the analysis of the X-ray structure may be due to the possibility that the crystal structure represents a different conformation of the enzyme, since it is complexed with the product.

c. Synthetase Activity of Glucosamine-6-phosphate Synthetase. The synthetase domain of glucosamine-6-phosphate synthetase catalyzes the actual coupling of NH_3, derived from the hydrolysis of

Scheme 9



glutamine, to D-fructose-6-phosphate (**40**). The proposed mechanism of this reaction is analogous to that of the ketose/aldose isomerases in which a *cis*-enediol-like intermediate (**46**) is formed following the abstraction of a proton from fructose-6-phosphate (Scheme 9).[118] The stereochemistry of the deprotonation step was established to be *pro-R* stereospecific, since the majority of the isotope label was washed out in the medium when (1*R*)-D-[1-^{3}H]fructose-6-phosphate was incubated with the enzyme. However, the retention of a small amount of tritium in (2*R*)-[2-^{3}H]glucosamine-6-phosphate was also noted. Results from this study indicated that the deprotonation and reprotonation must be mediated by a single active-site base and the reprotonation occurs at the *re* face of the enolamine intermediate (**46**).[113] On the basis of the studies of the tritium incorporation into both the substrate and product from tritiated water, it was concluded that the slowest step of this enzymatic conversion is the formation of fructosimine-6-phosphate (**45b**). In addition, the energy barrier for product release was determined to be lower by only 1.9 kcal/mol. Therefore, these two processes may both be partially rate-determining.

The phosphoglucoisomerase-like activity of glucosamine-6-phosphate synthetase was also addressed using [2-^{13}C]fructose-6-phosphate as the starting material.[119] In the absence of glutamine, glucose-6-phosphate was produced, albeit at an efficiency much lower than that of the normal catalytic turnover (K_m = 7.6 mM and k_{cat} = 0.2 min^{-1} vs K_m = 0.42 mM and k_{cat} = 931 min^{-1}). It was found that the K603R mutant showed enhanced phosphoglucoisomerase activity. Lys603 has been proposed to anchor the substrate at the enzyme active site by forming a Schiff base (**45a**) with the 2-keto group of fructose-6-phosphate. If glutamine is present, this Schiff base (**45a**) is exchanged with NH_3 to form the desired fructosimine-6-phosphate (**45b**).[119,120] On the basis of the results obtained with the K603R mutant, it seems that the postulated Schiff base favors the amination reaction over the phosphoglucoisomerase-like reaction in the presence of glutamine during normal turnover.[119]

d. Coupling between the Glutaminase Activity and the Synthetase Activity. Since exogenous NH_3 cannot substitute for glutamine in the reaction catalyzed by glucosamine-6-phosphate synthetase, the glutamine-hydrolyzing activity and sugar-aminating activity of this enzyme must be tightly coupled. In fact, based on the Bi−Bi mechanism established for this enzyme,[103] it has been proposed that the synthetase undergoes a conformational change upon binding of fructose-6-phosphate (**40**) to initiate the hydrolysis of glutamine. The nascent NH_3 is then channeled to the highly shielded acceptor site where the amination reaction occurs.[103] The close proximity of the glutamine and fructose-6-phosphate binding sites in glucosamine-6-phosphate synthetase has been probed by anhydro-1,2-hexitol-6-phosphate, a known irreversible inhibitor for phosphoglucoisomerase. Incubation of this compound, which should bind at the synthetase domain, results in the covalent modification of the *N*-terminal cysteine, probably due to the trapping of Cys-1 by the electrophilic epoxide ring of the inhibitor.[121]

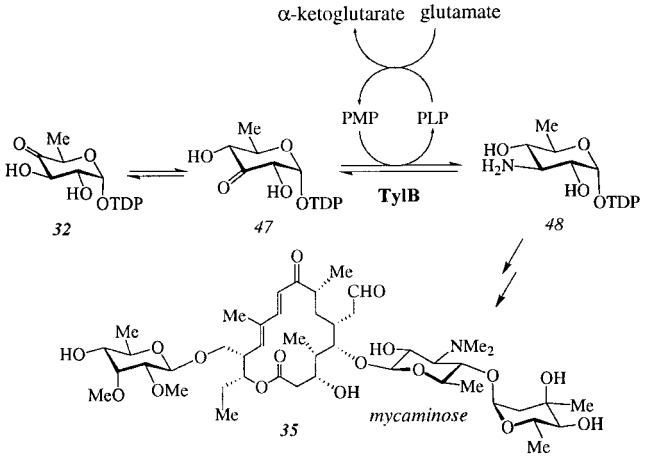
Additional evidence indicating the proximity of these domains comes from the inhibitory studies of this enzyme by *N*-iodoacetylglucosamine-6-phosphate. The structural core of this sugar derivative resembles product glucosamine-6-phosphate. It also contains an electrophilic amino branched chain. While this compound should be bound at the synthetase domain, it is able to inactivate the enzyme irreversibly by alkylating the thiol group of Cys-1 at the adjacent glutaminase domain.[122] It should be noted that fructose-6-phosphate effectively protects the enzyme from inhibition by *N*-iodoacetylglucosamine-6-phosphate. The dissociation constant for fructose-6-phosphate determined from the competitive protection experiments using this inhibitor was found to be 3 orders of magnitude less than that obtained from the initial velocity data.[109,122] Therefore, an alternative scenario in which the binding of glutamine reduces the affinity of the enzyme for fructose 6-phosphate has been suggested.[122]

While the proximity between the two domains of this enzyme during catalysis has been demonstrated, the interactions between them remains a subject of interest. In particular, details on the collaborative action of the NH_3 donor and acceptor sites have yet to be uncovered. A more definitive mechanistic model must therefore await more structural and mutagenesis data, especially the X-ray crystallographic information on the synthetase domain.

2. TylB and DesV: C–N Bond Formation via Transamination

Many aminoglycoside antibiotics produced by *Streptomyces* contain aminosugars, which serve as the key elements for interaction with their targets.[123] Elucidation of the biosynthetic pathways for the formation of these unusual sugars has become an integral part of the recent efforts to generate new antibiotics for fighting infectious diseases through microbial genetic manipulations. Mycaminose, a 3-amino-3,6-dideoxyhexose, is one of the sugar components of the macrolide antibiotic tylosin (**35**) produced by *Streptomyces fradiae*.[124] Extensive genetic and phenotypic complementation studies have led to the tentative assignment of various genes in the tylosin biosynthetic gene cluster. As shown in Scheme 10, the gene

Scheme 10



tylB has been assigned to encode the enzyme responsible for introducing an amino group into a ketosugar precursor (**47**) to yield an aminosugar (**48**).[79,80,125] On the basis of sequence alignment analysis, it was believed that the gene product of *tyl*B is a pyridoxal 5′-phosphate (PLP)-dependent transaminase.

To determine the function of its encoded protein, *tyl*B was subcloned and overexpressed in *E. coli* BL21 (DE3).[125] The recombinant enzyme was found to be homodimeric with a subunit molecular mass of 42 kDa. The purified enzyme appeared to be devoid of its putative cofactor PLP, since the UV−vis spectrum of the protein was transparent at wavelengths above 300 nm. Initially, the transaminase activity of this protein could not be detected, despite attempts to reconstitute it with PLP under native/denaturing conditions. However, when TylB was incubated with PLP, α-ketoglutarate, and its putative product **48**, the formation of a new species was observed by HPLC. Structural analysis of the new species indicated that it corresponds to the 3-ketosugar **47**, which is the proposed substrate of TylB. Therefore, the function of TylB was confirmed by assaying its activity in the reverse direction.[125]

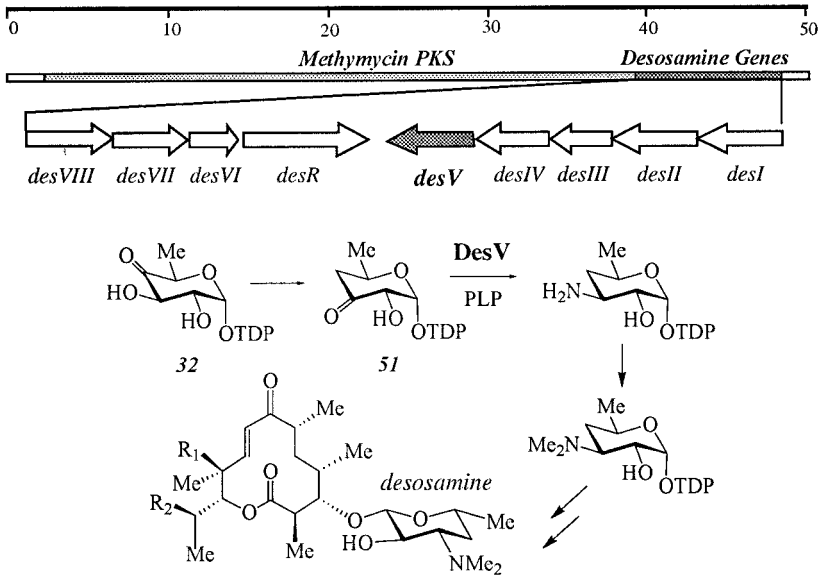
Interestingly, TylB recovered from this reaction displayed a typical spectrum of a PLP-containing protein: a fingerprint absorption at 418 nm. It was also fully active without adding exogenous PLP and had a maximal activity at pH 7.5. Although both α-ketoglutarate and pyruvate could accept the amino group derived from **48**, the former was found to be 5-fold more competent. This indicates that L-glutamate probably serves as the amino donor for the forward reaction in vivo. The TylB-catalyzed reaction is clearly reversible and favors the formation of the aminosugar **48** by a factor of 10 at 24 °C. Despite a previous observation that upon treatment of **32** with Dowex-1 ion-exchange resin the C-4 keto group could migrate to C-3 and form **47** in a nonenzymatic fashion,[126] compound **32** was shown not to be a substrate of TylB. Therefore, enzyme TylB is specific for a 3-ketosugar substrate, which has important implications for the biosynthetic pathway of mycaminose.[125]

Sequence alignment has revealed that *tylB* is homologous to a number of genes assigned to participate in the biosynthesis of unusual sugars associated with other macrolide antibiotics, such as *nleN2* of the oleandomycin pathway,[127] *eryC1* of the erythromycin pathway,[128] *dnrJ* of the daunorubicin pathway,[129] and *desV* of the methymycin/neomethymycin (**49**, **50**) pathway.[130] The biochemical confirmation of the role of TylB as a PLP-dependent transaminase strongly suggests that the products of the other genes must assume similar roles. Initial evidence supporting DesV as a TylB homologue was obtained from the heterologous expression of *tylB* in a mutant of the methymycin/neomethymycin producing strain *Streptomyces venezuelae* in which the *desV* gene had been deleted. When the coding sequence of *tylB* was introduced into *S. venezuelae* to substitute *desV*, production of both methymycin and neomethymycin was resumed by the recombinant strain.[131] Recently, DesV has been overexpressed and purified from *E. coli* BL21 (DE3). This enzyme has been characterized to exist as a homodimer with a subunit molecular mass of 41 kDa. Purified recombinant DesV was shown to exhibit transaminase activity and was able to process the ketosugar **51** even in the absence of exogenous PLP (Scheme 11), indicating a higher affinity for its cofactor as compared to TylB.[131] The biochemical characterization of DesV and TylB has thus confirmed that they represent a family of transaminases essential for the biosynthesis of aminosugars in nature. The catalytic mechanisms of these enzymes merit further investigation.

B. C–O Bond-Formation Reaction

Many transformations and interconversions of carbohydrate molecules involve the formation of C−O bonds. Included in this category are the construction or hydrolysis of glycosidic linkages in which the formation of one C−O bond is accompanied by the cleavage of another. Another example is the oxidation of the hydroxymethyl group in a hexose, as seen in the conversion of glucose to glucuronic acid. The nucleophilic hydroxyl groups in sugars also frequently participate in C−O bond-formation reactions. Transformations of this type include esterification reactions with long-chain fatty acids and the formation of *O*-methyl sugars by methyltransferase-catalyzed reactions. Reactions with electrophiles such as phosphoenol pyruvate (PEP) that result in *O*-alkyl-

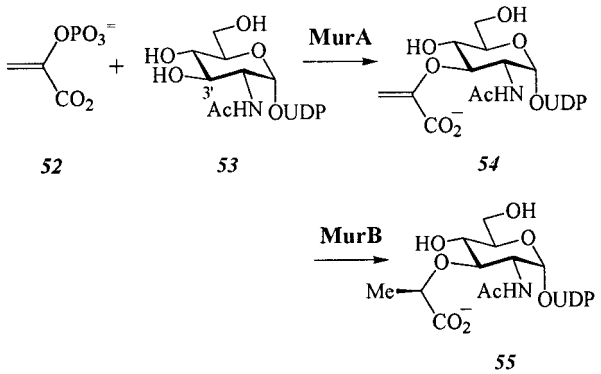
Scheme 11



R_1=OH, R_2=H: methymycin (*49*)
R_1=H, R_2=OH: neomethymycin (*50*)

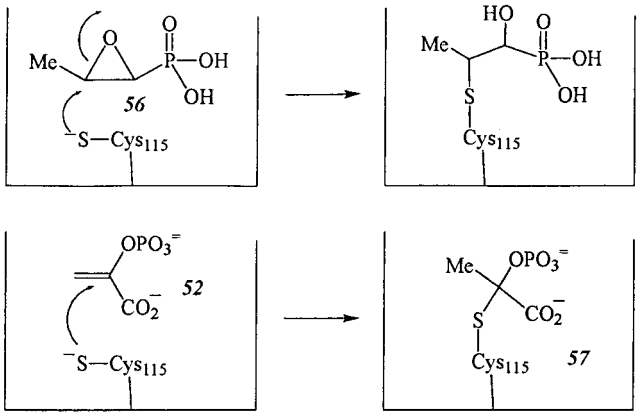
ation also fall in this class. In the following section, the enolpyruvyl transferase involved in the formation of *N*-acetylmuramic acid is selected as an example. The mechanism of its catalysis has been studied in detail.

UDP−*N*-Acetylglucosamine Enolpyrurył Transferase. The bacterial peptidoglycan is an essential cellular component that maintains the shape and the rigidity of the cell, provides strength to the bacterial cell wall,[132] and offers protection against osmotic shock lysis.[133] This layer is assembled from repeating disaccharide units consisting of *N*-acetylglucosamine and *N*-acetylmuramic acid. The formation of *N*-acetylmuramic acid from *N*-acetylglucosamine (GlcNAc) is accomplished in two reactions catalyzed by UDP−*N*-acetylglucosamine enolpyruvyl transferase (MurA) and UDP−*N*-acetylenolpyruvylglucosamine reductase (Mur B). The MurA protein catalyzes the coupling of an enolpyruvate unit from phosphoenolpyruvate (PEP, **52**) to the 3′-OH of UDP−GlcNAc (**53**) to form UDP−GlcNAc-enolpyruvate (**54**), which is subsequently reduced by MurB to form *N*-acetylmuramic acid (**55**). The activity of MurA is essential for the



survival of the bacterial cells, as demonstrated by a lethal chromosomal deletion of the encoding gene *murA* in *E. coli*.[133] Fosfomycin (**56**), an antibiotic produced by *Streptomyces* spp., also exerts its toxicity by blocking the biosynthesis of the peptidoglycan via inactivation of this enzyme (Scheme 12).[134] The assembly of the peptidoglycan layer offers a unique target for intervention by antimicrobial agents; therefore, as a key enzyme for making the building blocks that comprise the peptidoglycan, MurA has been studied extensively to enable the design of potentially useful inhibitors.

Scheme 12



1. UDP–N-Acetylglucosamine Enolpyruvyl Transferase as a Member of the PEP-Utilizing Enzyme Family

UDP−*N*-acetylglucosamine enolpyruvyl transferase was first purified to homogeneity from *Enterobacter cloacae*. This enzyme was found to be monomeric with a molecular mass of approximately 41 000 Da,[135] which was later revised to 44 800 Da based on the calculated nucleotide sequence.[136] This enzyme has now been purified from a variety of bacterial sources, and the encoding gene has been cloned and sequenced from various organisms.[135−137] The *Enterobacter cloacae* and *E. coli* enzymes are two of the best studied enzymes.[136,138]

MurA is a member of the PEP-utilizing enzyme family; while most enzymes in this class utilize PEP by cleaving its high-energy P−O bond, in MurA-catalyzed reaction, it is the C−O bond of PEP that is cleaved. The enolpyruvyl moiety (EP) is then transferred to the sugar receptor to form the enol ether linkage with an overall retention of configuration about the double bond. A similar regioselectivity of PEP degradation has also been found for 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, which catalyzes the addition of shikimate-3-phosphate to PEP in the shikimic acid pathway.[139] The intermediacy of a noncovalently bound ketal species in the mechanism of EPSP synthase has been well established.[140] Since MurA and EPSP synthase share moderate sequence homology (18.3% identity),[136] whether MurA adopts an analogous strategy to accomplish its chemical transformation has been the subject of extensive investigations in recent years.

2. Identification of an Enzyme–PEP Adduct and Its Relevance to the Catalytic Mechanism

The formation of an enzyme−PEP covalent adduct (**57**) in the MurA-catalyzed reaction was first detected in a study performed with partially purified enzyme from *Micrococcus leisodeikticus* using subsaturating amounts of the cosubstrate UDP−GlcNAc (**53**).[141] The same adduct was also found in the reverse reaction by incubating the enzyme with UDP−GlcNAc−enolpyruvate (**54**, UDP−GlcNAc-EP) and inorganic phosphate. It has been shown that this enzyme−PEP adduct (**57**) could be used as a PEP donor to react with UDP−GlcNAc (**53**) to generate UDP−GlcNAc−enolpyruvate (**54**). Furthermore, while the PEP-modified enzyme was found to be immune to the action of fosfomycin (**56**) and other thiol-directed agents such as *N*-ethylmaleimide (NEM) and Ellman's reagent (DTNB), it became vulnerable to these reagents upon exposure to UDP−GlcNAc due to the release of the bound PEP.[138,141] Since coupling between an active-site cysteine (Cys-115 in the case of *Enterobacter cloacae* enzyme) to C-2 of fosfomycin has been shown to be the cause of enzyme inactivation,[138] the above observations led to a conclusion that covalent modification of MurA by PEP may follow a similar mode of action involving the trapping of the same active-site cysteine by PEP (Scheme 12). The fact that this adduct is sufficiently stable to survive multistep purification but becomes labile in an acidic environment is consistent with the general properties of thioketals under similar conditions.

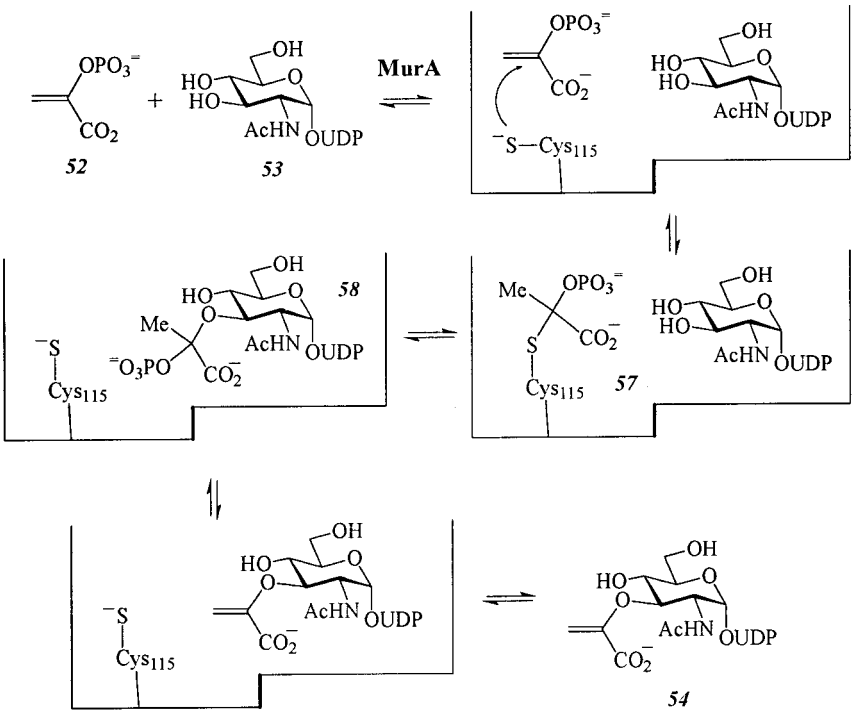
A more detailed characterization of the enzyme−PEP adduct was carried out using the recombinant *E. coli* enzyme.[142] It was found that a significant portion of MurA, when purified, already contained a covalently bound PEP. It was also noted that the uptake of PEP by PEP-free enzyme was enhanced considerably in the presence of UDP−GlcNAc. A favorable change of MurA conformation induced by binding with UDP−GlcNAc was suggested to account for this phenomenon. Using [2-^{13}C]PEP in the incubation with PEP-free enzyme, this enzyme−PEP adduct (**57**) was characterized by NMR as a tetrahedral phospholactoyl species derived from the attachment of an enzyme nucleophile at C-2 of PEP. Pre-steady-state kinetics showed that the formation and decay of this covalent adduct in the presence of UDP−GlcNAc paralleled the rate of consumption of the substrate and that of the appearance of the UDP−GlcNAc−enolpyruvate product. The inclusion of UDP−GlcNAc or 3′-deoxy-UDP−GlcNAc in the incubation mixture was also found to accelerate the time-dependent inactivation of MurA by fosfomycin, probably by exerting a favorable influence on the active-site conformation of the enzyme.[143] The remarkable similarity between the formation of an enzyme−PEP adduct and the inactivation by fosfomycin provided further support for the role of this phospholactoyl enzyme intermediate in the catalytic mechanism of the enzyme.

3. Isolation of a Free Tetrahedral Intermediate and a Revision of the Mechanism of MurA

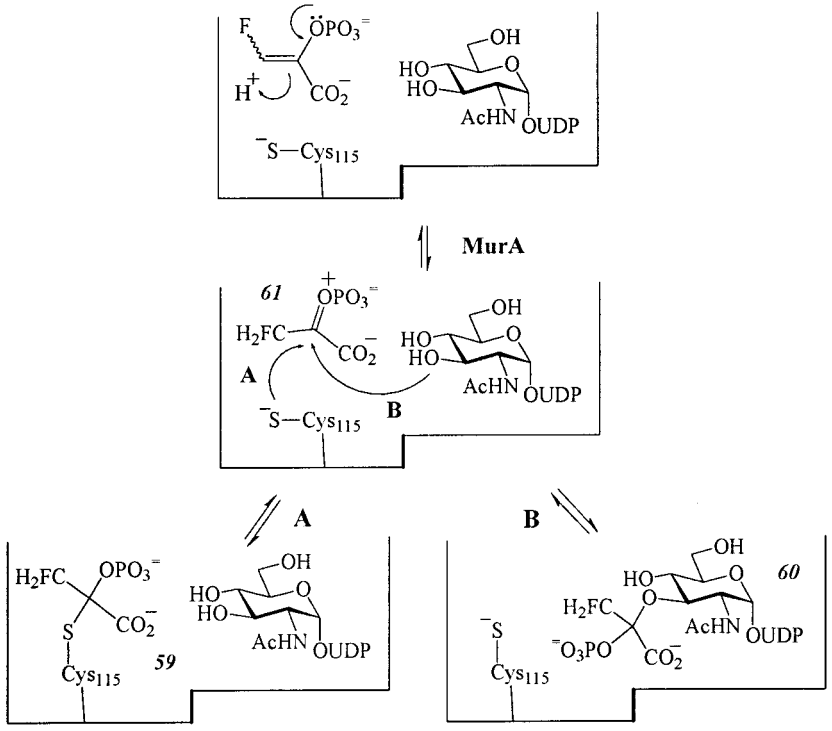
An important development in the mechanistic studies of MurA was the isolation of a relatively stable and noncovalently bound tetrahedral intermediate, phospholactoyl−UDP−GlcNAc (**58**), from a rapid quenching experiment.[144] Although the phospholactoyl enzyme **57** could also be detected, it was found to be in rapid equilibrium with **58** under single-turnover conditions.[142,144,145] Since the decomposition of **58** led to the formation of the turnover products of both the forward and reverse reactions, a sequential mechanism in which **57** is the precursor for **58** was proposed (Scheme 13).[145] Interestingly, both the (*E*)- and (*Z*)-isomers of 3-fluoro−PEP (F−PEP) are time-dependent inhibitors of MurA.[145] ^{19}F NMR analysis of the incubation mixture had identified two stable intermediates, fluorophospholactoyl enzyme (**59**) and fluorophospholactoyl−UDP−GlcNAc (**60**). Their accumulation was a result of the large rate retardation (10^6-fold) of the elimination reaction caused by the fluorine substitution at PEP.

Since adducts **59** and **60** are the corresponding mimics of the transient intermediates **57** and **58** in normal catalysis, studies of these two more stable adducts have provided important insights into the mechanism of MurA. A detailed kinetic analysis of their formation, interconversion, and decomposition back to the starting material revealed that intermediates **59** and **60** were formed from the enzyme·UDP−GlcNAc·inhibitor complex in parallel pathways, in contrast to the previously believed sequential formation of **57** followed by **58**.[146] As illustrated in Scheme 14, these two intermediates slowly interconverted at the MurA active site (0.1 min^{-1}) to reach a final ratio of 1.2 (**59**:**60**). However, it was determined that this interconversion did not involve the formation of FPEP. Therefore, an oxocarbenium ion (**61**) that would allow free rotation along the C_2-C_3 bond of PEP was proposed as an intermediate in this transformation. It was also postulated that since the formation of **60** did not require prior formation of **59**, the primary intermediate in the actual catalytic mechanism of the enzyme was **58**, which is analogous to **60**. Therefore, it appears that the formation of intermediates **57** and **59** upon incubation of the enzyme with PEP and FPEP, respectively, are likely the results of adventitious reaction with C115.[146]

Scheme 13



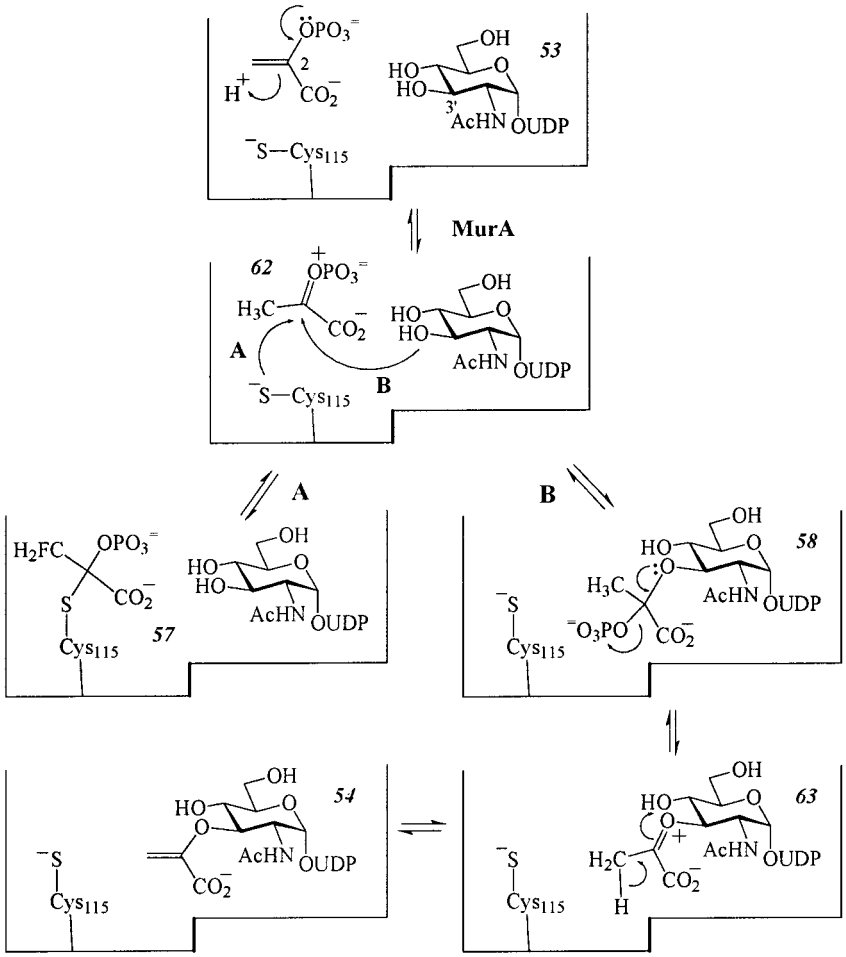
Scheme 14



According to the new mechanism shown in Scheme 15,[146] the first step is the protonation of PEP at C-3 to form an oxocarbenium ion **62**, which reacts with the 3′-OH of UDP−GlcNAc at C-2 to produce the tetrahedral intermediate **58**. The highly reactive **62** could also capture an active-site nucleophile (presumably Cys-115) to form the phospholactoyl enzyme adduct **57**. The breakdown of **58** is initiated by the release of inorganic phosphate, and the desired product UDP−GlcNAc enolpyruvate is formed from the deprotonation of the oxocarbenium ion **63** by an active-site base. The formation of the oxocarbenium ions **62** and **63** accounts for the lower pK_a value of the methyl group and would assist in its deprotonation. This was also supported by the observed reduction in the rate of the reaction with FPEP analogues due to the inductive destabilization of the oxocarbenium ions.

One important issue needed to be addressed is the function of Cys-115 which had earlier been implicated to act as a nucleophile in the catalysis. Since the formation of **57** has no direct role in the revised

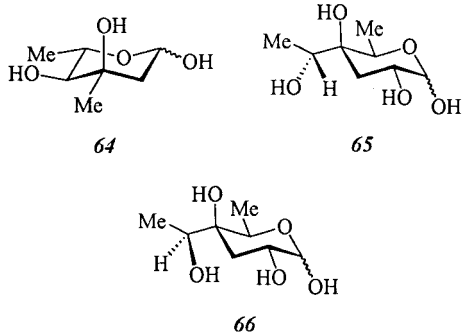
Scheme 15



catalytic mechanism of the enzyme, Cys-115 has now been suggested to participate in the reaction as a general acid/base that may also help to stabilize the oxocarbenium ions through its side-chain thiolate. The fact that mutants lacking ionizable side chains at this position (C115A and C115S) are devoid of enzyme activity and mutants bearing a carboxylate side chain (C115D and C115E) remain active appears to support the newly proposed role for this residue.[147] However, the postulated function of Cys-115 as a proton-transfer catalyst is challenged by the results from an X-ray crystallographic study of a complex between fluorophospholactoyl UDP−GlcNAc and the C115A mutant of MurA.[148] According to the structural data, the loop bearing Cys-115 adopts a flexible conformation and a major relocation is necessary in order to allow the thiolate to participate in the general acid/base catalysis. It is suggested that one of the phosphate oxygen atoms, which is only 2.5 Å away from the C-3 of the fluoromethyl group of **60** (**58** in normally catalysis), is a good candidate to promote an intramolecular proton transfer. Clearly, this issue remains to be resolved.

C. C–C Bond-Formation Reactions

The formation of C−C bonds in carbohydrates is commonly encountered in the biosynthesis of branched-chain sugars.[27,31,149,150] These sugars can be divided into two groups: the Group I sugars are pyranose derivatives carrying a side chain of one or two carbon atoms that are derived from exogenous donors, while the Group II sugars are furanose derivatives with a formyl/hydromethyl side chain that are formed by intramolecular rearrangements of the corresponding pyranose precursors.[31,149] Two-carbon-branched sugars and methyl-branched sugars, such as L-mycarose (**64**), are found predominantly in secondary metabolites, especially glycoside antibiotics.[27a,31] While their presence in other biological systems is less common, two hydroxyethyl-branched 3,6-dideoxyhexoses, yersiniose A (**65**) and B (**66**), were recently isolated from the *O*-antigens of *Yersinia pseudotuberculosis*.[151,152] The biosynthesis of the Group I branched-chain sugars involves the formation of a C−C bond between a hexulose precur-



sor with a C_1 or C_2 unit from appropriate donors. In this section, studies on the chain attachment of two Group I branched-chain sugars will be summarized. In the first case, the two-carbon side chain of yersiniose A (**65**) is installed by the thiamine pyrophosphate (TPP)-catalyzed addition of a C_2 unit to a 4-ketosugar, followed by reduction to generate a 1-hydroxyethyl side chain. The second example relates to the installation of a methyl branch derived from *S*-adenosylmethionine during the formation of mycarose (**64**), a 2,6-dideoxy branched-chain sugar found in the antibiotic tylosin. Preliminary studies on these enzymes that catalyze the branch installation will be described.

1. YerE/YerF: Two-Carbon Branched-Chain Attachment

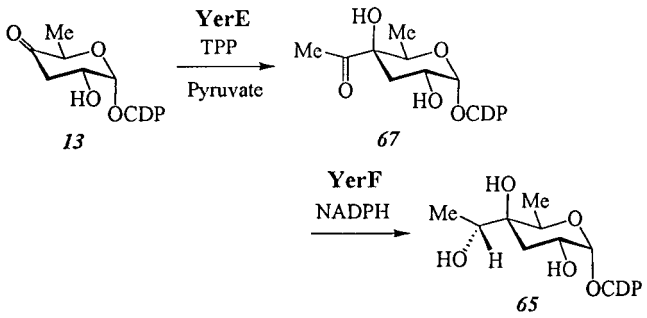
The mode of side-chain attachment in the biosynthesis of two-carbon branched-chain sugars has long been speculated. It has been proposed that the coupling reaction may be thiamine pyrophosphate (TPP) dependent, involving the formation of hydroxyethyl−TPP as the nucleophilic two-carbon donor.[27,31,149,150] However, the inability of well-known inhibitors of TPP-dependent enzymes to affect the production of these two-carbon branched-chain sugars and the failure to detect radioactive products in the incubation of 1-([1-^{14}C]-hydroxyethyl)−TPP with cell-free extracts of the producing strain had cast doubts on the proposed mechanism.[153−155] Hence, the mechanism of attachment of the C_2 side chain remained obscure until the recent overproduction and characterization of two enzymes responsible for the biosynthesis of the branched-chain sugar yersiniose A, a prototypical hydroxyethyl-branched 3,6-dideoxyhexose found in the *O*-antigen of *Yersinia pseudotuberculosis* VI.[151,152]

a. YerE as the Branched-Chain Coupling Enzyme in the Biosynthesis of Yersiniose A. Yersiniose A (**65**) is a 3,6-dideoxyhexose carrying a 1-(*R*)-hydroxyethyl branch.[151,152] Studies on the biosynthesis of 3,6-dideoxyhexoses have identified 3,6-dideoxy-4-hexulose (**13**) as a common intermediate for the formation of this class of unusual sugars, from which different isomers are formed by epimerization and reduction.[9,11,24−26] The knowledge gained from early work on the biosynthesis of ascarylose (**15**, Scheme 1) produced by a related strain aided the design of a suitable probe to screen the genomic library of *Y. pseudotuberculosis* VI and in the isolation of the yersiniose biosynthetic gene cluster (*yer*).[156] Among the eight open reading frames (ORFs) present in this cluster, the *yerE* and *yerF* genes were found to be essential for the installation of the oxyethyl branched chain in yersiniose A.[156]

The translated sequence of *yerE* in this cluster strongly resembles that of the large subunit of the FAD-containing acetolactate synthases (32% identity), whose activity is known to be TPP-dependent.[157] Sequence alignment further revealed the presence of a TPP-binding motif constituted by a glycine-aspartate-glycine (GDG) triplet and an asparagine doublet (NN). These consensus residues are located in a region highly conserved among acetolactate synthases and TPP-dependent decarboxylases.[158,159] Studies of this class of acetolactate synthases have shown that TPP is directly involved in the generation of an "acetyl carbanion" from pyruvate and its subsequent condensation with another molecule of pyruvate or 2-ketobutyrate. In view of the high sequence homology as well as the chemical relevance of the catalysis, the *yerE*-encoded protein (YerE) must be a TPP-dependent flavoenzyme which catalyzes the branched-chain attachment in the biosynthesis of yersiniose A (**65**).

b. Biochemical Properties and Catalytic Mechanism of YerE. The recombinant protein YerE overproduced in *E. coli* BL21(DE3) cells was indeed found to contain tightly bound FAD, which produces a typical flavin absorption spectrum with two maxima at 370 and 450 nm.[156] YerE is a homodimeric enzyme with a subunit molecular mass of roughly 58.5 kDa. In the presence of TPP and Mg^{2+}, YerE is able to couple the 4-ketosugar **13** and pyruvate to form **67** (Scheme 16), whose structure has been confirmed by

Scheme 16

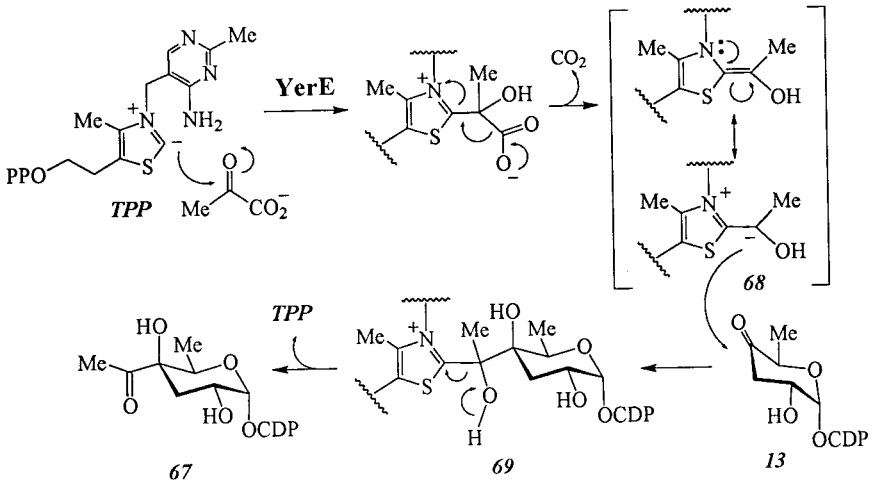


both ^{1}H and ^{13}C NMR.[156] The Michaelis constants (K_m) of sugar substrate **13** and pyruvate are 3.11 ± 0.24 and 7.9 ± 1.8 mM, respectively. Under saturating concentration of pyruvate, the dependence of reaction rate on the concentration of substrate **13** follows classical Michaelis−Menten saturation kinetics with a rate constant (k_{cat}) of 72.3 ± 2.8 min^{-1}.

Although the crystal structure of acetolactate synthase is yet to be determined, the known structures of TPP-dependent enzymes including pyruvate oxidase, transketolase, and pyruvate decarboxylase display a very similar TPP-binding motif.[160] The coenzyme is located at the interface of enzyme subunits, and a divalent metal ion (Mg^{2+} or Ca^{2+}) is necessary for anchoring the TPP pyrophosphate moiety to the protein core. The incorporation of TPP may have a crucial role inducing the formation of correct quaternary structure of the protein. As a result, this family of enzymes is generally sensitive to reaction conditions such as pH, ionic strength, and the presence of certain anions. Indeed, the activity of YerE was found to be dependent on the presence of Mg^{2+} with an optimal pH of 8.0.

Enzyme YerE facilitates a de facto "umpolung" (charge-reversal) reaction in catalyzing the attachment of the two-carbon side chain to make CDP−yersiniose A. As depicted in Scheme 17, the coupling of TPP and pyruvate generates a nucleophilic carbanionic adduct **68**, which subsequently attacks the 4-keto group of **13**. The collapse of adduct **69** produces **67** and regenerates the coenzyme. Since the activity

Scheme 17



of YerE was not altered in the presence of the reducing agent dithionite under aerobic or anaerobic conditions, it appears that the FAD does not play a redox role in this transformation.[156] Interestingly, acetolactate synthase is not a redox enzyme either. The flavin coenzyme in the latter case has been suggested to mainly play a structural role in stabilizing the active conformation of this enzyme.[157] Considering its significant sequence identity to acetolactate synthase, YerE may also be an evolutionary descendant of pyruvate oxidase, an enzyme that requires FAD and ubiquinone-40 (Q_8) for shuttling electrons and shielding reaction intermediates from solvent during catalysis. However, the FAD cofactor in YerE may be important solely for maintaining the integrity of the active site of this enzyme, as seen in acetolactate synthase.

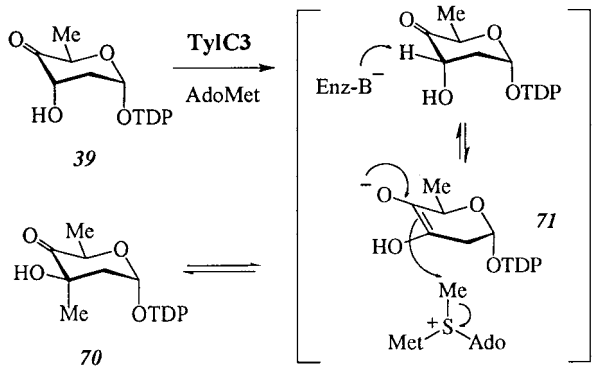
c. Biochemical Properties of YerF. The open reading frame designated *yerF* is downstream of the gene *yerE* in the yersiniose biosynthetic gene cluster. The translated sequence of *yerF* shows moderate homology to NAD(P)$^+$ binding enzymes such as CDP−D-tyvelose 2-epimerase and UDP−D-galactose-4-epimerase.[161] Thus, it was speculated that this gene encodes an enzyme for the reduction of sugar intermediate **67**. When this gene was overexpressed in *E. coli* BL21 (DE3), a homodimeric protein with a subunit molecular mass of 65.5 kDa was isolated. Incubation of YerF with NADPH and **67** produced CDP−yersiniose A (**65**), as confirmed by NMR. This enzyme works more efficiently in a slightly acidic environment and displays maximal activity at pH 6.5. Although YerF lacks a conventional Rossmann fold for binding the nicotinamide cofactor, this protein definitely utilizes nicotinamide dinucleotides for the reduction of the oxoethyl side chain in **67**, with a preference for NADPH over NADH by a factor of 12. The stereochemical course of the hydride transfer from NADPH to **67** was determined to be *pro*-S specific. The relatively small subunit size (309 amino acids) and its stereospecificity of hydride transfer places YerF in the short-chain alcohol dehydrogenase family. It is not clear if YerE and YerF form a tight complex in vivo for the synthesis of **65**, since a yeast two-hybrid assay failed to detect such an interaction.[162]

2. TylC3: One-Carbon Branched-Chain Attachment

Mycarose, a 2,6-dideoxy-D-*threo*-hexose with a C-3 methyl branch (**64**), has been found in several macrolide antibiotics, such as tylosin produced by *Streptomyces fradiae*.[124] As mentioned earlier, little is known about the attachment of a one-carbon unit to make methyl-branched sugars. Although *S*-adenosylmethionine (*S*-AdoMet) has long been proposed to be the methyl donor according to early tracer experiments[31,149] and the acceptor has been proposed to be a nucleotide−hexulose, specific information on the actual pathways leading to the formation of these unusual carbohydrates is sparse. In fact, coupling of the hexulose precursor with a nucleophilic methyl group supplied by methylcobalamin is an appealing alternative that has precedence in fosfomycin biosynthesis.[163] To elucidate the mechanism of the *C*-methylation reaction in unusual sugar biosynthesis, the methyl branched-chain attachment step in the mycarose biosynthetic pathway was investigated.

a. Biochemical Properties and Catalytic Mechanism of TylC3. Early genetic and phenotypic complementation studies have led to the identification of the entire gene cluster responsible for the biosynthesis of tylosin (**35**), including the genes involved in the biosynthesis of mycarose.[124] Sequencing results and analysis have helped identify *tylC3* as the gene likely to encode the *C*-methyltransferase required for the attachment of the methyl branched chain to the hexulose precursor **39** (Scheme 18).[162,164] Although TylC3 did not display significant sequence homology to any known AdoMet-dependent methyltransferase on the basis of a BLAST search, a closer inspection did reveal the presence of three localized sequences characteristic for the binding motif of *S*-AdoMet.[165] It should be noted that a TylC3 equivalent, EryBIII, which shows high sequence homology (68% identity and 80% similarity) to TylC3, has also been found in the biosynthetic gene cluster of erythromycin,[81] which contains a mycarose derivative (cladinose) as one of its appended sugars. Since *eryBIII* found in the erythromycin cluster has been implicated to encode the *C*-methyltransferase involved in the biosynthesis of cladinose based on gene-knockout studies,[166] the high sequence homology

Scheme 18



between TylC3 and EryBIII strongly suggests an analogous role of the former enzyme in mycarose biosynthesis.

The purified TylC3 is a monomeric protein with a molecular mass of 46 423 Da deduced from its amino acid sequence. It displays no significant UV−vis absorption above 300 nm and has a moderate affinity for *S*-AdoMet (1.5 ± 0.2 μM). When this enzyme was incubated with [C^3H_3]AdoMet and sugar substrate **39**, a new compound was isolated by HPLC that was identified as the C-3 methylated sugar **70** by ^{1}H NMR. This newly installed C-3 methyl group must adapt an axial position since a positive nuclear Overhauser effect (NOE) was observed between the methyl hydrogens and the C-5 axial hydrogen. It has also been established that the catalysis of TylC3 leads to an overall inversion of configuration at C-3.[162]

The determined k_{cat} value of 1.4 ± 0.1 min^{-1} is typical for methyltransferases. The K_m for sugar substrate **39** could only be estimated as <1 μM.[164] The mechanism of this reaction is likely initiated by a proton abstraction from C-3 to generate an enediol anion intermediate (**71**). As shown in Scheme 18, subsequent nucleophilic attack to capture the electrophilic methyl group in *S*-AdoMet leads to the formation of a new C−C bond. However, the activity of this enzyme shows no correlation to the content of Zn^{2+} or Mg^{2+}. Thus, the manner in which the enolate intermediate **71** is stabilized by TylC3 remains unclear. Nevertheless, this study has established TylC3 as the first biochemically characterized *C*-methyltransferase that is involved in branched-chain sugar biosynthesis. The mechanistic insights obtained by the study of this enzyme may be extrapolated to the formation of other methyl-branched sugars.

IV. Epimerization Reactions

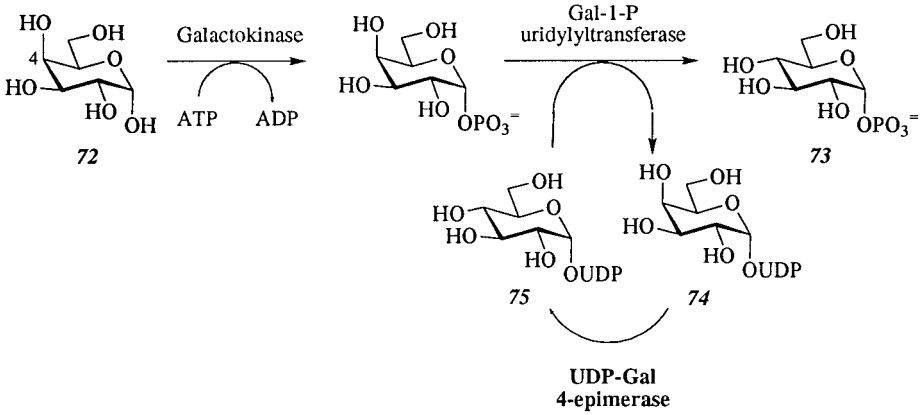
Many enzymatic transformations of carbohydrates involve the inversion of configuration at one or more stereogenic centers. Such an epimerization has been commonly used in the conversion of D-sugars to the corresponding L-sugars. It is also a convenient mechanism for accessing the structural diversity derived from a handful of common sugar precursors. The reactions of most epimerases take place at a chiral carbon adjacent to an activating moiety such as a carbonyl group, and the catalysis typically involves a simple deprotonation-reprotonation mechanism. For example, enzymes such as the hexose 3,5-epimerases and hexose 5-epimerases use a 4-hexulose as the substrate, where the presence of a keto group at C-4 facilitates the epimerization by lowering the pK_a of the adjacent chiral center(s).[28,30,69a] However, the four reactions discussed in this section stand apart due to the fact that their catalyses involve epimerization at unactivated centers. The C-4 epimerization of UDP−glucose to UDP−galactose mediated by UDP−galactose 4-epimerase is the best studied enzyme-catalyzed stereoinversion. The second reaction, catalyzed by ribulose-5-phosphate 4-epimerase, also involves C-4 epimerization, albeit via a different mechanism. The last two examples are the interconversion of UDP−*N*-acetylmannosamine and UDP−*N*-acetylglucosamine and the transformation between CDP−paratose and CDP−tyvelose catalyzed by the corresponding 2-epimerases. Again, both cases involve the inversion of stereochemistry at the unactivated C-2; however, their mechanisms are distinctly different. As will be evident in the following sections, nature has clearly evolved a diverse range of catalyses to accomplish epimerization at chemically inert centers.

A. UDP–Galactose 4-Epimerase

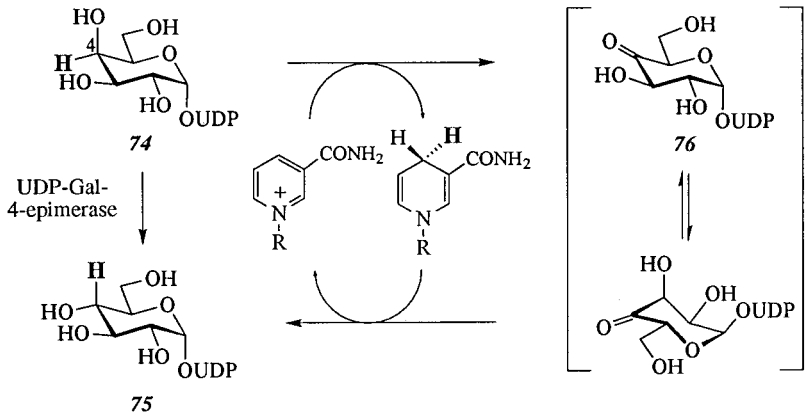
The Leloir pathway of galactose metabolism exists in most organisms including animals, plants, and microorganisms, where it plays an important physiological role in metabolism. This pathway consists of three enzymes: galactokinase, galactose-1-phosphate uridylyltransferase, and UDP−galactose 4-epimerase (Scheme 19).[167−170] Through the actions of these enzymes, galactose (**72**) enters metabolic pathways in the form of glucose-1-phosphate (**73**), which is used as the starting material for glycogen biosynthesis and glycolysis. The same set of enzymes acting in the reverse direction can also convert the glucosyl units to galactosyl units, which are utilized for the glycosylation of proteins and lipids and the biosynthesis of cell walls. The deficiency of galactose-1-phosphate uridylyltransferase or UDP−galactose 4-epimerase in humans causes metabolic disorders such as galactosemia and thrombocytopenia.[170−172] The most interesting chemical transformation in the Leloir pathway is the interconversion of UDP−galactose (**74**) and UDP−glucose (**75**) via stereoinversion at C-4, which is catalyzed by UDP−galactose 4-epimerase. The lack of a carbonyl group adjacent to C-4 in the substrate makes this reaction an intriguing example of epimerization at an unactivated center.

a. Biochemical Properties and Catalytic Mechanism. UDP−galactose 4-epimerase has been purified from a variety of sources such as *E. coli*,[173−175] *Saccharomyces fragilis*,[176−178] and bovine liver.[179,180] The *E. coli* enzyme, which is the most thoroughly investigated, is a homodimeric protein with a subunit molecular mass of 79 kDa. Early analysis showed that the purified enzyme contains only one NAD^+ per dimer.[173] However, the recent X-ray crystal structure of the enzyme at 2.5 Å resolution clearly demonstrated that the dimeric enzyme contained two nucleotide-binding pockets which are located at the

Scheme 19



Scheme 20



N-terminal domain of each polypeptide.[181] The enzyme active site is located at a cleft between the *N*- and *C*-terminal domains of each subunit. Early studies had shown that the tightly bound NAD^+ functions as a true prosthetic group mediating an intramolecular oxidation−reduction reaction in the catalysis as shown in Scheme 20.[182−185] The formation of 4-ketoglucose (**76**) as the intermediate is the key step of this reaction, during which the reaction center (C-4) changes from an sp^3 tetrahedral configuration to an sp^2 planar configuration. Subsequent enzyme-controlled reduction may allow the hydride to be transferred from NADH to either the *re* or the *si* face of the C-4 carbonyl group.

This mechanism is supported by many lines of evidence.[47,186−191] For example, incubation of the 6-deoxy analogue of the putative intermediate **76** with (4′*S*)-[4′-^{3}H]NADH-containing epimerase prepared by NaB^3H_4 reduction of the wild-type enzyme resulted in the formation of two expected products, the 4-^{3}H-labeled fucose (6-deoxy-D-galactose) and 4-^{3}H-labeled quinovose (6-deoxy-D-glucose).[47] Upon treatment with the oxidized form of the epimerase, the reverse transfer of tritium from [4-^{3}H]fucose to the 4-*pro-S* position on NAD^+ was also observed.[47] The kinetic isotope effects ranging from 1.7 to 3.5 detected for the epimerization of a series of nucleoside diphospho-[4-^{3}H]hexoses lent further credence to the proposed mechanism. The latter data also indicated that C−H bond cleavage at C-4 is at least partially rate limiting.[192,193]

b. Stereochemical Issues Surrounding Hydride Transfer to the Hexulose Intermediate. The central issue surrounding the catalytic mechanism of this enzyme is the stereochemical fidelity of the NAD^+-mediated intramolecular hydrogen transfer. While this process is stereospecific with respect to hydride transfer to and from the nicotinamide coenzyme, the oxidation and reduction at C-4 of the sugar substrates is clearly nonstereospecific. This unusual characteristic distinguishes UDP−galactose 4-epimerase from other pyridine nucleotide-dependent oxidoreductases.[35] To investigate the structural basis of this unusual lack of stereochemical preference, extensive studies on the environment of the active site and its interactions with bound substrate have been carried out.[191,194] All data indicate that the nicotinamide ring is buried in a hydrophobic environment,[194] as seen in other oxidoreductases. Since chemical reduction of the enzyme-bound NAD^+ by $NaBH_4$ was found to be *pro-S* stereospecific, the binding of NAD^+ must be reasonably tight, with only one face of the cofactor being exposed to the sugar substrates.[186,187] Thus, the nonstereospecific reduction of the 4-ketosugar catalyzed by this enzyme is less likely to be the result of an easy rotation of its nicotinamide coenzyme and more due to the flexible binding of the sugar nucleotides.

The interactions between UDP−galactose 4-epimerase in its oxidized/reduced forms and UMP, UDP, and other uridylyl sugars have been quantitatively analyzed.[190] The free energy changes for binding of

UMP and glucose to E·NAD$^+$ were determined to be −3.8 and +0.7 kcal/mol, respectively, suggesting that the major contribution to the binding interactions is made by the nucleotide moiety of the substrate.[190] A free energy change from −8.8 to −9.4 kcal/mol was also determined for the binding of UDP−4-keto-pyranoses to the reduced epimerase (E·NADH). Since the contribution of the interaction of the UDP moiety is expected to be −7 kcal/mol,[191] the above data indicated that the glycosyl moiety is not tightly bound to the enzyme and may possess a reasonable degree of conformational flexibility.[195] Thus, it was proposed that a rotation of the hexose ring along its C_1−O bond and the P_β−O linkage of the pyrophosphoryl group may allow both sides of the sugar to be presented to the nicotinamide coenzyme.[190]

NADH·UDP−Glc and NADH·UDP−Gal bound to a Y149F·S124A double mutant clearly shows that opposite faces of the pyranose ring are exposed to the si face of the nicotinamide ring.[196] However, inspection of the structure of data from the recently solved crystal structure of the abortive complex formed by the binding of UDP−glucose to the reduced enzyme (E·NADH·UDP−glucose) revealed that a simple 180° rotation about the oxygen−phosphorus bond may not be sufficient to place the opposite face of the keto intermediate in close proximity to the nicotinamide ring. Additional rotations along the phosphate backbone of UDP may be necessary to achieve the proper alignment for the hydride transfer to occur.[197] Overall, it is clear that the sugar substrate is bound to UDP−galactose 4-epimerase using UDP as the anchor. The loose interactions between the hexose portion of the substrate and the enzyme active site may allow the sugar ring to reorient itself toward the nicotinamide coenzyme, constituting the structural basis of a nonstereospecific reduction.

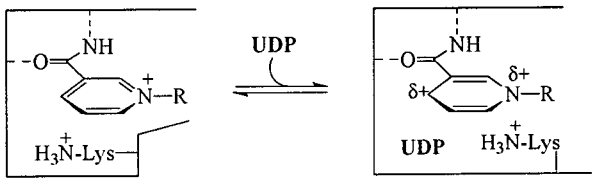
c. Activation of UDP−Galactose 4-Epimerase by a Nucleotide-Induced Conformational Change. Early studies showed that UMP was effective in accelerating the reaction of E·NAD$^+$ with glucose and in stabilizing E·NADH against oxidation.[197−202] Thus, it was speculated that the binding of UMP induces a different protein conformation (E*), which constrains the nicotinamide ring to make it a better hydride receptor.[35,203] The variation of the chemical shifts of the phosphorus atoms of enzyme-bound NAD$^+$ in the absence and presence of UMP provided spectral evidence for the postulated protein conformational change.[204] Interestingly, a comparison of the crystal structures of the oxidized and reduced epimerase from *E. coli* complexed with UDP failed to show any significant structural difference except for the nicotinamide ring.[205] The ring adopts a syn conformation in E·NAD$^+$·UDP but an anti conformation in the reduced enzyme complex.

The mechanism of NAD$^+$ activation induced by binding with uridine nucleotide was further investigated by ^{13}C and ^{15}N NMR analysis using isotopically labeled nicotinamide cofactors.[206] While the ^{15}N/^{13}C NMR signals of enzyme-bound [carboxamide-^{15}N]-NAD$^+$, [2-^{13}C]NAD$^+$, and [6-^{13}C]NAD$^+$ remained practically unchanged upon addition of UDP, those of [nicotinamide-1-^{15}N]NAD$^+$ and [nicotinamide-4-^{13}C]-NAD$^+$ shifted upfield by 3.0 ppm and downfield by 3.4 ppm, respectively.[170,206] Such a change of polarization of the nicotinamide ring reflected a decrease of the electron density at C-4 making the coenzyme a stronger oxidant that can accept a hydride from the substrate more readily. In fact, a perturbation leading to a 3.4 ppm downfield shift in the ^{13}C NMR signal corresponds to a 125−150 mV increase in the reduction potential of the nicotinamide ring, which could theoretically bring about a 3000- to 15 000-fold enhancement in the rate of oxidation.

Since no physical distortion of the nicotinamide ring was observed in the structure of E·NAD$^+$·UDP complex,[205] electrostatic repulsion between the positively charged nicotinamide ring at N-1 and a positively charged amino acid side chain has been rationalized as the cause of the electronic destabilization of the nicotinamide ring.[206] Lys153 is probably the residue involved in this process, since it is hydrogen-bonded to both the 2′- and 3′-OH groups of the nicotinamide riboside moiety of NAD$^+$ in the active form (E*) of the enzyme[207] and is conserved in all known epimerase sequences.[208] The pH−rate profile of the inactivation of the epimerase by P^1-5′-uridine-P^2-glucose-6-yl pyrophosphate (UGP) also suggests the participation of two groups with pK_a values between 9.2 and 9.3, one of which is likely to be Lys153.[209] Two mutants of the epimerase, K153M and K153A, had been constructed to test the role of Lys153.[210] Unlike the wild-type enzyme, these mutants could not react with glucose in the presence of UMP. Furthermore, the hydride reduction of the enzyme-bound NAD$^+$ in these mutants was insensitive to the addition of UMP to the reaction mixture. Evidently the removal of Lys153 dramatically reduced the susceptibility of NAD$^+$ toward reducing agents.

A ground-state destabilization model has been proposed to account for the activation of NAD$^+$ toward reducing agents upon binding of uridine nucleotides to UDP−galactose 4-epimerase.[170,206,210] As illustrated in Scheme 21, binding of uridine

Scheme 21



nucleotides may induce a conformational change of the enzyme that moves Lys153 near the N-1 of the nicotinamide ring. The destabilization of the nicotinamide ring due to charge repulsion results in a minimization of the energy barrier between the ground state and the transition state for the reduction of NAD$^+$. This model represents an uncommon case in which the enzyme accelerates a chemical transformation by selectively destabilizing the ground state along the reaction coordinate through conformational changes, as opposed to the widely held belief that enzymes exert their catalytic power by the selective stabilization of reaction transition states.

However, there are apparent inconsistencies between this model and the recently available structural information. In the X-ray crystal structure of the enzyme, Lys153 is not located near C-1′ of the nicotinamide ring, indicating that a large movement would be necessary for it to impose any significant effects on the redox properties of the cofactor.[211] Moreover, although it had been shown that the conformational changes of this enzyme during catalysis determine the course of the reaction, it appears that the enzyme can accommodate various sugars in its active site by shuffling water molecules rather than by changing the conformations of the amino acid side chains.[212] These apparent contradictions may arise due to the fact that structural information, while critical for the visualization of the binding site and gaining insights into the mechanism, is static in nature. Therefore, such information may not truly reflect the dynamic movements of the enzyme along the reaction coordinate. Clearly, more experiments are needed to help resolve these mechanistic issues.

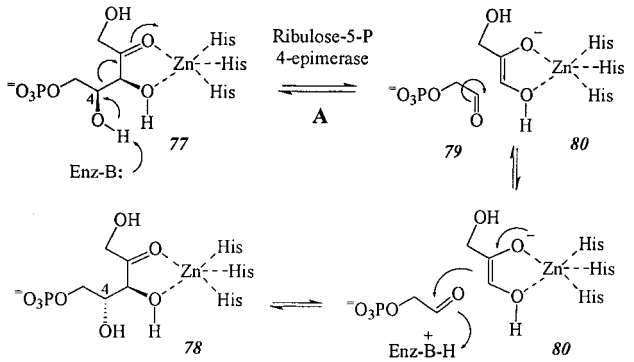
B. L-Ribulose-5-phosphate 4-Epimerase

L-Ribulose-5-phosphate 4-epimerase catalyzes the C-4 stereoinversion of L-ribulose-5-phosphate (**77**, L-Ru5P) to form D-xylulose-5-phosphate (**78**, D-Xu5P).[213,214] This enzyme activity is inducible in *E. coli*,[215] *Lactobacillus plantarum*,[216] *Bacillus subtilis*,[217] and *Aerobacter aerogenes*[218−220] under growth conditions where L-arabinose, L-arabitol, or L-xylose is used as the sole carbon source. The *E. coli* enzyme consists of four identical subunits, and each has a molecular mass of 25.5 kDa. Each subunit of this homotetrameric enzyme contains a metal-binding site.[221] It has also been shown that a divalent transition-metal cation is required for the activity of L-ribulose-5-phosphate 4-epimerase. The bound metal ion can be stripped off the enzyme by treatment with EDTA. The activity of the metal-reconstituted enzymes follows the order $Mn^{2+} > Co^{2+} > Ni^{2+} > Ca^{2+} > Zn^{2+} > Mg^{2+}$,[222] although Zn^{2+} has been found to be the most abundant divalent metal associated with the recombinant *E. coli* enzyme.[214] This enzyme links the metabolism of arabinose to the intermediates found in the pentose phosphate pathway, enabling the bacteria to utilize arabinose as a source for metabolic energy.[223]

1. Possible Mechanisms for C-4 Epimerization

The mechanism of this epimerization cannot rely on a direct deprotonation−reprotonation strategy because the inversion of configuration occurs at an unactivated stereocenter. The involvement of a rate-determining C−H bond-cleavage event in this catalysis can also be excluded, since no primary kinetic isotope effect was detected in the epimerization of D-[4-^{3}H]Xu5P or L-[4-^{3}H]Ru5P.[221,224] Furthermore, characterization of this epimerase failed to detect the presence of enzyme-bound NAD^+, and the addition of any form of the nicotinamide cofactor showed no effect on the activity of the enzyme.[225] Therefore, it seems unlikely that L-ribulose-5-phosphate 4-epimerase follows a mechanism analogous to that of the previously discussed UDP−galactose 4-epimerase.[226a]

Scheme 22

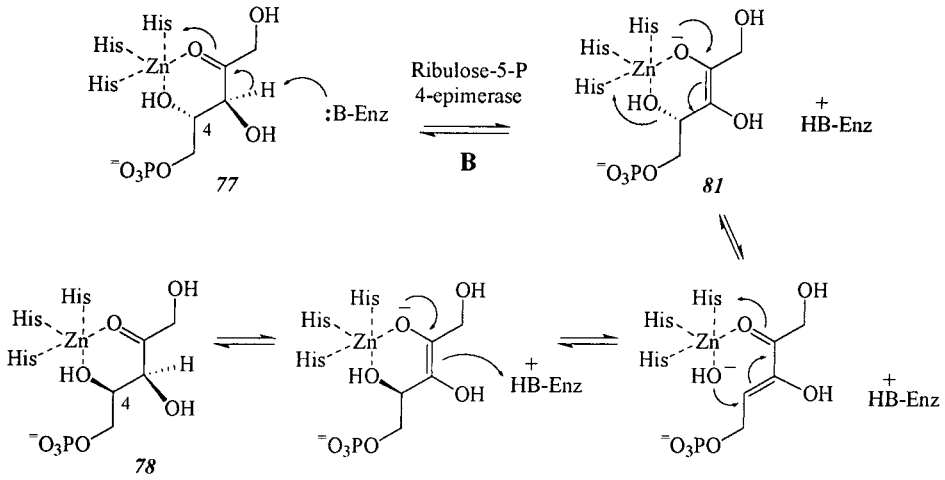


Several mechanisms can be envisioned that are consistent with the above experimental observations (Schemes 22−24). In Mechanism A (Scheme 22), the reaction is initiated by a retro-aldol-type C_3-C_4 bond cleavage to give glycolaldehyde phosphate (**79**) and dihydroxyacetone in its enediolate form (**80**). A rotation of glycolaldehyde phosphate (**79**) followed by an aldol condensation with **80** would generate the epimerized product.[214,222] In this mechanism, the divalent metal ion at the enzyme active site serves as an electrophilic catalyst to stabilize the developing negative charge during the formation of the enediolate intermediate. Mechanism B (Scheme 23) proceeds with deprotonation at C-3 by an active-site base followed by the elimination of a hydroxide anion from C-4. Here, the role of the divalent metal is to accept this hydroxide and to deliver it back to the opposite face of the enediolate intermediate (**81**).[214,222] However, a recent study failed to detect the presence of a significant deuterium primary kinetic isotope effect that should be associated with this mechanism.[226b] The epimerization reaction can also proceed via a stepwise migration of a carbonyl group through proton relays as depicted in Mechanism C (Scheme 24).[226a] However, neither solvent-derived oxygen nor hydrogen atoms are incorporated into the product or the recovered starting material in the enzyme-catalyzed reaction, suggesting that the hydroxide anion and protons removed from the substrate would have to be sequestered from the bulk solvent if this mechanism were operative.[227]

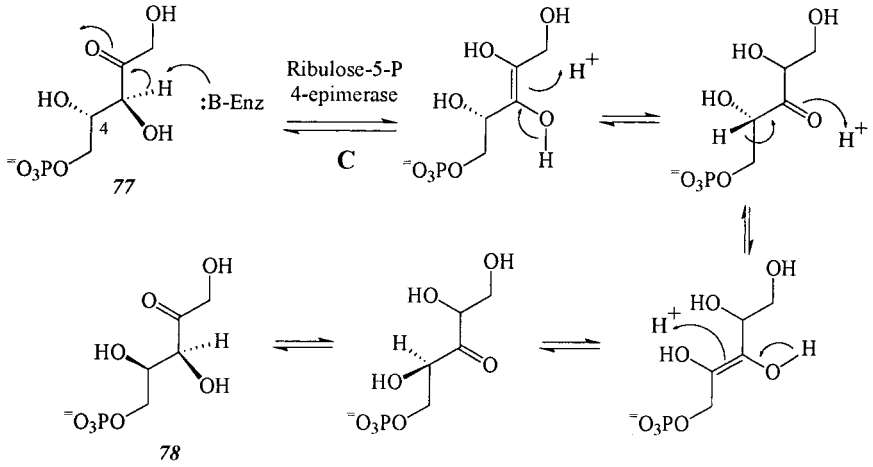
2. Similarity of L-Ribulose-5-phosphate 4-Epimerase to Class II Aldolases

Sequence alignments have shown that L-ribulose-5-phosphate 4-epimerase and the class II bacterial aldolases are highly homologous.[214,228] In fact, the *N*-terminal domains of L-ribulose-5-phosphate 4-epimerase and L-ribulose-1-phosphate aldolase (FucA) were found to be 35% identical. More importantly, the 4-epimerase and the class II aldolases are both divalent metal-dependent. It has been well established that, in contrast to the class I aldolases where an essential lysine residue forms a Schiff base with the substrate carbonyl group to serve as an electron sink, the class II aldolases utilize a divalent metal ion as a Lewis acid catalyst to stabilize the electron-rich reaction intermediates.[229,230] In view of the high sequence homology between L-ribulose-5-phosphate and class II aldolases, it is likely that their mecha-

Scheme 23



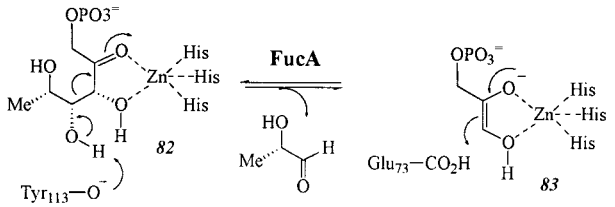
Scheme 24



nisms are also closely related.[214,228] Namely, L-ribulose-5-phosphate 4-epimerase may achieve its catalysis via the metal-assisted cleavage and reformation of a C−C bond as depicted in Mechanism A.

Interestingly, FucA is the only protein among class II aldolases whose structure has been determined at high resolution.[228,231] Three histidine residues (His92, His94, and His155) and a glutamate residue (Glu73) have been found to coordinate the active site Zn^{2+} in the resting state of this enzyme.[231] Residue Glu73, which is subsequently displaced by the substrate (**82**), also serves as a general acid in the formation of the product, dihydroxyacetone phosphate (**83**, Scheme 25).[214,228,231,232a] The counterparts of the above metal

Scheme 25



ligands in L-ribulose-5-phosphate 4-epimerase are probably Asp76, His95, His97, and His171.[214,232b] Individual replacement of the first three (Asp76, His95, and His97) with asparagine provided mutant enzymes with almost unchanged K_m values. However, the catalytic efficiencies of these mutant proteins were dramatically reduced, especially in the absence of exogenous Zn^{2+}.[214] These data confirmed that the aforementioned residues are indeed involved in the metal ion coordination or the general base catalysis but do not participate in the binding of substrate.[214]

3. Aldolase Activity of H97N Mutant

Since the overall reactions and mechanisms of L-ribulose-5-phosphate 4-epimerase and class II aldolases are closely related, the epimerase may also possess aldolase activity of its own, resulting in the formation of L-ribulose or D-xylose from dihydroxyacetone and glycolaldehyde phosphate. However, the wild-type epimerase does not show a significant level of such activity nor are these compounds potent inhibitors of the enzyme.[214] On the contrary, the H97N mutant of L-ribulose-5-phosphate 4-epimerase exhibits good aldolase activity while retaining a substantial amount of epimerase activity (~33%).[214] Incubation of this mutant protein with the epimeric sugar phosphate substrates, L-ribulose-5-phosphate (**77**) or D-xylulose-5-phosphate (**78**), led to the formation of dihydroxyacetone. It is possible that the enzyme may employ two spatially distinct bases to initiate the retro-aldol reaction with these epimeric substrates.[214] The H97N mutant also accepts dihy-

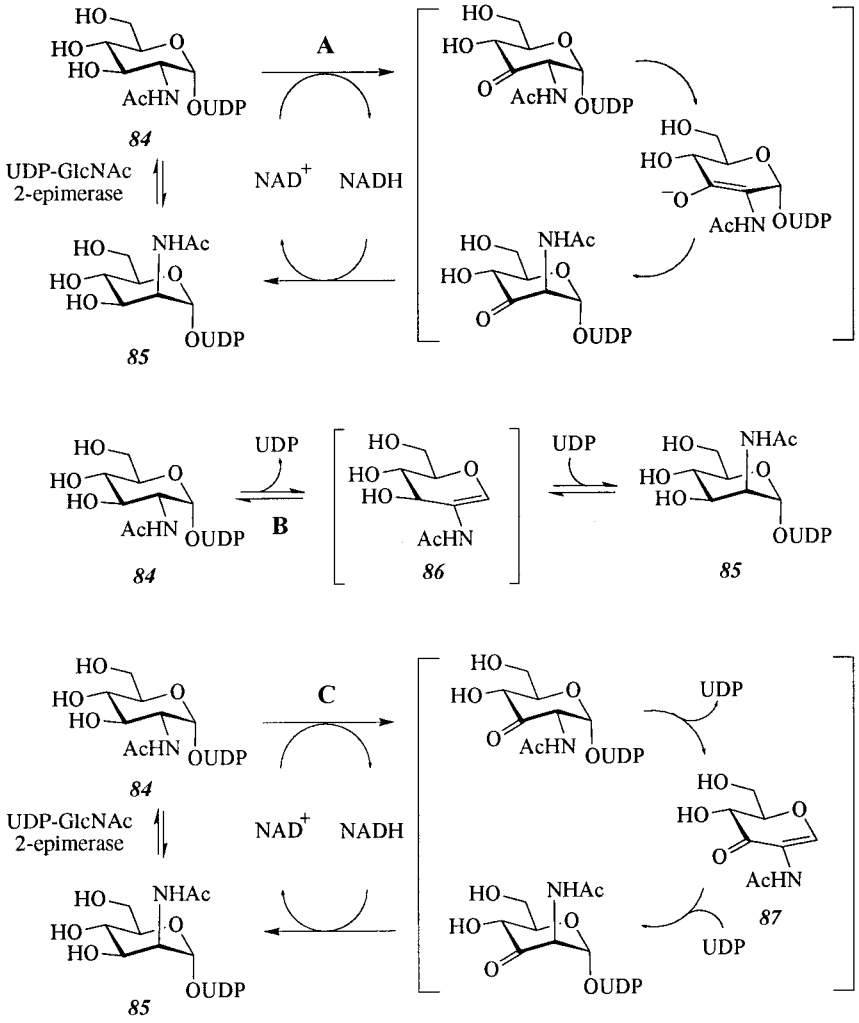
droxyacetone (**80**) and glycolaldehyde phosphate (**79**) to produce L-ribulose-5-phosphate and D-xylulose-5-phosphate. However, glycolaldehyde phosphate inhibits the epimerase activity of the H97N mutant in a competitive fashion (K_I = 0.37 mM).[214] Thus, the substitution of His97 with Asn not only slows down the epimerization reaction, but also loosens the sequestered nature of enzyme and the reaction intermediates. As a result, the solvent accessibility to the active site is increased at the expense of fidelity. Residue Asp76 is another important residue which is believed to function as the general base. It could either promote the C−C bond-cleavage process, analogous to the role played by Tyr113 in FucA, or facilitate the formation of a new C−C bond between the intermediates as mediated by Glu73 in FucA.[231]

A major distinction between L-ribulose-5-phosphate 4-epimerase and the aldolases is the stereochemical stringency of their catalyses. With a few exceptions, aldolases exhibit high stereoselectivity of bond formation by controlling the rotational freedom of their reaction intermediates either through Schiff base formation or metal coordination.[229,233−240] However, L-ribulose-5-phosphate 4-epimerase catalyzes a nonstereospecific C−C bond-cleavage/formation process to achieve epimerization, presumably because its intermediates have a certain degree of rotational flexibility.[214] It is expected that dihydroxyacetone (**80**), as a bidentate ligand of the divalent metal ion, would experience significant steric restraints while glycolaldehyde phosphate (**79**), which is not tightly bound, may undergo rotation without introducing too much conformational hindrance in the active-site.[214] As illustrated by Mechanism A, such a rotation would expose the opposing faces of **79** to accept electrons from dihydroxyacetone to generate two epimeric products. Structural knowledge of L-ribulose-5-phosphate 4-epimerase will undoubtedly aid in the establishment of a more conclusive mechanistic model for its reaction.

C. UDP–*N*-Acetylglucosamine 2-Epimerase

The mammalian UDP−*N*-acetylglucosamine 2-epimerase catalyzes the conversion of UDP−*N*-acetylglucosamine (UDP−GlcNAc, **84**) to UDP and *N*-acetylmannosamine (ManNAc) in an essentially irreversible process. While this enzyme has been isolated from rat liver, only limited studies have been conducted on it due to its extreme instability. This murine enzyme is specific for UDP−GlcNAc and is inactive toward other glycosyl derivatives such as UDP−*N*-acetylgalactosamine, UDP−*N*-glycosylglucosamine, *N*-acetylglucosamine-1-phosphate, and *N*-acetylglucosamine.[241−244] A similar enzyme activity that interconverts the diastereomers UDP−*N*-acetylglucosamine (**84**) and UDP−*N*-acetylmannosamine (**85**, UDP−

Scheme 26



ManNAc) has also been found in various bacteria, such as *E. coli*,[245−247] *Bacillus cereus*,[248] or *Aerobacter cloacae*.[249] This enzymatic reaction is physiologically significant since its product, ManNAc, is used for the biosynthesis of a variety of bacterial surface polysaccharides, such as the capsule polysaccharide found in both the Gram-positive and Gram-negative encapsulated bacteria.[250−252] The ManNAc residue also serves as a bridge between the peptidoglycan and teichoic acids which are the source of negative charges on the cell surface that are important for cell division.[253,254]

UDP−*N*-Acetylglucosamine 2-epimerase purified from *E. coli* JM109 (DE3, pKI86) cells is a homodimer with a subunit molecular mass of 38 kDa. This enzyme shows optimal catalytic activity in the pH range of 7∼9. A Hill coefficient of 2.0 for the dependence of the rate of epimerization on the concentration of UDP−GlcNAc has been obtained. Since UDP−GlcNAc is also required for the reverse reaction, where UDP−ManNAc is the substrate, it has been suggested that either a modulator site is present in the enzyme or the active site of one subunit must be occupied by UDP−GlcNAc in order for the other subunit to function.[246,247,255] At equilibrium, the enzyme-catalyzed epimerization favors the formation of UDP−GlcNAc by a 9:1 margin.[247,255] The apparent K_m for UDP−GlcNAc is 0.63 mM, while that for UDP−ManNAc in the presence of 1 mM of UDP−GlcNAc is 0.5 mM.[246,255]

1. Possible Mechanisms for C-2 Epimerization

The reaction catalyzed by UDP−*N*-acetylglucosamine 2-epimerase is of great mechanistic interest, since the epimerization takes place at an unactivated stereocenter. Early studies on this enzyme have allowed the postulation of three possible mechanisms for the epimerization at C-2, which are highlighted in Scheme 26. Mechanism A is based on the assumption that this enzyme is NAD^+-dependent. This proposal involves the transient oxidation at C-3 of **84** mediated by the NAD^+ cofactor, deprotonation at C-2 followed by reprotonation from the opposite side, and the final reduction of the C-3 keto group to give **85** and regenerate the cofactor.[256] In Mechanism B, the substrate **84** undergoes an *anti*-elimination of UDP from UDP−GlcNAc to form a 2-acetamidoglucal intermediate **86**. A subsequent rebound of UDP in a syn addition could form the product UDP−ManNAc (**85**).[255] Elements from the these two mechanisms can be incorporated into a hybrid mechanism (Mechanism C) in which a tightly bound NAD^+ is also a prerequisite. The cofactor could be used for a transient oxidation at C-3 of **84**, which would promote the elimination of UDP.[255]

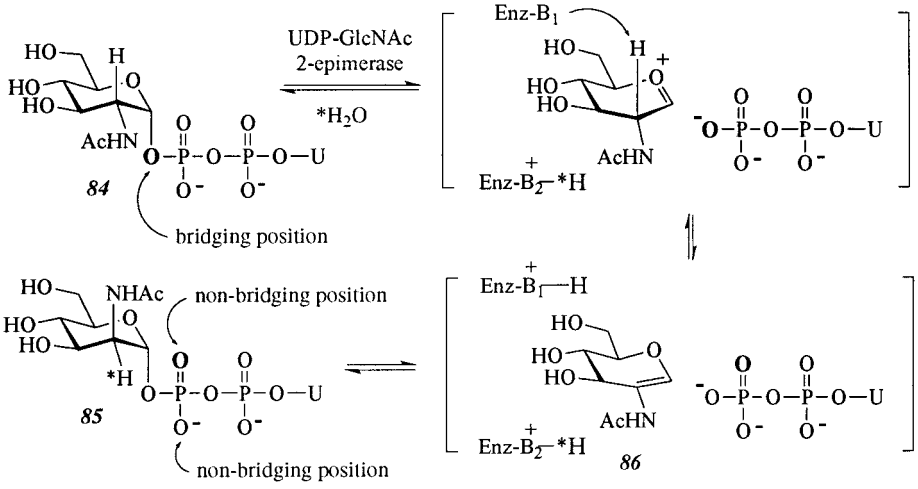
The identification and overexpression of the gene encoding UDP−*N*-acetylglucosamine 2-epimerase from *E. coli*[255,257,258] has greatly aided in unveiling the mechanistic details of this enzyme catalysis. The amino acid sequence of this enzyme does not contain any of the consensus sequences shared by the NAD^+ binding proteins.[54] Unlike the previously discussed UDP−galactose 4-epimerase, no associated NAD^+ coenzyme has been detected with the purified enzyme. In addition, the exogenous addition of nicotinamide cofactors has no effect on the enzyme activity.[255,259] Therefore, it is unlikely that Mechanisms A or C is involved in the catalytic process.

The above conclusion is also supported by the findings that isotope incorporation at C-2 was observed when the enzymatic reaction was carried out in D_2O or T_2O.[255,256] The incorporation of solvent-derived hydrogen at the C-2 position of both UDP−GlcNAc and UDP−ManNAc also argues against a hydride-transfer mechanism similar to that of UDP−galactose 4-epimerase catalysis.[255,256] In addition, enzymatic incubation with ^2H-labeled substrate suggested that the cleavage of the C_2''−H bond that occurs during epimerization is at least partially rate-limiting, as indicated by the primary kinetic isotope effect of 1.8 ± 0.1.[255]

2. Elimination–Addition Mechanism for UDP–N-Acetylglucosamine 2-Epimerase

One of the most informative studies to elucidate the mechanism of this enzyme was the positional-isotope exchange (PIX) experiment in which UDP−GlcNAc enriched with ^{18}O at the anomeric (bridging) position was incubated with *E. coli* UDP−*N*-acetylglucosamine 2-epimerase (Scheme 27). After incubation, isotope scrambling, leading to the incorporation of the ^{18}O label at the bridging as well as nonbridging

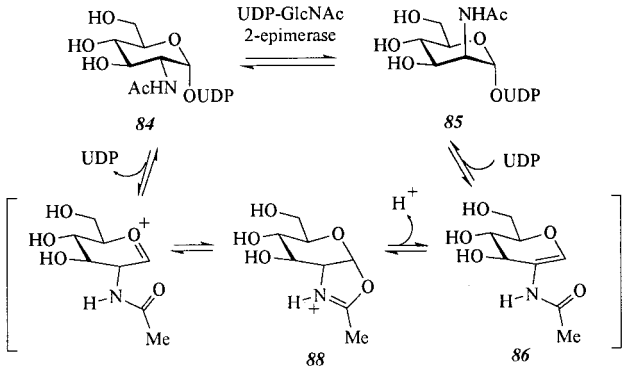
Scheme 27



positions of the phosphate group, was observed in the product and the recovered substrate. This observation indicated a mechanism involving cleavage of the anomeric C−O bond during catalysis.[255] The fact that UDP and 2-acetamidoglucal **86** gradually accumulate in the enzyme active site during the reaction lent further credence to Mechanism B. Although the products of the reaction catalyzed by the mammalian UDP−*N*-acetylglucosamine 2-epimerase are UDP and free ManNAc, 2-acetamidoglucal has also been speculated as an intermediate in this transformation. In this case, it is proposed that the ManNAc product is formed from an addition of a water molecule across the double bond of 2-acetamidoglucal.[244]

Taken together, the evidence supports Mechanism B as being most likely in the reaction catalyzed by UDP−*N*-acetylglucosamine 2-epimerase. The proposed β-elimination of UDP could proceed through either an E1cb, E2, or E1 mechanism.[255] The possibility of an E1cb mechanism is doubtful given the weak acidity of the proton at C-2. An E2 mechanism is also unlikely since the prerequisite *anti*-elimination is not possible in the case of ManNAc. It should be noted that neighboring-group participation by the acetamido functionality may facilitate the elimination reaction (Scheme 28), since nonenzymatic formation

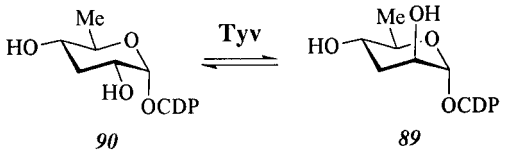
Scheme 28



of oxazoline is well-precedented in molecules derived from GlcNAc and ManNAc.[260] In addition, an oxazoline intermediate has also been proposed to exist in the catalytic mechanism of *N*-acetyl-β-hexosaminidase.[261] Therefore, inhibition studies of this enzyme by structural analogues of the possible oxazoline intermediates **88** merit further investigation. Interestingly, the analogue UDP−3-deoxy-GlcNAc is not a substrate but a modest inhibitor for the *E. coli* enzyme.[255] This result raised a possibility that accumulation of 2-acetamidoglucal **86** may arise from the premature reduction of the enone intermediate **87** during normal turnover as proposed in Mechanism C. However, since the involvement of NAD^+ in the catalysis is unlikely, the observed inhibition by UDP−3-deoxy-GlcNAc may simply result from destabilization of a positively charged intermediate (or transition state) formed during the reaction.

D. CDP–Tyvelose 2-Epimerase

Tyvelose (**5**) is a 3,6-dideoxyhexose present at the nonreducing end of the *O*-antigen of some Gram-negative bacteria, such as *Salmonella typhi*[262] and *Yersinia pseudotuberculosis* IVA.[263] The sugar nucleotide CDP−tyvelose (**89**) is the only 3,6-dideoxyhexose that is derived directly from another 3,6-dideoxyhexose. The precursor for **89** is CDP−paratose (**90**), which is converted to **89** by the enzyme CDP−tyvelose 2-epimerase.[9] This reaction is another intriguing example of an epimerization at an unactivated stereocenter.



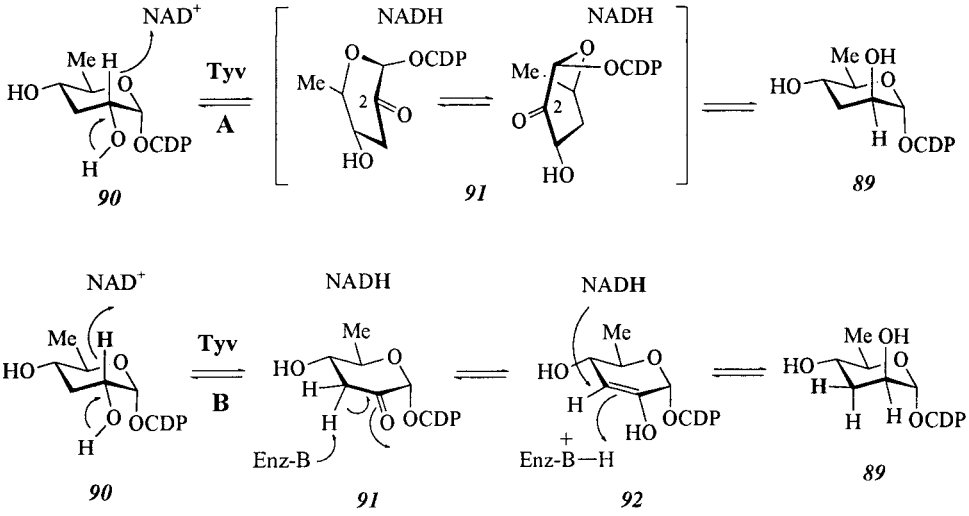
The gene encoding CDP−tyvelose 2-epimerase (*tyv*) has recently been identified and sequenced from *Yersinia pseudotuberculosis* IVA.[264] A BLAST search revealed strong sequence homology of this enzyme to the NAD^+-dependent enzymes, TDP−glucose 4,6-dehydratase from *Salmonella typhimurium* (29% identity and 47% similarity) and the previously discussed UDP−galactose 4-epimerase (see section IV.A) from *E. coli* (26% identity and 41% similarity). The *N*-terminus of its translated sequence contains a Rossmann fold (^{7}**GG**C**G**FL^{13}**G**) that is typical for nucleotide diphosphate-binding proteins.[54] Another conserved motif present in most short-chain dehydrogenases, **Y**XXX**K**, is also seen in this protein (^{164}**Y**GCS^{168}**K**). These pieces of evidence strongly suggested that CDP−tyvelose 2-epimerase is a member of the short-chain dehydrogenase family[87] and its activity depends on the presence of the nicotinamide cofactor.

1. Biochemical Properties of CDP–Tyvelose 2-Epimerase

The recombinant *Y. pseudotuberculosis* epimerase has been overproduced in *E. coli* BL21 (DE3) and purified for mechanistic studies.[265] Detailed quantitation established a stoichiometry of one NAD^+/NADH per monomer in the 152 kDa holoenzyme tetramer. The presence of bound NAD^+ and NADH in the purified enzyme is also evident in the UV−vis spectrum of this enzyme. The protein shows maximal absorption at approximately 275 nm with a broad band extending to 450 nm, a characteristic feature known as "Racker Bands"[266] resulting from a charge-transfer transition between the bound NAD^+ and a residue in the protein core.[265]

CDP−tyvelose 2-epimerase displays maximal activity at pH 7.5. The epimerization at C-2 is reversible, with the equilibrium slightly favoring the formation of CDP−tyvelose (K_{eq} = 1.22 at pH 7.5). The K_m of CDP−paratose (**90**) was determined to be 6.8 ± 0.4 μM, and that of CDP−tyvelose (**89**) was determined as 170 ± 20 μM. Interestingly, the reverse reaction that results in the formation of CDP−paratose from CDP−tyvelose is almost 10 times faster with a k_{cat} of 240 ± 10 min^{-1} as compared to a k_{cat} value of 22 ± 1 min^{-1} for the forward reaction. No divalent metal ion was found to be necessary for the activity of the epimerase, consistent with the observations made for the short-chain dehydrogenases.[87]

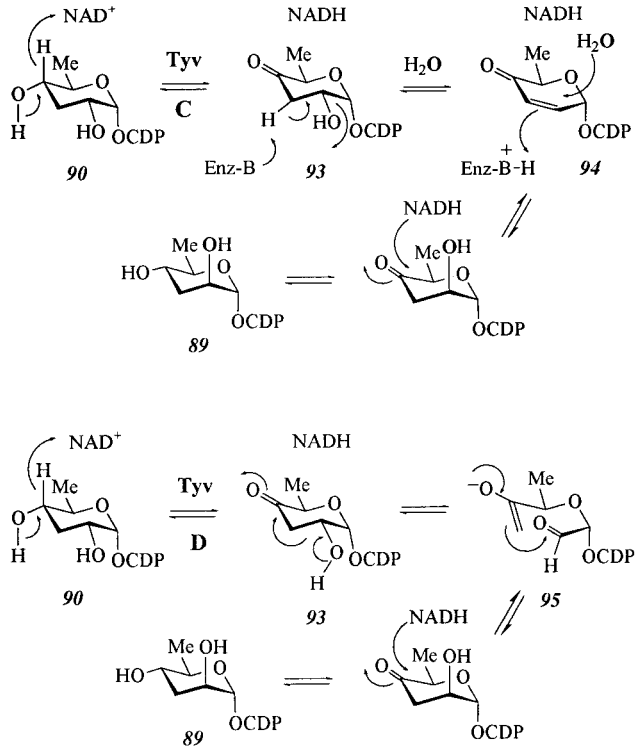
Scheme 29



2. Possible Mechanisms for C-2 Epimerization

The presence of NAD^+/NADH in purified CDP−tyvelose 2-epimerase indicates that this enzyme may follow a redox mechanism analogous to that used by UDP−galactose 4-epimerase (Mechanism A) (see section IV.A). As depicted in Scheme 29, this mechanism is highlighted by the formation of a 2-ketohexose intermediate **91**. However, three other mechanisms are also worth considering (Mechanisms B−D). Mechanism B involves an initial oxidation at C-2 which may sufficiently increase the acidity of the C-3 hydrogen to allow deprotonation by an active-site base. Subsequent reduction of the enol/enolate intermediate **92** at C-3 via an intramolecular hydride transfer would result in the racemization of the C-2 stereocenter (Scheme 29). Mechanisms C and D (Scheme 30) proceed through the formation of a 4-ketohexose intermediate **93** arising from C-4 oxidation. The key step in Mechanism C is a reversible dehydration executed by a general base on the enzyme. The nonstereoselective addition of water to the enolate intermediate **94** at C-2 could produce a mixture of epimers. Mechanism D involves the formation of a 4-ketohexose intermediate **93** that could first undergo a retro-aldol ring opening. The resulting acyclic species **95** may rotate about its C-1/C-2 bond prior to an aldol-type ring closure to give both epimers. A final reduction at C-4 by a hydride derived from the reduced nicotinamide cofactor completes the catalytic cycle in Mechanisms C and D.[266]

Scheme 30



3. Distinguishing between Mechanisms Involving C-2 or C-4 Oxidation

Various experiments were designed to differentiate among these possibilities in the catalytic mechanism of CDP−tyvelose 2-epimerase. When CDP−D-paratose **90** was incubated with the enzyme in buffers prepared with either ^2H_2O or $H_2{}^{18}O$ and the reaction products were analyzed by ^1H NMR and FAB-MS, no exchange of hydrogen or oxygen atoms with the solvent was observed.[265] This evidence argues against Mechanism B and Mechanism C, although it might be reasoned that the enzyme active site could be well-sequestered from the bulk solvent. However, when CDP−[2-^{2}H]paratose was used in the enzyme incubation, no hydrogen migration from C-2 to C-3 could be detected by ^1H NMR,[265] which rules out Mechanism B.

Initial attempts to distinguish between Mechanisms A and D were based on the measurement of a kinetic isotope effect (KIE) for the transfer of a deuterium from the C-2 or the C-4 position to NAD^+, respectively. The KIEs on V and V/K for the C-2 deuterium-labeled substrate were found to be 1.3 ± 0.1 and 1.4 ± 0.2, respectively, and those for the C-4 deuterium-labeled substrate were found to be 0.9 ± 0.02 and 1.2 ± 0.1, respectively. These values are relatively small for a primary KIE and are far from being conclusive to distinguish between Mechanisms

A and D. More informative evidence was obtained from the incubation of the putative Mechanism D reaction intermediate, 4-keto-3,6-dideoxy-D-glucose (**93**), with CDP−D-tyvelose 2-epimerase containing NADH, which resulted in the formation of a mixture of CDP−D-paratose and CDP−D-tyvelose. While these data initially appeared to support Mechanism D,[266] additional experiments cast doubts on the viability of this conclusion. For instance, the analogue CDP−4-deoxy-4-fluoroparatose was found to be a reasonably good substrate for the epimerase.[265b] Since this compound cannot form a 4-keto intermediate, it should not be a substrate for the enzyme if Mechanism D were to be operational.

Additional experiments with the epimerase revealed that prolonged incubation of the epimerase with the substrates resulted in a slow accumulation of NADH, presumably via the formation of a dead-end complex due to the exchange of a reaction intermediate for the substrate in a ternary complex. This phenomenon was exploited to determine whether the C-2 or C-4 hydrogen was being transferred to NAD^+. The outcome was expected to unravel the regiochemistry of the initial oxidation and shed light on the reaction mechanism.

After prolonged incubation of the enzyme with either CDP−[2-^{2}H]paratose or CDP−[4-^{2}H]paratose, the nicotinamide cofactor was released from the enzyme. Due to the difficulties in separating NADH from the excess substrate, the accumulated NADH was converted to NAD^+ prior to FPLC isolation. Since the epimerase is a member of the short-chain dehydrogenase family, as discussed earlier, the hydride being transferred to NAD^+ during catalysis is predicted to reside at the *pro-S* position of C-4 on the nicotinamide ring.[87] Therefore, a *pro-R*-specific dehydrogenase, L-lactate dehydrogenase, was chosen to convert the released NADH to NAD^+ with the expectation that the *pro-S* hydrogen of NADH derived from the substrate would be retained in the resulting NAD^+. Analysis of the isolated NAD^+ by mass spectroscopy found that while incubation of the enzyme with CDP−[4-^{2}H]paratose resulted in no incorporation of deuterium into the cofactor, significant deuterium label was incorporated when CDP−[2-^{2}H]paratose was used as the substrate.[265b] Therefore, this experiment has provided convincing evidence that the first step in the catalytic mechanism of CDP−tyvelose 2-epimerase is C-2 oxidation and strongly supports Mechanism A.

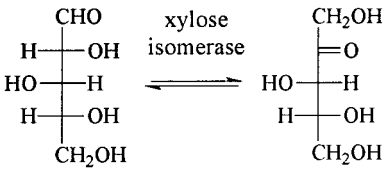
This mechanism is reminiscent of that of UDP−galactose 4-epimerase in which the 4-ketohexulose reaction intermediate rotates about the P_β and glycosyl oxygen bond to present alternate faces to the reducing agent. However, the nonstereospecific oxidation−reduction at C-2 catalyzed by CDP−D-tyvelose epimerase must invoke a different motion of the 2-ketohexose intermediate and perhaps involves additional conformational changes of the active-site scaffold. A better understanding of this intriguing catalyst must await further mechanistic investigations and structural information from X-ray crystallographic studies.

V. Reactions Involving Carbonyl (1,2) Rearrangements

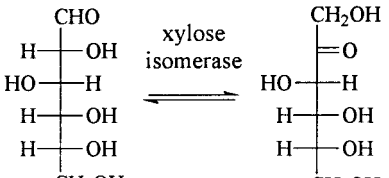
The following section describes the reaction mechanisms of D-xylose isomerase, D-glucarate dehydratase, and D-galactonate dehydratase. D-Xylose isomerase catalyzes the conversion of an aldopentose to a 2-pentulose. D-Glucarate dehydratase and D-galactarate dehydratase, which will be discussed together, catalyze dehydration reactions using aldonic sugars as substrates. The key transformation in these reactions involves functional group manipulation at vicinal centers. While the reaction catalyzed by D-xylose isomerase involves the migration of a carbonyl group from C-1 to C-2, the reactions catalyzed by D-glucarate dehydratase and D-galactonate dehydratase involve the formation of an enolate followed by the elimination of water. In each case, the net result is a de facto internal redox reaction involving vicinal carbons.

A. D-Xylose Isomerase

D-Xylose isomerase is a bacterial enzyme that catalyzes the interconversion of D-xylose (**96**) and D-xylulose (**97**). It is inducible under conditions where xylose serves as the sole carbon source for growth and metabolism.[267] However, this enzyme displays a broad specificity, accepting the α-anomers of many other pentoses, hexoses, sugar alcohols, sugar phosphates, and deoxysugars as substrates.[268,269] Of particular importance is the fact that D-xylose isomerase is able to catalyze the conversion of D-glucose (**98**) to D-fructose (**99**).[270] Therefore, this enzyme is also



96 *97*



98 *99*

known as D-glucose isomerase and is one of the most widely used industrial enzymes. In fact, it is used as the catalyst for an annual production of more than a billion pounds of high-fructose corn syrup, a common food and drink sweetener.[270]

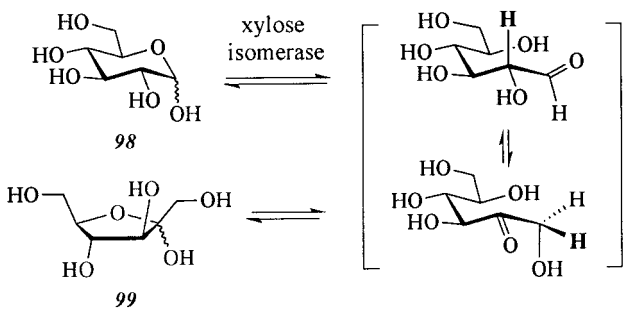
The chemical transformation catalyzed by D-xylose isomerase is intriguing, and the elucidation of this enzymatic reaction has attracted a lot of attention due to the important industrial applications of this enzyme. D-Xylose isomerase has been isolated from a variety of *Actinomycetaceae* and many other Gram-negative and Gram-positive bacterial species. The K_m values for D-xylose, D-glucose, and D-ribose depend

on the source of the enzyme but are usually in the millimolar range. Most isoforms of this enzyme show optimal activity between 80 and 90 °C and pH 7.0 and 9.0.[270] The active enzymes, with a subunit molecular mass varying from 52 to 191 kDa, exists as a dimer or a tetramer.[271,272] The presence of divalent metal cations is essential for the activity and stability of D-xylose isomerase.[273] Under physiological conditions, it is believed that Mg^{2+} is the preferred metal cofactor for this enzyme.[271] The family of xylose isomerases can be classified into two groups based on their metal-dependency. Group I isomerases are usually isolated from the *Actinomycetaceae* species and contain two metal ions per monomer (designated Mg-1 and Mg-2) in their active forms. Group II enzymes have a stoichiometry of 1 mol of metal per mol of monomer and are usually obtained from other bacterial sources.[274]

Most isolated D-xylose isomerases have shown a high degree of sequence homology to each other. With the availability of overproduced recombinant proteins, the X-ray crystal structures of the enzymes from several *Streptomyces* species and from *Arthrobacter* have been solved. Not surprisingly, given the high degree of sequence homology among them, the three-dimensional structures of these proteins have been found to be very similar.[275−286] Each subunit of the isomerase folds into an eight-stranded α/β barrel that is better known as the "triose−phosphate isomerase (TIM) barrel" motif. This motif is characteristic for almost all other aldose−ketose interconverting enzymes. The active site is located near the center of the TIM barrel. Each subunit also has a *C*-terminal helical domain that interacts with the neighboring subunit.[281,287−289] Although the three-dimensional structure of D-xylose isomerase is very similar to that of triose phosphate isomerase, it employs a different strategy to accomplish the isomerization of simple sugars.

Through biological assays and X-ray crystallographic studies, it has been established that the D-xylose isomerases catalyze the interconversion of only the α-anomers of aldoses and ketoses.[268,269,284,285,290−295] Interestingly, the open-chain forms of these sugars have been found at the enzyme active site, indicating that they may be the reaction intermediates.[284,285,296,297] Therefore, the mechanism for the D-xylose isomerase-catalyzed reaction appears to consist of at least three chemical transformations: (1) ring opening, (2) hydrogen transfer between C-1 and C-2, and (3) ring closure (Scheme 31). The first and last steps in this proposed sequence are supported by a substantial body of experimental evidence. However, the detailed mechanism regarding the hydrogen shift between C-1 and C-2 has remained controversial. The results of many site-directed mutagenesis, chemical modeling, kinetic measurements, X-ray crystallography, and other spectroscopic studies aimed at determining the details of these transformations facilitated by Group I isomerases are highlighted here.

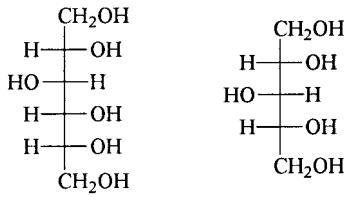
Scheme 31



98 *99*

1. Enzyme-Catalyzed Ring Opening of α-Aldose/Ketose Substrates

The existence of an extended-chain sugar substrate in the enzyme active site as revealed by X-ray crystallography has prompted the hypothesis that an enzyme-facilitated ring opening might be the first step of the D-xylose isomerase-catalyzed reaction.[275,280,284,296,298] This mechanistic postulation is supported by the fact that while D-xylose isomerases have a low affinity for D-xylose and D-glucose with K_m values of 3.3 and 225 mM, respectively, the open-chain sugar analogues, such as D-sorbitol (**100**) and D-xylitol (**101**), are potent competitive inhibitors of this enzyme with K_I values of 0.3 and 6.5 mM, respectively. These K_I values are in the sub-K_m range



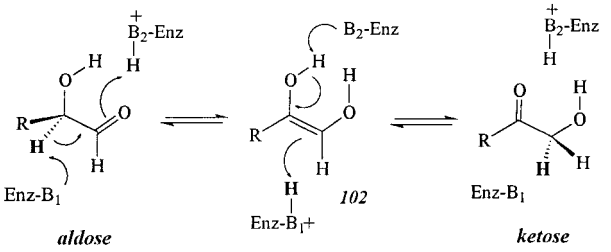
100 *101*

and thus indicate that these acyclic polyols are good mimics of the reaction intermediate(s).[284,299] The formation of ring-opened intermediates as part of the catalytic mechanism is also supported by the study of the E180K mutant of the *Streptomyces rubiginosus* enzyme. It was found that this mutant, which contains only one metal ion at the active site, is unable to catalyze the overall isomerization but retains the ability to catalyze the ring opening of the substrate.[300] Among the residues that have been implicated to play a role in this process are His-53[280,284,285,296,301−304] and Lys-182.[284,298,305,306] It is also believed that a hydroxide bound to Mg-2 may facilitate this transformation.[300]

2. Enzyme-Catalyzed Aldose−Ketose Isomerization

The aldose−ketose isomerization of the substrate in its open-chain form is the rate-limiting step of the overall catalysis.[284,301,307,308] It was determined that an enediol intermediate **102** is involved in all aldose−ketose isomerizations, including those of phosphosugars and simple sugars (Scheme 32).[309,310] Initial evidence for the existence of a *cis*-enediol intermediate in the catalytic mechanism of D-xylose isomerase came from the determination of the stereochemical course of the reaction.[311] It was found that the proton exchange between substrate and solvent was limited, and the C-2 hydrogen of D-xylose was transferred to the 1-*pro*-*R* position of D-xylulose in a suprafacial fashion. This result is consistent with a *re* face

Scheme 32

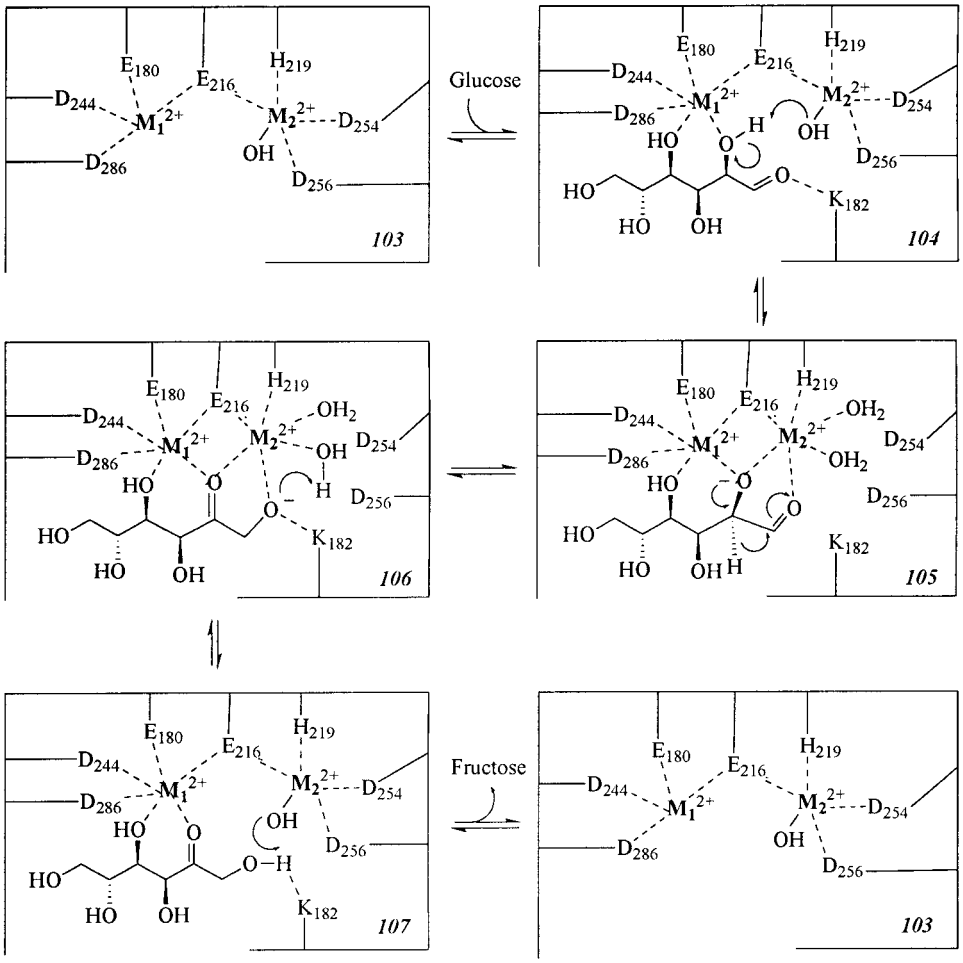


deprotonation and reprotonation mechanism of a planar *cis*-enediol mediated by a monoprotic active-site base.

The X-ray crystal structures of D-xylose isomerase from *Streptomyces olivochromogenes* have been determined in its free state as well as in states complexed with glucose or the substrate analogue 3-*O*-methyl-D-glucose at resolutions of 1.81, 1.96, and 2.19 Å, respectively.[305] This has helped in determining the precise orientation of the substrate at the active site and the interactions of the substrates with the divalent metals and active-site residues. The presence of the two divalent metal ions, Mg-1 and Mg-2, appears to be important for protein's structural/thermal stability and catalytic activity.[275,284,300,312,314] It has been shown that selective removal of Mg-1 in the E180K mutant produced a totally inactive enzyme.[298,300] In the free enzyme, Mg-1 is found in a tetracoordinated state complexed with Glu180, Asp244, Asp286, and Glu216 (**103**, Scheme 33).[305] The binding of substrate changes the coordination state to octahedral with the participation of the O-2 and O-4 oxygen atoms from the substrate (**104**).[305] On the other hand, in the absence of the substrate, Mg-2 is octahedrally coordinated with the active-site residues Glu216, His219, Asp254, Asp256, and a hydroxide anion (**103**).[298,305] The hydroxide bound to Mg-2 is believed to act as a base for the proton abstraction at 2-OH of the substrate, a process that also leads to further movement of this metal.[310,315] It is proposed that the protein ligands Asp254 and Asp256 dissociate from Mg-2 at this stage and are replaced by a water molecule, O-1, and O-2 of the substrate (**105**).[284,305] Thus, the developing negative charge at O-2 is stabilized by both Mg-1 and Mg-2, which in turn pulls the metal centers closer to establish a proper geometry to facilitate the isomerization step (**105**).[305,308]

As depicted in Scheme 33, after tautomerization the partial negative charge generated at O-1 may be stabilized by Lys182 (**106**).[298,301,305,316] This lysine residue together with the metal ions confines the extended reaction intermediate in a *syn*-conformation and thus determines the stereochemical course of the hydride shift process.[284,285] The Mg-2-bound water molecule has been proposed to be the proton donor to O-1 (**106**).[277,285,310,317] Lys182 may be an alternative candidate for the active-site acid according to a

Scheme 33



theoretical study.[318] Each of these transformations is reversible, and the isomerization can also be initiated by a deprotonation of 1-OH of D-xylulose (**107**). Therefore, D-xylose isomerase is able to interconvert aldoses and ketoses. The final step of the enzymatic catalysis is the cyclization of the linear sugar, which is the reversal of the ring-opening reaction, presumably involving the same set of active-site residues. The α-cyclic product is then released into solvent, and the enzyme reverts back to its resting state.[305]

Overall, the reaction catalyzed by Group I D-xylose isomerase relies on the electrophilic catalysis performed by these two metal ions to lower the pK_a of the 2-OH of the substrate and to polarize the hydride−receptor carbonyl of the aldose/ketose substrate.[282,305] The hydrophobic environment at the active site of D-xylose isomerase is also important to enhance the coordination between the metal ions and the sugar hydroxyl groups. In addition, the hydrophobic surface consisting of Trp-136, Phe-93, and Phe-25′ from the neighboring subunit may shield the enzyme active site from solvent.[284]

Clearly the knowledge of the peptide sequences and the three-dimensional structures has made the catalytic mechanism of D-xylose isomerases better understood. Information from structural as well as mechanistic studies has been proved invaluable for the efforts to engineer mutant enzymes with better binding affinity and higher reactivity toward D-glucose.[320−324]

B. D-Galactonate Dehydratase and D-Glucarate Dehydratase

D-Galactonate dehydratase and D-glucarate dehydratase catalyze the dehydration of aldonic sugars. D-Galactonate dehydratase (GalD), which converts D-galactonate (**108**) to 2-keto-3-deoxygalactonate (**109**), is an important enzyme in the metabolic pathways of galactose in both bacteria and mammals.[325] D-

108 *109*

110 *111*

Glucarate dehydratase (GlucD) catalyzes the elimination of a water molecule from D-glucaric acid (**110**) to give 3-deoxy-L-*threo*-2-hexulosarate (**111**). The biological activities of these two proteins were detected several decades ago; however, their mechanistic details had not been studied in detail until recently, when sequence alignment revealed that both GalD and GlucD belong to the mandelate racemase (MR) subgroup of the "enolase superfamily".[326,327] Enzymes that belong to this superfamily catalyze a broad range of reactions on structurally diverse substrates. Interestingly, in each case, catalysis is always initiated by the abstraction of the α-proton of a carboxylic acid. In this section, a brief account will be provided regarding the identification of the encoding sequences for GalD and GlucD from previously unassigned open reading frames of the *E. coli* and *Pseudomonas putida* genomes, the speculations on the mechanism of the enzymatic catalysis judged by the conserved amino acid residues in these polypeptides, and the supporting evidence from biochemical studies of the purified enzymes.

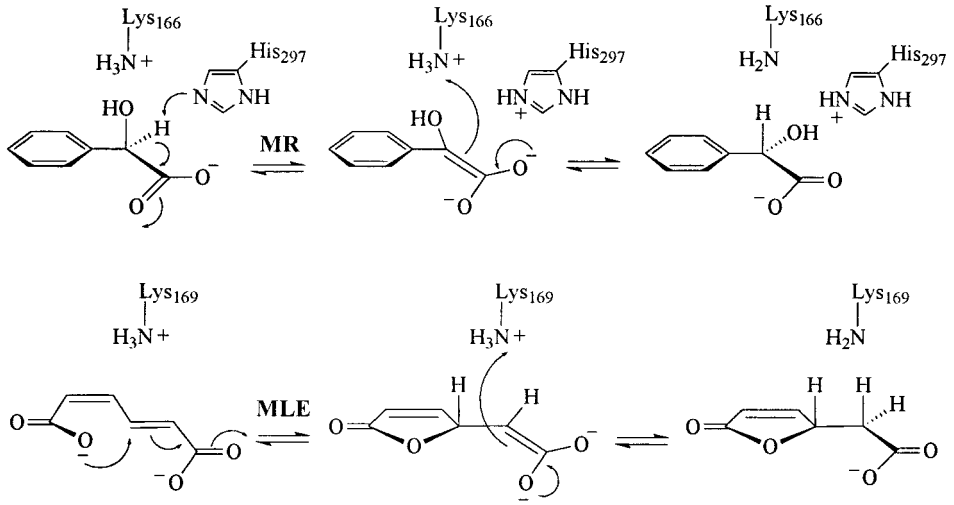
1. Identification of the Gene galD and Mechanistic Study of the galD-Catalyzed Reaction

In a BLAST search of MR homologues from various DNA and protein data banks, five open reading frames of unknown functions were assigned as MR homologues.[326] One sequence, ORF f587, was located in the vicinity of the *dgo* operon involved in the metabolism of D-galactonate in *E. coli*. This operon contains four genes including *dgoD*, which is a candidate to encode the galactonate dehydratase.[325] Detailed sequence analysis showed that the *C*-terminal two-thirds of the f587 translated polypeptide sequence is homologous to MR and other members of the enolase superfamily. When f587 DNA was cloned into the vector pUC18, the host *E. coli* DH5α cells indeed exhibited the desired galactonate dehydratase activity.[328]

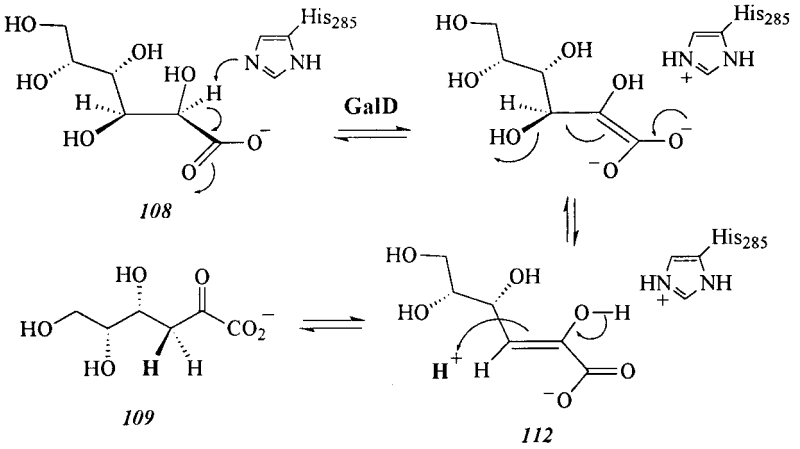
At this point, it would be pertinent to discuss some properties of MR and the muconate-lactonizing enzyme (MLE). Mandelate racemase is a divalent metal Mg^{2+}-dependent enzyme that catalyzes the interconversion of the mandelate enantiomers using a general base−general acid mechanism (Scheme 34). The racemization proceeds by stereospecific α-proton abstraction of one mandelic acid enantiomer followed by reprotonation of the enolic intermediate from the opposite face.[328] The X-ray crystallographic studies of MR have revealed that His297 is responsible for the proton abstraction from (*R*)-mandelate, while Lys166 is the (*S*)-mandelate-specific base.[230,329,330] Another important active-site residue is Lys164 that presumably serves as an electrophilic catalyst. These two lysine residues constitute a diagnostic "KXK" catalytic motif.[326] Residue Glu317 is believed to be the general acid catalyst that delivers a proton to the substrate carboxyl group and helps lower the energy barrier of the α-proton abstraction dramatically.[326,331] The carboxylic side chains of Asp195, Glu221, and Glu247 as well as the carboxyl group of mandelate serve as ligands to the Mg^{2+} ion.[326]

Muconate-lactonizing enzyme (MLE) catalyzes the conversion of *cis*,*cis*-muconate to muconolactone (Scheme 34). Although Mn^{2+} ions instead of Mg^{2+} have been found to be associated with MLE, the primary sequence and three-dimensional structure of this enzyme are strikingly similar to those of MR.[332−334] A major difference between these proteins is the absence of a residue corresponding to His297 in MLE. The direct consequence of this alteration is the stringent stereospecificity displayed by MLE. The reverse reaction is initiated by abstraction of the

Scheme 34



Scheme 35



α-*pro-R* proton by Lys169, a homologue of Lys166 in MR, to carry out a *syn*-*β*-elimination. The primary sequence of MLE contains amino acids corresponding to the metal-coordinating residues, Lys164 and Glu317, that are found in MR, suggesting a common active-site structural framework. In light of their structural and mechanistic similarities, it has been proposed that MR and MLE may share a common ancestry.[328]

The translated sequence of GalD contains 382 amino acids, a size similar to that of MR and muconate-lactonizing enzyme (MLE) (358 and 372 amino acids, respectively). This protein has a histidine residue (His285) corresponding to His297 of MR. The putative metal ion ligands, D183, E209, and E235, have also been identified in the GalD sequence. Although the three-dimensional structure of GalD is not yet available, it has been postulated that this enzyme catalyzes the elimination of a water molecule through the initial abstraction of a proton by His[285] from C-2 that bears an *R*-configuration, a chemical step that is shared with the MR-catalyzed transformation (Scheme 35).[328] It is worth mentioning that the consensus sequence K164−X−K166 of MR is replaced by a K144−X−N146 motif in GalD. The substitution of lysine166 in MR with an asparagine in GalD may have facilitated the vinylogous *β*-elimination of the enolic intermediate to give **112** instead of racemization following the α-proton abstraction observed in the MR-catalyzed reaction.

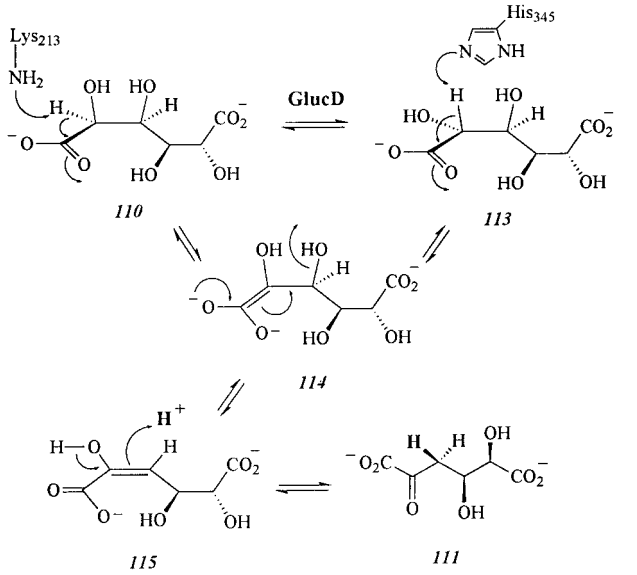
When the enzyme incubation was performed in D_2O, the 3-*pro-S* hydrogen of the product was found to be deuterated by ^1H NMR.[335] Hence, the enzymatic transformation results in a retention of configuration at C-3. A direct assessment of the stereochemical course of the *β*-elimination is not feasible due to the facile tautomerization of the proposed enolic intermediate **112**. However, based on the assumption that the substrate is bound in an extended conformation in the active site of GalD, it has been proposed that this reaction is an anti elimination that occurs via a two-base mechanism.[331] Despite the identification of several critical amino acid residues in GalD, residues that act as the second general base and the homologue of Glu317 in MR are yet to be determined. A complete elucidation of the stereochemical course of this enzyme must await more information from structural and mutagenesis studies.

2. Identification of the Gene glucD and Mechanistic Study of glucD-Catalyzed Reaction

Sequence comparisons using BLAST also led to the functional identification of a glucarate dehydratase from *P. putida*. The desired enzyme activity was observed when the tentative gene was cloned and recombinant protein was purified to homogeneity.[326]

Two metal ligands are conserved in the primary sequence of the putative GlucD active site, and the presence of the KXK motif is also noted. Moreover, the active site of GlucD contains Lys213 and His345, which are the counterparts of K166 and H297 in MR. To probe the stereospecificity of GlucD, the epimer of D-glucarate, L-idarate (**113**), was incubated with D-glucarate dehydratase. The product from this incubation, **111**, was identical to that seen with the incubation of the native substrate. The dehydration reactions of both epimers have comparable K_m and k_{cat} values (k_{cat} = 3 s^{-1} and K_m = 65 μM for D-glucarate and k_{cat} = 4 s^{-1} and K_m = 180 μM for L-idarate), indicating that the binding affinities and reactivities of these compounds are similar.[336] Thus, this enzyme-catalyzed transformation is stereorandom.

Scheme 36



It has been shown that except for a few cases such as the 3-carboxymuconate cycloisomerase-catalyzed reaction,[337] almost all enzyme-catalyzed β-eliminations from a carboxylate α-anion proceed through an anti stereochemical pathway involving a two-base mechanism. The necessity to recruit a second base has been rationalized by the fact that the conjugated acid of the first base, which is responsible for abstraction of the α-proton with high pK_a, is too weak to facilitate the departure of the leaving group through protonation.[331] However, the stereorandom feature of GlucD presents an interesting challenge to the above argument. The utilization of both D-glucarate and L-idarate by GlucD indicates that this enzyme binds both substrates in an identical geometry. Incubations with either D-galactonate (**110**) or L-idarate (**113**) in D_2O yielded the same product (3-deoxy-L-*threo*-2-hexulosarate, **111**) with a deuterium label at the 4-*pro*-*S* hydrogen.[326,335,336] The retention of configuration with respect to the departing 4-OH in both cases suggests that the enzyme-catalyzed α-proton abstraction from either D-glucarate or L-idarate leads to an identical enolic intermediate (**115**).[336] It appears that both Lys213 and His345 are equally competent in initiating proton abstraction, leading to this common enolic intermediate. The above data establish that GlucD can catalyze both syn and anti dehydration reactions with distinct active-site bases and have also prompted the suggestion that D-glucarate dehydratase may have evolved from a racemase ancestor.

The structural and functional similarities of GalD and GlucD to the rest of the enolase superfamily, especially MR and MLE, support the hypothesis that the optimization and evolution of enzymes with alternative substrate specificities or new activities takes advantage of the active-site architectures of existing proteins.[326] Enzymes with diverse substrate specificities and catalytic functions may evolve from proteins catalyzing the same class of chemical reaction by strategic replacements of the active-site residues. Thus, it is likely that the chemistry employed in the transformation overcomes the substrate specificity as one of the determining factors in the course of enzyme evolution.

VI. Reactions Involved in the Formation/ Rearrangement of Hexose Skeletons

So far, the enzymes that have been discussed in this review generate structural variations in sugars by catalyzing functional group transformations, although none of these reactions involves a dramatic change in the hexose skeletal framework itself. However, the final section of this review features two enzymes which catalyze the formation/rearrangement of the sugar backbone. The first enzyme, dehydroquinate synthase, facilitates the rearrangement of a pyranose skeleton to a cyclohexane-based framework. The second enzyme, 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (KDO8P) synthase, catalyzes the de novo synthesis of an eight-carbon sugar by the condensation of a C_5 open-chain sugar unit (arabinose 5-phosphate) with PEP.

A. Dehydroquinate Synthase

3-Dehydroquinate (DHQ) synthase catalyzes the conversion of 3-deoxy-D-arabinoheptulosonic acid 7-phosphate (DAHP, **116**) to dehydroquinate (DHQ, **117**),[338,339] which can be further transformed into an aryl group by downstream processing enzymes. This enzymatic reaction is the second step in the shikimate pathway for the biosynthesis of the three common aromatic amino acids, phenylalanine, tyrosine, and tryptophan, and many other primary and secondary metabolites in plants and microorganisms.[340,341] Since the corresponding pathway does not exist in mammals, inhibitors directed against the enzymes of the shikimate pathway have the potential to be used as antibacterial agents and herbicides. Therefore, DHQ synthase has received much attention over the years as an attractive candidate for the design of potentially useful inhibitors.

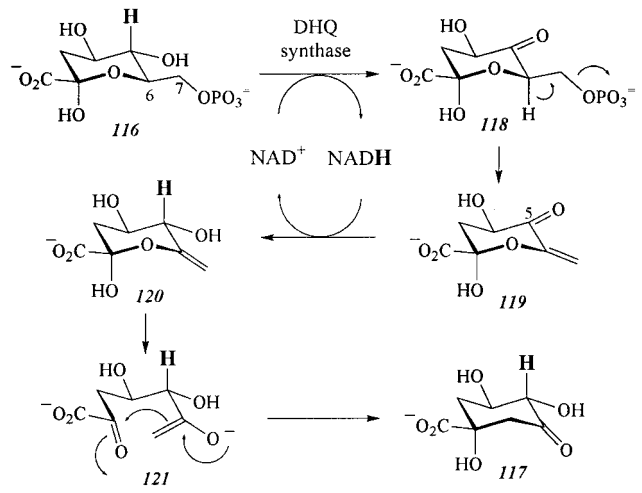
1. Catalytic Properties and Proposed Mechanism

DHQ synthase has been purified from various bacterial, fungal, and plant sources. The genes encoding this enzyme have been subsequently cloned and sequenced.[342−349] An *E. coli* strain has been

constructed to overproduce recombinant DHQ synthase at a level of 1000-fold greater than that of the wild-type strain.[342,344,345] The purified enzyme is a monomeric protein composed of 362 amino acids, with a calculated molecular mass of 38 880 Da.[344] It contains 1 equiv of tightly bound NAD^+ at the active site,[344] which is crucial to the structural stability and catalytic activity of the enzyme. Like many well-known NAD^+-dependent oxidoreductases, 3-dehydroquinate synthase is a metalloprotein.[350−356] It was originally discovered that the presence of Co(II) is necessary for restoring the activity of the partially purified DHQ synthase from *E. coli*.[338,357] Removal of Co(II) from the recombinant enzyme by dialysis against EDTA resulted in an inactive apoenzyme. The activity of this apoenzyme could be reconstituted by the addition of Co(II) and other divalent metal ions such as Zn(II).[356] Under physiological conditions, it is believed that Zn(II) acts as the metal cofactor for DHQ synthase.

The mechanism of the reaction catalyzed by DHQ synthase may be dissected into at least five consecutive transformations: (1) the oxidation of the secondary hydroxyl group at C-5 of **116** with concomitant reduction of the cofactor NAD^+, (2) the elimination of inorganic phosphate across C-6 and C-7 in **118**, (3) the reduction of the keto group at C-5 of **119**, (4) the ring opening of the enol pyranose **120**, and (5) the intramolecular aldol reaction (see **121**) to produce the saturated six-membered ring of DHQ (**117**, Scheme 37).[338] The manner in which this monomeric enzyme of a relatively small size can fulfill the demands for the catalysis of such diverse transformations has puzzled investigators for many years.

Scheme 37

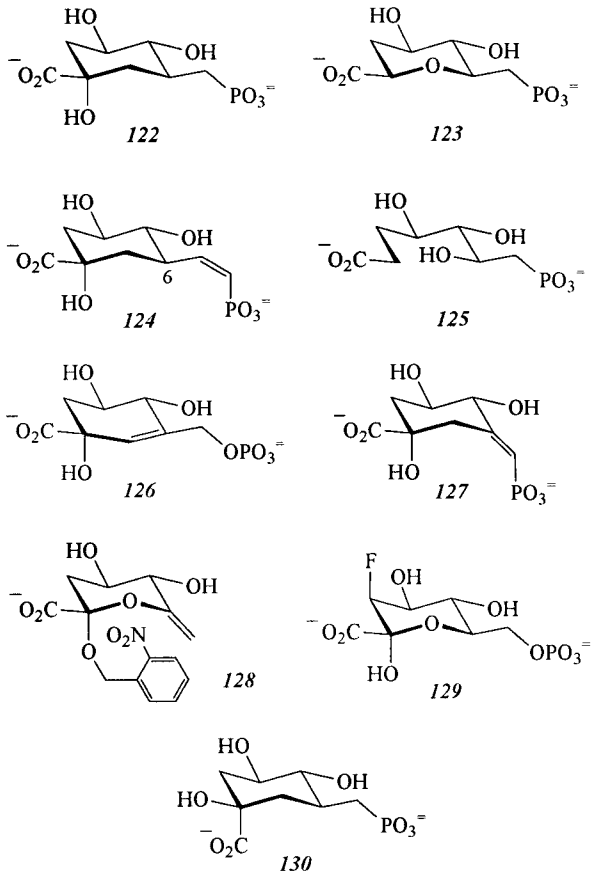


2. Role of NAD^+ in the Catalysis of DHQ Synthase

As illustrated in Scheme 37, NAD^+ is essentially a prosthetic group of DHQ synthase and is responsible for the initial oxidation and subsequent reduction at C-5.[35] This oxidation is pH-dependent, indicating that the hydride transfer from substrate to NAD^+ may be initiated by the deprotonation of 5-OH mediated by an active-site base.[358] Interestingly, although DHQ synthase has a high affinity for NAD^+ with a K_s of 80 nM,[356] the cofactor could dissociate from the enzyme active site during the course of the reactions, leading to a gradual loss of enzyme activity. However, the rate of cofactor dissociation is much slower than that of substrate turnover ($k_{cat}/k_{off} \sim 300$).[356] The regulatory functions and physiological consequences of this cofactor dissociation during enzyme catalysis are not yet completely understood.[358]

3. β-Elimination of Inorganic Phosphate

After the C-5 oxidation of substrate, the next step in the mechanism of DHQ synthase is the elimination of the phosphate group at C-7. Studies on the stereochemistry of this process have shown that this transformation is a *syn*-elimination.[357−361] On the basis of precedents derived from enzymes catalyzing β-elimination reactions of ketones, it appears that the elimination of phosphate proceeds through an E1cb pathway. Probing this enzyme's catalytic mechanism with compounds **122** and **123** led to the conclusion that the release of phosphate anion occurs prior to the interconversion between the cyclic hemiketal and the acyclic keto forms of DAHP.[359]



The elimination of phosphate to form **119** has been proposed to be assisted in an intramolecular fashion by the leaving phosphate group itself. The syn stereochemistry of elimination observed in the enzyme-catalyzed reaction is consistent with this hypothesis. In fact, the direct abstraction of the C-6 proton by the phosphate group at C-7 offers many catalytic advantages. For instance, the C-6 proton of DAHP may be well-shielded by the neighboring negatively charged phosphate group and the axial hydroxyl

group at C-2, making its removal by an enzyme general-base difficult. In this context, the dianionic phosphate ester, which is a strong base at physiological pH, is properly situated and is readily available. Furthermore, protonation of the phosphate would make it a better leaving group, eliminating the need for the assistance of a general base.

The conformational issues surrounding the β-elimination reaction were studied using carba-*cis*-vinylhomophosphate (**124**), which binds to DHQ synthase 25-fold more tightly than DAHP. The lowest energy conformation of **124** has its C-6 proton located in the plane of the side-chain double bond. The tight binding of **124** to the enzyme suggests that it mimics the conformation of the reaction intermediate prior to elimination, in which the methylene phosphate group must be forced into a gauche orientation at the active site in preparation for the subsequent elimination of inorganic phosphate.[358] In support of this hypothesis, the acyclic analogue **125** was found to be neither a substrate nor an inhibitor for the enzyme, indicating that the orientation of the substrate is crucial for its interaction with the enzyme active site.[357,359,360] Although the involvement of enzyme residues in the elimination of inorganic phosphate seems to be unnecessary, studies on **126**, **127**, and other olefinic analogues have indicated that DHQ synthase may accelerate the E1cb elimination of inorganic phosphate by restricting the conformational flexibility of the phosphorylmethyl ester in intermediate **118** and by stabilizing the transition state(s)/intermediate(s) bearing an sp^2-character during the E1cb elimination.[362]

4. Aldol Condensation in the DHQ Synthase Mechanism

The most intriguing transformation in the DHQ synthase mechanism is the ring-opening and recyclization step that occurs after the elimination of phosphate. As shown in Scheme 37, the ring-opening reaction leads to the formation of an enolate anion **121**. Rotation about the C5/C6 bond presents the C-end of the enolate toward the C-2 keto group on intermediate **121**. Nucleophilic attack by the enolate results in the formation of a cyclohexane ring, in which a new C−C bond is formed instead of the original C−O bond. Studies on the nonenzymatic generation of **117** by photochemical cleavage of *O*-nitrobenzyl ketal **128** suggested that ring opening and aldol condensation may not be an enzyme-catalyzed event.[363] The tendency for **120** to dissociate from the enzyme active site prior to subsequent rearrangement was probed by using the fluorine-substituted DAHP analogue **129**.[364] Two products that differed in their configurations at C-1 were observed after **129** was incubated with DHQ synthase, which most likely arose from reaction intermediates with distinctive conformations. It is unlikely that this enzyme uses different strategies to facilitate the formation of these diastereomers. Rather, the introduction of an axial fluorine next to the hemiketal center may stabilize the enol pyranose intermediate, which dissociates from the enzyme active site and undergoes a rearrangement of its carbon skeleton.

However, a more detailed investigation of this rearrangement revealed that the DHQ synthase active site may play a role in controlling the configuration of the final product through interactions with various functional groups present in the reaction intermediates, such as the carboxylate moiety. The ring opening of **120** may be promoted by a proton abstraction based on the evidence that carbaphosphate **130** (K_i = 7.3 nM) is an equally good inhibitor as its epimer **122** (K_i = 5.4 nM).[365] The interaction between the carboxylate group in the analogue and a protonated enzymatic residue may be responsible for the tight binding of **130** to the enzyme.

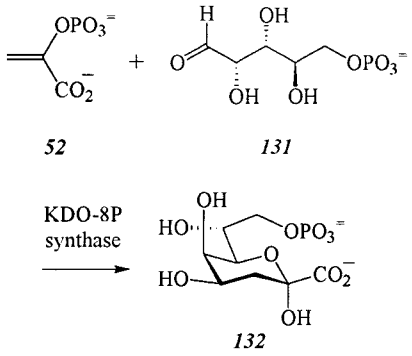
5. Design of DHQ Synthase Inhibitors as Herbicidal or Antimicrobial Agents

The extensive mechanistic investigations of DHQ synthase have resulted in the development of a large number of inhibitors of this enzyme. Interestingly, nonisosteric phosphonate analogues of DAHP have been found to be better inhibitors than the isosteric homophosphates.[358,361,366] In addition, nanomolar-level inhibition has been observed for cyclohexyl analogues in which the substitution of oxygen by a methylene group dramatically increases the stability of the compounds.[356,358,359,361] Mimics of reaction intermediates, such as a ketocarbaphosphonate analogue of **116**, have been reported to be an irreversible inhibitor of DHQ synthase.[367] The C-2 or C-6 epimers as well as the 2,6-anhydro[368] and cyclohexyl[365,369,370] analogues have also been shown to be DHQ synthase inhibitors. However, removal of hydroxyl groups at C-3 or C-4 or changes in the ionization states of the phosphoester/phosphonate moiety at near neutral pH could lead to a dramatic reduction or even a total loss of the inhibition potency.[371−374]

The cyclohexenyl and cyclohexylidene analogues are by far the best inhibitors for DHQ synthase. They can effectively abolish the enzyme activity at subnanomolar concentrations in a slowly reversible manner.[362] Despite the 33−55% sequence homology between the microbial and plant enzymes, large discrepancies have been observed in their responses to various inhibitors.[348,349,360] However, the majority of the currently known in vitro DHQ synthase inhibitors cannot penetrate the plant/bacterial membrane due to their incompatible ionization state at physiological pH. A prodrug strategy holds promise as a solution to this problem.

B. 3-Deoxy-D-manno-2-octulosonate-8-phosphate (KDO8P) Synthase

The enzyme KDO8P synthase catalyzes the stoichiometric condensation of phosphoenol-pyruvate (PEP, **52**) and D-arabinose-5-phosphate (Ara5P, **131**) to form KDO (3-deoxy-D-manno-2-octulosonate-8-phosphate, **132**), an unusual sugar found in the lipopolysaccharide (LPS) of Gram-negative bacteria.[375−377] This eight-carbon sugar serves as a bridge in LPS to link the complex lipid A structure and the inner core oligosaccharide to which the antigenic *O*-polysaccharide is attached.[378,379] Since LPSs are essential for the growth and virulence of Gram-negative bacteria and are absent in mammalian



52 *131* *132*

cells,[380−382] enzymes involved in the biosynthesis of KDO have been considered as attractive targets for the design of antibacterial agents.[381]

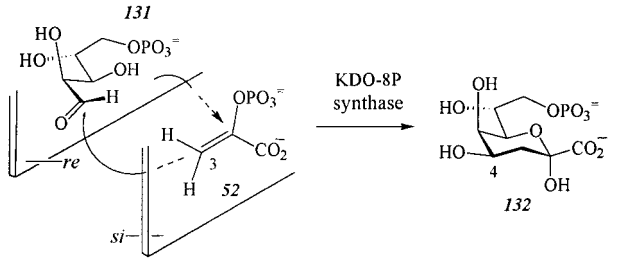
KDO8P synthase was first purified from *Pseudomonas aeruginosa*[375] and subsequently from *E. coli*.[379] The *E. coli* enzyme has a molecular mass of about 90 kDa, which consists of three identical subunits of 32 kDa each. The catalytic mechanism of this enzyme does not require the assistance of any metal ions or cofactors.[379] In fact, the enzyme activity is inhibited by 1 mM Cd^{2+}, Cu^{2+}, Zn^{2+}, or Hg^{2+}. The gene encoding this enzyme, *kdsA*, has been cloned and overexpressed in *E. coli*.[383] Although KDO8P synthase falls into the family of PEP-utilizing enzymes, this protein distinguishes itself from most other members by cleaving a C−O bond of PEP instead of the P−O bond.

1. Substrate Specificity and Stereospecificity of KDO8P Synthase

KDO8P synthase exhibits an absolute specificity for PEP ($K_m = 6\ \mu$M).[379] Structural analogues of PEP such as aldose phosphate, alditol phosphate, or aldonic acid phosphate are neither substrates nor potent inhibitors of this enzyme ($IC_{50} \geq 5$ mM),[384] indicating a strict recognition of the carboxylate and phosphate functional groups of PEP by the enzyme. Such strict substrate specificity is very different from that of the dimeric divalent-metal-ion-dependent DAHP synthase (see section VI.A).[385] A relatively less strict substrate stringency was found for Ara5P, which is the pentose phosphate utilized by KDO8P synthase ($K_m = 20\ \mu$M).[379] Interestingly, this enzyme also accepts the open chain analogues of Ara5P, such as 4-deoxy-D-arabinose-5-phosphate ($K_m = 330 \pm 30\ \mu$M) and 2-deoxy-2-fluoro-D-arabinose-5-phosphate ($K_i < 1$ mM),[386] and discriminates against compounds with a locked furanose ring.[384] Thus, the substrate of KDO8P synthase must be the acyclic form of Ara5P.[386,387] Steady-state inhibition kinetics further revealed that the catalysis proceeds via an ordered Bi−Bi sequential mechanism in which the enzyme binds phosphoenolpyruvate prior to the binding of Ara5P and releases KDO8P only after the dissociation of inorganic phosphate.[386] However, pre-steady-state kinetics under single-turnover conditions showed that Ara5P and PEP are bound by KDO8P synthase in a random order.[388]

The stereochemical course of the enzymatic reaction with respect to Ara5P (**131**) can be easily established as *re* face specific according to the configuration of the C-4 hydroxyl of product KDO8P (**132**), which is derived from the C-1 carbonyl of Ara5P. As shown in Scheme 38, the C-4-*manno* configuration of KDO8P (**132**) is a result of the attack by the C-3 of PEP on the *re* face of Ara5P at C-1. To determine the stereospecificity with respect to PEP, the labeled (*Z*)- and (*E*)-[3-^{2}H]PEP as well as (*Z*)- and (*E*)-[3-F]PEP were incubated with KDO8P synthase. Judged by the *R* configuration at C-3 of the labeled products, it was concluded that the condensation of PEP and Ara5P takes place on the *si* face of PEP (**52**),[389] which is similar to the stereospecificity observed in related PEP-utilizing enzymes,[390,391] including DAHP synthase.[392]

Scheme 38

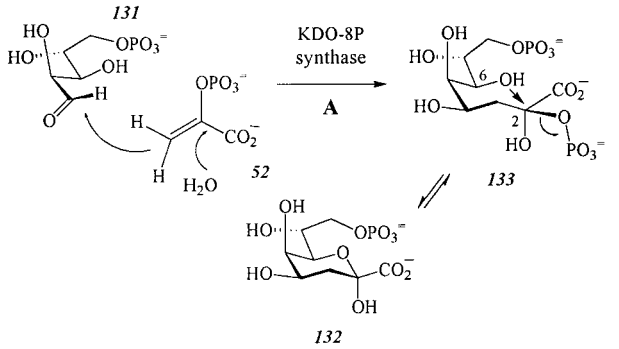


131 *52* *132*

2. Possible Catalytic Mechanisms for KDO8P Synthase

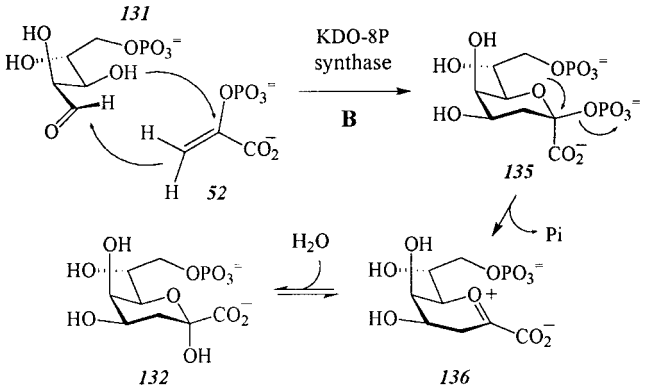
Two distinct, chemically feasible mechanisms have been proposed for the KDO8P synthase catalysis. An earlier hypothesis (Mechanism A) involved the formation of an acyclic tetrahedral intermediate **133**,[393] which results from the attack of a water molecule on PEP, followed by its coupling with Ara5P. A subsequent displacement of the C-2 phosphate by the C-6 hydroxyl gives the final product **132** (Scheme 39).

Scheme 39



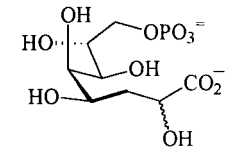
131 *52* *133* *132*

Scheme 40



131 *52* *135* *132* *136*

However, since compound **134**, which mimics the putative acyclic intermediate **133**, is not an inhibitor of the enzyme, a second mechanism has also been postulated. This mechanism (Mechanism B) includes the formation of a cyclic intermediate **135**. The breakdown of this species may lead to the formation of KDO8P (**132**) and inorganic phosphate via an oxocarbenium ion **136** (Scheme 40).[394,395]



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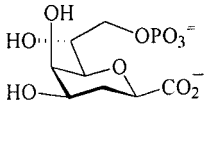
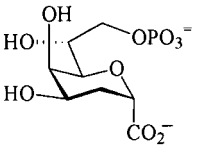
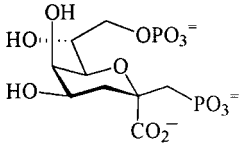


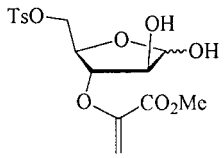
137



138



139



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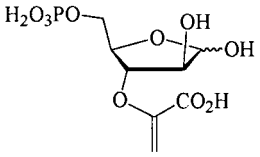
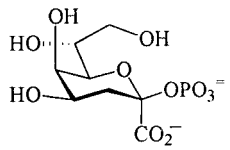
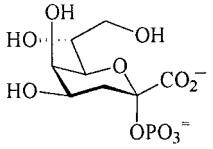


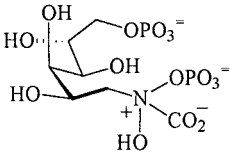
141



142



143



145

3. Mechanistic Studies Using Mimics of Putative Reaction Intermediates

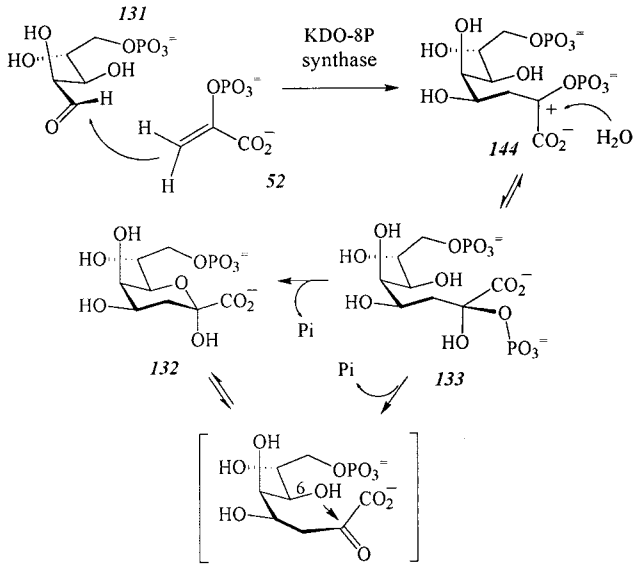
Support for Mechanism B was derived from the enzyme inhibition studies using probes that resemble the proposed cyclic intermediate **135**. For example, compounds **137** and **138** are two 2-deoxy analogues of KDO8P having their structures locked into the pyranose form without the possibility of ring opening. These compounds showed moderate inhibitory activity against the enzyme with K_i values of 470 and 303 μM, respectively.[395] Studies on the isosteric phosphonate **139**, which is a good topological and electrostatic mimic of the proposed intermediate **135**, provided more compelling evidence for this mechanism. This analogue is able to inhibit KDO8P synthase competitively against both PEP and Ara5P, with inhibition constants ($K_i = 4.9 \pm 0.1$ and 27.7 ± 0.8 μM) that are more than 2 orders of magnitude better than that of KDO8P itself (K_i for a mixture of 2-epimers of KDO8P is 590 μM).[394] These studies suggested that **139** is a better mimic of a transient species, such as **135**, that appears during the course of the catalysis. A chemical model was also designed to explore the feasibility of similar chemistry occurring in solution. It was found that compounds **140** and **141**, in equilibrium with their acyclic anomers, were converted into KDO8P diastereospecifically in the presence of Lewis acids such as $SnCl_4$ and $ZnCl_2$.[396] Such a nonenzymatic synthesis of KDO8P lent further credence to the mechanistic proposal involving a cyclic intermediate.

To elucidate the details of the hydrolysis of the anomeric phosphate group in **135**, two simplified analogues of **135** (**142** and **143**) were synthesized. These compounds were found to be competitive inhibitors with respect to PEP with K_i values of 160 and 1300 μM, respectively.[397,398] Studies of the hydrolysis of **142** and **143** under acidic conditions showed that the carboxylate functional group did not directly participate in the hydrolysis of the anomeric phosphate. However, esterification of the carboxylate groups slowed the rate of hydrolysis. Thus, it was suggested that the carboxylate may stabilize the positive charge on the putative oxocarbenium ion intermediate **136** by an inductive effect.[398]

One may argue that the above analogues are not the true mimics of intermediate **135**. The successful chemical synthesis of **135** by D'Souza et al.[399] made it possible to evaluate whether it is a real intermediate in the biosynthesis of KDO8P. The competence of KDO8P synthase to convert **135** into KDO8P and inorganic phosphate was investigated at pD 7.0. Surprisingly, no rate enhancement by the action of this enzyme could be observed when the release of inorganic phosphate was monitored by ^{31}P NMR.[399] Obviously, compound **135** cannot be processed by KDO8P synthase. It is not even an effective enzyme inhibitor with a modest K_i of 35 μM. Moreover, when rapid chemical-quench flow experiments were performed on KDO8P synthase, no new species with the proposed structure of **135** could be identified.[388,400] These data have cast doubts on the existence of such a cyclic intermediate during the course of the enzymatic reaction.[388]

The unexpected experimental results described above prompted a revisitation of Mechanism A (Scheme 39).[393] In fact, a modified pathway (Mechanism C) in which the formation of **133** via a stepwise process with an intermediate carrying positive charge at C-2 (**144**) has been proposed (Scheme 41). An amino phosphonate analogue **145**, which is a mimic of the proposed intermediate **144**, was synthesized to test this mechanism. The K_i value for this compound was determined to be 3.3 ± 0.3 μM, making it the most potent inhibitor of this enzyme thus far.[388] This result provided the preliminary evidence supporting Mechanism C. However, the inherent chemical lability of **144** makes it difficult to be chemically synthesized and subjected to the same type of mechanistic scrutiny as **135**. Recent REDOR studies revealed that the interactions between compound **145** and KDP8P synthase do not mimic those existing in

Scheme 41



the normal enzyme−PEP−Ara5P ternary complex, which does not sustain the argument that **144** is an intermediate in the enzyme catalysis.[401] A true understanding of the catalytic mechanism of KDO8P synthase must therefore await further in-depth studies. Nevertheless, the postulation of Mechanism C and the discovery of potent inhibitor **145** have provided new leads for the design of antibiotics targeting on the biosynthesis of LPS in Gram-negative bacteria.

VII. Summary

Nature has devised many elegant methods to amplify carbohydrate structural diversity, which is the underlying basis for the many biological roles played by these sugars. Glucose, the primary product from plant photosynthesis and the most popular energy vehicle in living organisms, can be transformed into unusual sugars with unique structural features and activities. The replacements of its hydroxyl groups are realized through C−O bond-cleavage reactions, C−N bond-formation reactions, C−O bond-formation reactions, and C−C bond-formation reactions. More complicated monosaccharides, such as KDO8P, are synthesized from simple building blocks such as PEP and Ara5P. Sugar molecules can also undergo rearrangement of their skeletons and enter other important biosynthetic pathways. Common hexoses can be interconverted through structural isomerizations and epimerizations at specific stereogenic centers. Thus, cells are able to obtain the sugars they need for specific objectives at each stage of their lifecycles. This review is not intended to be an exhaustive description of all enzyme catalyses involved in the metabolism of carbohydrates. Instead, we have attempted to select a few representative examples with the aim to illustrate the many facets of carbohydrate enzymology and its practical applications.

The paradigms for C−O bond-cleavage reactions at C-6, C-3, and C-2 positions of a hexose have been well-established through detailed characterizations of the participating enzymes. So far, most of the better understood enzymatic reactions are involved in the biosynthesis of dideoxysugars. Among them, the C-3 deoxygenation catalyzed by E_1 and E_3 represents a novel example of a coenzyme B_6 radical in biological catalysis. The corresponding pathways for the formation of tri- and tetradeoxysugars have not been studied to the same extent. Apart from the subtle variations in the properties of individual enzymes, the order in which multiple C−O bond-cleavage reactions occur is an aspect worth investigation. The regulation of the biosynthesis of these unusual sugars is also a relatively unexplored area. A thorough understanding of the regulatory mechanisms is essential if these pathways are to be used to mass-produce glycoconjugates for future clinical and/or commercial applications.

The studies on the C−N bond-formation reactions catalyzed by TylB/DesV have offered many interesting perspectives. Since the substrate of TylB is unstable and difficult to access by synthetic methods, the function of this enzyme was actually confirmed by assaying it in the reverse direction. The function of DesV was first established genetically by knocking out the gene encoding this protein and examining the resulting product generated in vivo by a heterologous strain. This study highlights a more recent trend in the biosynthetic research of polyketide antibiotics and has far-reaching implications for the tailored design of a new generation of antimicrobial agents. However, information on the C−N bond-formation reactions in the biosynthesis of sugars containing an amino substituent at a quarternary carbon (such as vacosamine) and sugars containing a nitro functional group (such as kijanose) is still limited. It would be interesting to find out if the installation of these nitrogen-containing groups follows any of the three mechanisms known for C−N bond formation in the biosynthesis of aminosugars, namely, nucleophilic displacement, transamidation, or transamination.

While the epimerization catalyzed by UDP−galactose 4-epimerase has been well-studied, the recent discovery that CDP−tyvelose 2-epimerase might employ a similar mechanism is intriguing. The epimerization at C-4 caused by the 180° rotation of the hexulose ring in the case of UDP−galactose 4-epimerase is intuitively appealing. However, it is rather difficult to envision a similar movement at molecular level when the epimerization occurs at the C-2 position. Clearly, further structural studies of this enzyme are absolutely necessary for unveiling the mechanistic details of its catalysis. The isomerization catalyzed by D-xylose isomerase is another well-characterized enzymatic reaction. Knowledge of the peptide sequence, three-dimensional structure, and catalytic mechanism of this enzyme has prompted efforts to engineer mutant enzymes with better catalytic properties.

Extensive studies of DHQ synthase have unraveled a fascinating multistep transformation from DAHP to DHQ. A similar mechanistic scheme may be followed by a few other enzymes. For example, the proposed mechanism for 2-deoxy-*scyllo*-inosose synthase also involves the series of ring-cleavage, rota-

tion, and recyclization events to form a six-membered ring, accompanied by the redox recycling of an NAD^+ cofactor.[402,403] The final product of this enzymatic reaction serves as the precursor for the formation of 2-deoxystreptamine, an important component of aminoglycoside antibiotics. Likewise, a multistep mechanism is believed to be operative in the catalysis of *myo*-inositol-1-phosphate synthase.[35] However, the details of these enzyme catalyses remain relatively scarce.

In-depth knowledge of enzymes participating in the biosyntheses of carbohydrates is a prerequisite to the rational design of methods to control and/or mimic their physiological roles. The successful design of an active-site-directed inhibitor of E_{od}, CDP−6-deoxy-6,6-difluoro-α-D-glucose, can be attributed to the comprehensive mechanistic studies on this enzyme. Similarly, investigations of the DHQ synthase have led to the discovery of potent enzyme inhibitors which may be further developed into herbicides. Studies on the bacterial enzyme KDO8P synthase have also boosted efforts to synthesize more effective inhibitors with a pharmaceutical potential. The identifications of the myriad biosynthetic pathways for unusual sugar formation have allowed the genetic manipulation of the producer microorganisms for the construction of tailored carbohydrate moieties, both natural and unnatural. The diversity of saccharide structures produced by the engineered mutants may be further expanded via recruitment of genes from a variety of biosynthetic pathways to form composite clusters. It is worth mentioning that the glycosyltransferases responsible for the final glycosylation of certain secondary metabolites appear to have a high degree of promiscuity toward their sugar substrates. These encouraging discoveries have opened the door to the possibility of generating structurally diverse secondary metabolite scaffolds with natural or unnatural glycosylation patterns. Since many of these glycoconjugates have been shown to have attractive antimicrobial/antitumor activities, such a combinatorial approach bears the promise of producing new chemical entities that will be useful in clinical situations where the current arsenal of antibiotics is ineffective.

VIII. Acknowledgments

The authors acknowledge the many valuable contributions made by members of the Liu group, both past and present, whose efforts are described in this review. Research in H.-w.L.'s laboratory in the area of carbohydrate biochemistry has been supported by grants from the National Institutes of Health (GM35906 and GM54346). We owe a special gratitude to Svetlana Borisova, Erich Molitor, Beth Paschal, and Drs. Lishan Zhao and Qibo Zhang for their help in preparing this manuscript. H.-w.L. also thanks the National Institute of General Medical Sciences for a MERIT Award.

IX. References

(1) *Carbohydrate Chemistry*; Kennedy, J. F., Ed.; Clarendon Press: Oxford, 1988.
(2) *Bioorganic Chemistry: Carbohydrates*; Hecht, S. M., Ed.; Oxford University Press: New York, 1999.
(3) Robyt, J. F. *Essentials of Carbohydrate Chemistry*; Springer: New York, 1997.
(4) Pinto, B. M. *Carbohydrates and Their Derivatives, Including Tannins, Cellulose, and Related Lignins*. In *Comprehensive Natural Products Chemistry*; Barton D., Nakanishi, K., Eds.; Elsevier: New York, 1999; Vol. 3.
(5) Feizi, T.; Bundle, D. R. *Curr. Opin. Struct. Biol.* **1996**, *6*, 659.
(6) Rini, J.; Drickamer, K. *Curr. Opin. Struct. Biol.* **1997**, *7*, 615.
(7) *Glycosciences: Status and Perspectives*; Gabius, H.-J., Gabius, S., Eds.; Chapman & Hall: Weinhein, 1997.
(8) *Molecular Glycobiology*; Fukuda, M., Hindsgaul, O., Eds.; Oxford University Press: Oxford, 1994.
(9) Hallis, T. M.; Liu, H.-w. *Acc. Chem. Res.* **1999**, *32*, 579.
(10) Laine, R. A. In *Glycosciences: Status and Perspectives*; Gabius, H.-J., Gabius, S., Eds.; Chapman & Hall, Weinham; New York, 1997; p 1.
(11) Johnson, D. A.; Liu, H.-w. In *Comprehensive Natural Products Chemistry*; Barton, D., Nakanishi, K., Eds.; Elsevier: New York, 1999; Vol. 3, p 311.
(12) Leeden, R. W.; Yu, R. K. *Methods Enzymol.* **1982**, *83*, 139.
(13) Wiegandt, H. *New Compr. Biochem.* **1985**, *10*, 199.
(14) *Biological Function of Gangliosides, Progress in Brain Research*; Svennerholm, L., Asbury, A. K., Resifeld, R. A., Sandhoff, K., Suzuki, G., Tettamanti, G., Toffano, G., Eds.; Elsevier: Amsterdam, 1994; Vol. 101.
(15) (a) Jenkins, N.; Parekh, R. B.; James, D. C. *Nat. Biotechnol.* **1996**, *14*, 975. (b) Crout, D. H.; Vic, G. *Curr. Opin. Chem. Biol.* **1998**, *2*, 98.
(16) (a) Henrissat, B. *Biochem. Soc. Trans.* **1998**, *26*, 153. (b) Birsan, C.; Johnson, P.; Joshi, M.; MacLeod, A.; McIntosh, L.; Monem, V.; Nitz, M.; Rose, D. R.; Tull, D.; Wakarchuck, W. W.; Wang, Q.; Warren, R. A.; White, A.; Withers, S. G. *Biochem. Soc. Trans.* **1998**, *26*, 156. (c) Gottschalk, T. E.; Fierobe, H. P.; Mirgorodskaya, E.; Clarke, A. J.; Tull, D.; Sigurskjold, B. W.; Christensen, T.; Payre, N.; Frandsen, T. P.; Jude, N.; McGuire, K. A.; Cottaz, S.; Roepstorff, P.; Driguez, H.; Williamson, G.; Svensson, B. *Biochem. Soc. Trans.* **1998**, *26*, 198.
(17) (a) Stubbe, J.; van der Donk, W. A. *Chem. Rev.* **1999**, *98*, 705. (b) Stubbe, J.; Riggs-Gelasco, P. *Trends Biochem. Sci.* **1998**, *23*, 438. (c) Stubbe, J.; van der Donk, W. A. *Chem. Biol.* **1995**, *2*, 793. (d) Stubbe, J. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1990**, *63*, 349. (e) Stubbe, J. *J. Biol. Chem.*, **1990**, *265*, 5329. (f) Stubbe, J. A. *Annu. Rev. Biochem.* **1989**, *58*, 257. (g) Stubbe, J. *Biochemistry* **1988**, *27*, 3893.
(18) Fessner, W. D. *Curr. Opin. Chem. Biol.* **1998**, *2*, 85.
(19) (a) Moss, J.; Zolkiewska, A.; Okazaki, I. *Adv. Exp. Med. Biol.* **1997**, *419*, 25. (b) Alvarez-Gonzalez, R.; Watkins, T. A.; Gill, P. K.; Reed, J. L.; Mendoza-Alvarez, H. *Mol. Cell Biochem.* **1999**, *193*, 19. (c) Moss, J.; Balducci, E.; Cavanaugh, E.; Kim, H. J.; Konczalik, P.; Lesma, E. A.; Okazaki, I. J.; Park, M.; Shoemaker, M.; Stevens, L. A.; Zolkiewska, A. *Mol. Cell. Biochem.* **1999**, *193*, 109.
(20) Zhao, L.; Sherman, D. H.; Liu, H.-w. *J. Am. Chem. Soc.* **1998**, *120*, 10256.
(21) Zhao, L.; Ahlert, J.; Xue, Y.; Thorson, J. S.; Sherman, D. H.; Liu, H.-w. *J. Am. Chem. Soc.* **1999**, *121*, 9881.
(22) Johnson, D. A.; Liu, H.-w. In *The Biology-Chemistry Iinterface: A Tribute to Koji Nakanishi*; Cooper, R., Snyder, J. D., Eds.; Marcel Dekker: New York, 1999; p 351.
(23) (a) Weymouth-Wilson, A. C. *Nat. Prod. Rep.* **1997**, *14*, 99. (b) Varki, A. *Glycobiology* **1993**, *3*, 97.
(24) Liu, H.-w.; Thorson, J. S. *Annu. Rev. Microbiol.* **1994**, *48*, 223.
(25) Johnson, D. A.; Liu, H.-w. *Curr. Opin. Chem. Biol.* **1998**, *2*, 642.
(26) Kirschning, A.; Bechthold, A. F.-W.; Rohr, J. *Top. Curr. Chem.* **1997**, *188*, 1.
(27) (a) Lindberg, B. *Adv. Carbohydr. Chem. Biochem.* **1990**, *48*, 279. (b) Williams, N. R.; Wander, J. D. In *The Carbohydrates: Chemistry and Biochemistry*; Pigman, W., Horton, D., Eds.; Academic Press: New York, 1980; Vol. 1B, pp 761−798.
(28) Thorson, J. S.; Lo, S. F.; Ploux, O.; He, X.; Liu, H.-w. *J. Bacteriol.* **1994**, *176*, 5483.
(29) Thorson, J. S.; Kelly, T. M.; Liu, H−w. *J. Bacteriol.* **1994**, *176*, 1840.
(30) Thorson, J. S.; Lo, S. F.; Liu, H.-w. *J. Am. Chem. Soc.* **1993**, *115*, 5827.
(31) Grisebach, H. *Adv. Carbohydr. Chem. Biochem.* **1978**, *35*, 81.
(32) Glaser, L.; Zarkowsky, R. In *The Enzymes*, 3rd ed.; Boyer, P., Ed.; Academic Press: New York, 1971; Vol. 5, pp 465−480.
(33) Gabriel, O. In *Carbohydrates in Solution*; Gould, R., Ed.; Advances in Chemistry Series 117; American Chemical Society, Washington, D. C., 1973; p 387.
(34) Gabriel, O.; van Lenten, L. *Biochemistry of Carbohydrates II*; University Park Press: Baltimore, 1978; Vol. 16, p 1.
(35) Frey, P. A. In *The Pyridine Nucleotide Coenzymes*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley-Interscience: New York, 1987; Part B, p 461.
(36) He, X.; Thorson, J. S.; Liu, H.-w. *Biochemistry* **1996**, *35*, 4721.
(37) Wang, S.-F.; Gabriel, O. *J. Biol. Chem.* **1969**, *244*, 3430.
(38) Zarkowsky, H.; Glaser, L. *J. Biol. Chem.* **1969**, *244*, 4750.

(39) Chiknas, C.; Gabriel, O. *Fed. Proc.* **1977**, *36*, 3169.
(40) Zarkowsky, H.; Lipkin, E.; Glaser, L. *J. Biol. Chem.* **1970**, *245*, 6599.
(41) Melo, A.; Elliott, W. H.; Glaser, L. *J. Biol. Chem.* **1968**, *243*, 1467.
(42) Gabriel, O.; Lindquist, L. C. *J. Biol. Chem.* **1968**, *243*, 1479.
(43) Zarkowsky, H.; Lipkin, E.; Glaser, L. *Biochem. Biophys. Res. Commun.* **1970**, *38*, 787.
(44) Herman, K.; Lehman, J. *Eur. J. Biochem.* **1968**, *3*, 369.
(45) Wang, S. F.; Gabriel, O. *J. Biol. Chem.* **1970**, *245*, 8.
(46) Hallis, T. M.; Liu, H.-w. *Tetrahedron* **1998**, *54*, 15975.
(47) Nelsestuen, G. L.; Kirkwood, S. *J. Biol. Chem.* **1971**, *246*, 7533.
(48) Byun, S.; Jenness, R. *Biochemistry* **1981**, *20*, 5174.
(49) Snipes, C. E.; Brillinger, G.-U.; Sellers, L.; Mascaro, L.; Floss, H. G. *J. Biol. Chem.* **1977**, *252*, 8113.
(50) Oths, P. J.; Mayer, R. M.; Floss, H. G. *Carbohydr. Res.* **1990**, *198*, 91.
(51) Russell, R. N.; Liu, H.-w. *J. Am. Chem. Soc.*, **1991**, *113*, 7777.
(52) Yu, Y.; Russell. R. N.; Thorson, J. S.; Liu, L.-d.; Liu, H.-w. *J. Biol. Chem.* **1992**, *267*, 5868.
(53) Gonzalez-Porque, P.; Strominger, J. L. *J. Biol. Chem.* **1972**, *247*, 6748.
(54) Rossmann, M. G.; Liljas, A.; Brandon, C.-I.; Banaszak, L. J. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. 11, p 61.
(55) Chang, C.-w., T.; Chen, X. H.; Liu, H.-w. *J. Am. Chem. Soc.* **1998**, *120*, 9698.
(56) Gonzalez-Porqué, P. In *Pyridine Nucleotide Coenzymes*; Dolphine, D., Poulson, D. R., Avramovic, O., Eds.; Wiley: New York, 1987; Vol. B, pp 391−419.
(57) Rubenstein, P. A.; Strominger, J. L. *J. Biol. Chem.* **1974**, *249*, 3776.
(58) Rubenstein, P. A.; Strominger, J. L. *J. Biol. Chem.* **1974**, *249*, 3782.
(59) Lo, S. F.; Miller, V. P.; Lei, Y.; Thorson, J. S.; Liu, H.-w. *J. Bacteriol.* **1994**, *176*, 460.
(60) Weigel, T. M.; Liu, L.-d.; Liu, H.-w. *Biochemistry* **1992**, *31*, 2129.
(61) Lei, Y.; Ploux, O.; Liu, H.-w. *Biochemistry* **1995**, *34*, 4643.
(62) Thorson, J. S.; Liu, H.-w. *J. Am. Chem. Soc.* **1993**, *115*, 7539.
(63) Johnson, D. A.; Gassner, G. T.; Bandarian, V.; Ruzicka, F. J.; Ballou, D. P.; Reed, G. H.; Liu, H.-w. *Biochemistry* **1996**, *35*, 15846.
(64) Miller, V. P.; Thorson, J. S.; Ploux, O.; Lo, S. F.; Liu, H.-w. *Biochemistry* **1993**, *32*, 11934.
(65) Ploux, O.; Lei, Y.; Vatanen, K.; Liu, H.-w. *Biochemistry* **1995**, *34*, 4159.
(66) Gassner, G. T.; Johnson, D. A.; Liu, H.-w.; Ballou, D. P. *Biochemistry* **1996**, *35*, 7752.
(67) Pape, H.; Strominger, J. L. *J. Biol. Chem.* **1969**, *244*, 3598.
(68) Thorson, J. S.; Lo, S. F.; Liu, H.-w.; Hutchinson, C. R. *J. Am. Chem. Soc.* **1993**, *115*, 6993.
(69) (a) Jiang, X. M.; Neal, B.; Santiago, F.; Lee, S. J.; Romana, L. K.; Reeves, P. R. *Mol. Microbiol.* **1991**, *5*, 695. (b) Kessler, A. C.; Haase, A.; Reeves, P. R. *J. Bacteriol.* **1993**, *175*, 1412. (c) Stroeher, U. H.; Jedani, K. E.; Manning, P. A. *Gene* **1998**, *223*, 269. (d) Ichinose, K.; Bedford, D. J.; Tornus, D.; Bechthold, A.; Bibb, M. J.; Revill, W. P.; Floss, H. G.; Hopwood, D. A. *Chem. Biol.* **1998**, *5*, 647. (e) Westrich, L.; Domann, S.; Faust, B.; Bedford, D.; Hopwood, D. A.; Bechthold, A. *FEMS Microbiol. Lett.* **1999**, *170*, 381. (f) Skurnik, M.; Peippo, A.; Ervela, E. unpublished results. The gene bank accession number is CAB63273.
(70) Weigel, T. M.; Miller, V. P.; Liu, H.-w. *Biochemistry* **1993**, *31*, 2140.
(71) Thorson, J. S.; Liu, H.-w. *J. Am. Chem. Soc.* **1993**, *115*, 12177.
(72) Pieper, P. A.; Guo, Z.; Liu, H.-w. *J. Am. Chem. Soc.* **1995**, *117*, 5158.
(73) Burns, K. D.; Pieper, P. A.; Liu, H.-w.; Stankovich, M. T. *Biochemistry* **1996**, *35*, 7879.
(74) (a) Fields, S.; Song, O.-k. *Nature* **1989**, *340*, 245. (b) Vojtek, A. B.; Hollenberg, S. M.; Cooper, J. A. *Cell* **1993**, *74*, 205.
(75) Pieper, P. A.; Yang, D.-y.; Zhou, H.-q.; Liu, H.-w. *J. Am. Chem. Soc.* **1997**, *119*, 1809.
(76) Chang, C.-w. T.; Johnson, D. A.; Bandarian, V.; Zhou, H.-q.; LoBrutto, R.; Reed, G. H.; Liu, H.-w *J. Am. Chem. Soc.* **2000**, *122*, 4239.
(77) Draeger, G.; Park, S.-H.; Floss, H. G. *J. Am. Chem. Soc.* **1999**, *121*, 2611.
(78) Chen, H.; Agnihotri, G.; Guo, Z.; Que, N. L. S.; Chen, X. H.; Liu, H.-w. *J. Am. Chem. Soc.* **1999**, *121*, 8124.
(79) Chen, H.; Guo, Z.; Liu, H.-w. *J. Am. Chem. Soc.* **1998**, *120*, 9951.
(80) (a) Merson-Davies, L. A.; Cundliffe, E. *Mol. Microbiol.* **1994**, *13*, 349. (b) Gandecha, A. R.; Large, S. L.; Cundliffe, E. *Gene* **1997**, *184*, 197. (c) Fish, S. A.; Cundliffe, E. *Microbiology* **1997**, *143*, 3871. (d) Bulter, A. R.; Bate, N.; Cundliffe, E. *Chem. Biol.* **1999**, *6*, 287.
(81) (a) Gaisser, S.; Bohm, G. A.; Cortés, J.; Leadley, P. F. *Mol. Gen. Genet.* **1997**, *256*, 239. (b) Salah-Bey, K.; Doumith, M.; Michel, J. M.; Haydock, S.; Cortés, J.; Leadley, P. F.; Raynal, M.-C. *Mol. Gen. Genet.* **1998**, *257*, 542. (c) Summers, R. G.; Donadio, S.; Staver, M. J.; Wendt-Pienkowski, E.; Hutchinson, C. R.; Katz, L. *Microbiology* **1997**, *143*, 3251.
(82) Pissowotzki, K.; Mansouri, K.; Piepersberg, W. *Mol. Gen. Genet.* **1991**, *231*, 113.
(83) Westrich, L.; Domann, S.; Faust, B.; Bedford, D.; Hopwood, D. A.; Bechthold, A. *FEMS Microbiol. Lett.* **1999**, *170*, 381.
(84) van Wageningen, A.; Kirkpatrick, P.; Williams, D.; Harris, B.; Kershaw, J.; Lennard, N.; Jones, M.; Jones, S.; Solenberg, P. *Chem. Biol.* **1998**, *3*, 155.
(85) Torkkell, S.; Ylihonko, K.; Hakala, J.; Skurnik, M.; Mantsala, P. *Mol. Gen. Genet.* **1997**, *256*, 203.
(86) Scotti, C.; Hutchinson, C. R. *J. Bacteriol.* **1996**, *178*, 7316.
(87) (a) Jörnvall, H.; Persson, B.; Jeffrey, J. *Eur. J. Biochem.* **1987**, *167*, 195. (b) Reid, M. F.; Fewson, C. A. *Crit. Rev. Microbiol.* **1994**, *20*, 13.
(88) Andruszkiewicz, R.; Chmara, H.; Milewski, S.; Zieniawa, T.; Borowski, E. *J. Med. Chem.* **1990**, *33*, 2755.
(89) Auvin, S.; Cochet, O.; Kucharczyk, N.; Le Goffic, F.; Badet, B. *Bioorg. Chem.* **1991**, *19*, 143.
(90) Ghosh, S.; Blumenthal, H. J.; Davidson, E.; Roseman, S. *J. Biol. Chem.* **1960**, *235*, 1265.
(91) Bates, C. J.; Adams, W. R.; Handschumacher, R. E. *J. Biol. Chem.* **1966**, *241*, 1705.
(92) Kornfeld, R. *J. Biol. Chem.* **1967**, *242*, 3135.
(93) Winterburn, P. J.; Phelps, C. F. *Biochem. J.* **1971**, *121*, 701.
(94) Miyagi, T.; Tsukui, S. *Biochim. Biophys. Acta* **1971**, *250*, 51.
(95) Miyagi, T.; Tsukui, S. *Cancer Res.* **1979**, *39*, 2783.
(96) Chmara, H. *J. Gen. Microbiol.* **1985**, *131*, 265.
(97) Chmara, H.; Zahner, H.; Borowski, E. *J. Antibiot.* **1984**, *27*, 1038.
(98) Norman, J.; Giddings, T. H.; Catino, E. *Phytochemistry* **1975**, *14*, 1271.
(99) Moriguchi, M.; Yamamoto, K.; Kawai, K.; Tochikura, T. *Agric. Biol. Chem.* **1976**, *40*, 1655.
(100) Milewski, S. *Biochim. Biophys. Acta* **1993**, *1161*, 279.
(101) Bachmann, B. J. *Microbiol. Rev.* **1983**, *47*, 180.
(102) Walker, J. E.; Gay, J.; Saraste, M.; Eberle, N. *Biochem. J.* **1984**, *224*, 799.
(103) Badet, B.; Vermoote, P.; Haumont, P.-Y.; Lederer, F.; Le Goffic, F. *Biochemistry* **1987**, *26*, 1940.
(104) Tso, J. Y.; Zalkin, H.; van Cleemput, M.; Yanofsky, C.; Smith, J. M. *J. Biol. Chem.* **1982**, *257*, 3525.
(105) Tso, J. Y.; Hermodson, M. A.; Zalkin, H. *J. Biol. Chem.* **1982**, *257*, 3532.
(106) Vollmer, S. J.; Switzer, R. L.; Hermodson, M. A.; Bower, S. G.; Zalkin, H. *J. Biol. Chem.* **1983**, *258*, 10582.
(107) Mei, B.; Zalkin, H. *J. Biol. Chem.* **1989**, *264*, 16613.
(108) Badet-Denisot, M.-A.; Badet, B. *Arch. Biochem. Biophys.* **1992**, *292*, 475.
(109) Badet, B.; Vermoote, P.; Le Goffic, F. *Biochemistry* **1988**, *27*, 2282.
(110) Kucharczyk, N.; Denisot, M.-A.; Le Goffic, F.; Badet, B. *Biochemistry* **1990**, *29*, 3668.
(111) Badet-Denisot, M.-A.; René, L.; Badet, B. *Bull. Soc. Chim. Fr.* **1993**, *130*, 249.
(112) Kort, M. J. *Adv. Carbohydrate. Chem. Biochem.* **1970**, *25*, 311.
(113) Golinelli-Pimpaneau, B.; Le Goffic, F.; Badet, B. *J. Am. Chem. Soc.* **1989**, *111*, 3029.
(114) Denisot, M.-A.; Le Goffic, F.; Badet, B. *Arch. Biochem. Biophys.* **1991**, *288*, 225.
(115) Bearne, S. L.; Wolfenden, R. *Biochemistry* **1995**, *34*, 11515.
(116) Obmolova, G.; Badet-Denisot, M.-A.; Badet, B.; Teplyakov, A. *J. Mol. Biol.* **1994**, *242*, 703.
(117) Isupov, M. N.; Obmolova, G.; Butterworth: S.; Badet-Denisot, M.-A.; Badet, B.; Polikarpov, I.; Littlechild, J. A.; Teplyakov, A. *Structure* **1996**, *4*, 801.
(118) Rose, I. A. *Brookhaven Symp. Biol.* **1962**, *15*, 293.
(119) Leriche, C.; Badet-Denisot, M.-A.; Badet, B. *J. Am. Chem. Soc.* **1996**, *118*, 1797.
(120) Golinelli-Pimpaneau, B.; Badet, B. *Eur. J. Biochem.* **1991**, *201*, 175.
(121) Leriche, C.; Badet-Denisot, M.-A.; Badet, B. *Eur. J. Biochem.* **1997**, *245*, 418.
(122) Bearne, S. L. *J. Biol. Chem.* **1996**, *271*, 3052.
(123) Hendrix, M.; Alper, P. B.; Priestley, E. S.; Wong, C.-H. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 95.
(124) (a) Baltz, R. H.; Seno, E. T. *Antimicrob. Agents Chemother.* **1981**, *20*, 214. (b) Fisherman, S. E.; Cox, K.; Larson, J. L.; Reynolds, P. A.; Seno, E. T.; Yeh, W.-K.; Van Frank, R.; Hershberger, C. L. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8248. (c) Baltz, R. H.; Seno, E. T. *Annu. Rev. Microbiol.* **1988**, *42*, 547.
(125) Chen, H.; Yeung, S.-M.; Que, N. L. S.; Müller, T.; Schmidt, R. R.; Liu, H.-w. *J. Am. Chem. Soc.* **1999**, *121*, 7166.
(126) (a) Stein, A.; Kula, M.-R.; Elling, L.; Verseck, S.; Klaffke, W. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1748. (b) Naundorf, A.; Klaffke, W. *Carbohydr. Res.* **1996**, *285*, 141.
(127) Quiros, L. M.; Aguirrezabalaga, I.; Olano, C.; Mendez, C.; Salas, J. A. *Mol. Microbiol.* **1998**, *28*, 1177.
(128) Dhillon, N.; Hale, R. S.; Cortes, J.; Leadlay, P. F. *Mol. Microbiol.* **1989**, *3*, 1405.

(129) Stutzman-Engwall, K. J.; Otten, S. L.; Hutchinson, C. R. *J. Bacteriol.* **1992**, *174*, 144.
(130) Xue, Y.; Zhao, L.; Liu, H.-w.; Sherman, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12111.
(131) Zhao, L.; Yamase, H.; Liu, H.-w. Unpublished data.
(132) Brock, T. D.; Madigan, M. T. Biology of Microorganisms, 6th ed.; Prentice Hall: Englewood Cliffs, 1991.
(133) Brown, E. D.; Vivas, E. I.; Walsh, C. T.; Kolter. R. *J. Bacteriol.* **1995**, *177*, 4194.
(134) Hendlin, D.; Stapley, E. O.; Jackson, M.; Wallick, H.; Miller, A. K.; Wolf, F. J.; Miller, T. W.; Chaiet, L.; Kahan, F. M.; Foltz, E. L.; Woodruff, H. B.; Mata, J. M.; Hernandez, S.; Mochales, S. *Science* **1969**, *166*, 122.
(135) Zemell, R.; Anwar, R. A. *J. Biol. Chem.* **1975**, *250*, 3185.
(136) Marquardt, J. L.; Siegele, D. A.; Kolter, R.; Walsh, C. T. *J. Bacteriol.* **1992**, *174*, 5748.
(137) Wanke, C.; Falchetto, R.; Amrhein, N. *FEBS Lett.* **1992**, *301*, 271.
(138) Wanke, C.; Amrhein, N. *Eur. J. Biochem.* **1993**, *218*, 861.
(139) (a) Steinrucken, H. C.; Amrhein, N. *Biochem. Biophys. Res. Comm.* **1980**, *94*, 1207. (b) Steinrucken, H. C.; Amrhein, N. *Eur. J. Biochem.* **1984**, *143*, 351.
(140) Anderson, K. S.; Johnson, K. A. *Chem. Rev.* **1990**, *90*, 1131.
(141) Cassidy, P.; Kahan, F. M. *Biochemistry* **1973**, *12*, 1364.
(142) Brown, E. D.; Marquardt, J. L.; Lee, J. P.; Walsh, C. T.; Anderson, K. S. *Biochemistry* **1994**, *33*, 10638.
(143) Marquardt, J. L.; Brown, E. D.; Lane, W. S.; Haley, T. M.; Ichikawa, Y.; Wong, C.-H.; Walsh, C. T. *Biochemistry* **1994**, *33*, 10646.
(144) Marquardt, J. L.; Brown, E. D.; Walsh, C. T.; Anderson, K. S. *J. Am. Chem. Soc.* **1993**, *115*, 10398.
(145) Kim, D. H.; Lees, W. J.; Walsh, C. T. *J. Am. Chem. Soc.* **1994**, *116*, 6478.
(146) Kim, D. H.; Lees, W. J.; Haley, T. M.; Walsh, C. T. *J. Am. Chem. Soc.* **1995**, *117*, 1494.
(147) Kim, D. H.; Lee, W. J.; Kempsell, K. E.; Lane, W. S.; Duncan, K.; Walsh, C. T. *Biochem.* **1996**, *35*, 4923.
(148) Skarzynski, T.; Kim, D. H.; Lees, W. J.; Walsh, C. T.; Duncan, K. *Biochemistry* **1998**, *37*, 2572.
(149) Grisebach, H.; Schmid, R. *Angew. Chem., Int. Ed. Engl.* **1972**, *11*, 159.
(150) Yoshimura, J. *Adv. Carbohydr. Chem. Biochem.* **1985**, *42*, 69.
(151) Gorshkova, R. P.; Zubkov, V. A.; Isakova, V. V.; Ovodov, Yu. S. *Carbohydr. Res.* **1984**, *126*, 308.
(152) Gorshkova, R. P.; Zubkov, V. A.; Isakov, V. V.; Ovodov, Yu. S. *Bioorg. Khim.* **1987**, *13*, 1146.
(153) Schmid, R.; Grisebach, H.; Karl, W. *Eur. J. Biochem.* **1970**, *14*, 243.
(154) Matern, U.; Grisebach, H. *Eur. J. Biochem.* **1972**, *29*, 5.
(155) Matern, U.; Grisebach, H. *Z. Naturforsch. Teil.* **1974**, *29*, 407.
(156) Chen, H.; Guo, Z.; Liu, H.-w. *J. Am. Chem. Soc.* **1998**, *120*, 11796.
(157) (a) Godon, J. J.; Chopin, M. C.; Ehrilich, S. D. *J. Bacteriol.* **1992**, *174*, 6580. (b) Chipman, D.; Barak, Z.; Schloss, J. V. *Biochim. Biophys. Acta* **1998**, *1385*, 401.
(158) Candy, J. M.; Duggelby, R. G. *Biochem. J.* **1994**, *300* (Part I), 7.
(159) Hawkins, C. F.; Borges, A.; Perham, R. N. *FEBS Lett.* **1989**, *255*, 77.
(160) Muller, Y. A.; Lingqvist, Y.; Furey, W.; Schultz, G. E.; Jordan, F.; Schneider, G. *Structure* **1993**, *1*, 95.
(161) Webster, T. D.; Dickson, R. C. *Nucleic Acids Res.* **1988**, *16*, 8192.
(162) Chen, H.; Zhao, Z.; Hallis, T. M.; Guo, Z.; Liu, H.-W. *Angew. Chem.*, in press.
(163) Kuzuyama, T.; Hidaka, T.; Kamigiri, K.; Imai, S.; Seto, H. *J. Antibiot. (Tokyo)* **1992**, *45*, 1812.
(164) Hallis, T. M. Ph.D. Thesis, 2000, University of Minnesota.
(165) Kagan, R. M.; Clark, S. *Arch. Biochem. Biophys.* **1994**, *310*, 417.
(166) Gaisser, S.; Bohm, G. A.; Doumith, M.; Raynal, M. C.; Dhillon, N.; Cortes, J.; Leadlay, D. F. *Mol. Gen. Genet.* **1998**, *258*, 78.
(167) Trucco, R. E.; Caputto, R.; Leloir, L. F.; Mittelman, N. *Arch. Biochem.* **1948**, *18*, 137.
(168) Wilkinson, J. F. *Biochem. J.* **1949**, *44*, 460.
(169) Caputto, R.; Leloir, L. F.; Trucco, R. E.; Cardini, C. E.; Paladini, A. C. *J. Biol. Chem.* **1949**, *179*, 497.
(170) Frey, P. A. *FASEB* **1996**, *10*, 461.
(171) Quimby, B. B.; Alano, A.; Almashanu, S.; DeSandro, A. M.; Cowan, T. M.; Fridovich- Keil, J. L. *Am. J. Hum. Genet.* **1997**, *61*, 590.
(172) Rosoff, P. M. *J. Pediatr.* **1995**, *127*, 605.
(173) Wilson, D. B.; Hogness, D. S. *J. Biol. Chem.* **1964**, *239*, 2469.
(174) Imae, Y.; Morikawa, N.; Kurahashi, K. *J. Biochem.* **1964**, *56*, 138.
(175) Wilson, D. B.; Hogness, D. S. *J. Biol. Chem.* **1968**, *244*, 2132.
(176) Maxwell, E. S.; Robichon-Szulmajster, H. *J. Biol. Chem.* **1960**, *235*, 308.
(177) Darrow, R. A.; Rodstrom, R. *Proc. Natl. Acad. Sci. U.S.A.* **1966**, *55*, 205.
(178) Darrow, R. A.; Rodstrom, R. *Biochemistry* **1968**, *7*, 1645.
(179) Maxwell, E. S. *J. Biol. Chem.* **1957**, *229*, 139.
(180) Geren, C. R.; Ebner, K. E. *J. Biol. Chem.* **1977**, *252*, 2082.
(181) (a) Bauer, A. J.; Rayment, I.; Frey, P. A.; Holden, H. M. *Proteins: Struct., Funct., Genet.* **1992**, *12*, 372. (b) Liu, Y. J.; Vanhooke, J. L.; Frey, P. A. *Biochemistry* **1996**, *35*, 7615.
(182) Kowalsky, A.; Koshland, D. E., Jr. *Biochim. Biophys. Acta.* **1956**, *22*, 575.
(183) Bevill, R. D.; Nordin, J. H.; Smith, F.; Kirwood, S. *Biochem. Biophys. Res. Commun.* **1963**, *12*, 152.
(184) Anderson, L.; Landel, A. M.; Diedrich, D. F. *Biochim. Biophys. Acta* **1956**, *22*, 573.
(185) Kalckar, H. M.; Maxwell, E. S. *Biochim. Biophys. Acta* **1956**, *22*, 588.
(186) Wee, T. G.; Davis, J.; Frey, P. A. *J. Biol. Chem.* **1972**, *247*, 1339.
(187) Wee, T. G.; Frey, P. A. *J. Biol. Chem.* **1973**, *248*, 33.
(188) Maita, U. S.; Ankel, H. *J. Biol. Chem.* **1973**, *248*, 1477.
(189) Glaser, L.; Ward, L. *Biochim. Biophys. Acta* **1970**, *198*, 613.
(190) Kang, U. G.; Nolan, L. D.; Frey, P. A. *J. Biol. Chem.* **1975**, *250*, 7099.
(191) Wong, S. S.; Frey, P. A. *Biochemistry* **1977**, *16*, 298.
(192) Nelsestuen, G. L.; Kirwood, S. *Biochim. Biophys. Acta* **1970**, *220*, 633.
(193) Adair, W. L.; Gabriel, O.; Ullrey, D.; Kalckar, H. M. *J. Biol. Chem.* **1973**, *248*, 4635.
(194) Wong, S. S.; Cassim, J. Y.; Frey, P. A. *Biochemistry* **1978**, *17*, 516.
(195) Blackburn, P.; Ferdinand, W. *Biochem. J.* **1976**, *155*, 225.
(196) Thoden, J. B.; Holden, H. M. *Biochemistry* **1998**, *37*, 11469.
(197) Thoden, J. B.; Frey, P. A.; Holden, H. M. *Biochemistry* **1996**, *35*, 5137.
(198) Bhaduri, A.; Christensen, A.; Kalchar, H. M. *Biochem. Biophys. Res. Commun.* **1965**, *21*, 631.
(199) Bertland, A. U.; Bugge, B.; Kalckar, H. M. *Arch. Biochem. Biophys.* **1966**, *116*, 280.
(200) Kalckar, H. M.; Bertland, A. U.; Bugge, B. *Proc. Natl. Acad. Soc. U.S.A.* **1970**, *65*, 1113.
(201) Bertland, A. U.; Seyama, Y.; Kalckar, H. M. *Biochemistry* **1971**, *10*, 1545.
(202) Seyama, Y.; Kalckar, H. M. *Biochemistry* **1972**, *11*, 36.
(203) Davis, J. E.; Nolan, L. D.; Frey, P. A. *Biochim. Biophys. Acta* **1974**, *334*, 442.
(204) Konopka, J. M.; Halkides, C. J.; Vanhooke, J. L.; Gorenstein, D. G.; Frey, P. A. *Biochemistry* **1989**, *28*, 2645.
(205) Thoden, J. B.; Frey, P. A.; Holden, H. M. *Biochemistry* **1996**, *35*, 2557.
(206) Burke, J. R.; Frey, P. A. *Biochemistry* **1993**, *32*, 13220.
(207) Frey, P. A.; Bauer, A. J.; Vanhooke, J. L.; Konopka, J. M.; Rayment, I.; Holden, H. M. *J. Nutr. Sci. Vitaminol. Special Issue, Proceedings of the First International Congress on Vitamines and Biofactors in Life Science*, Kobe, Japan, 1992, 461−464.
(208) Poolman, B.; Royer, T. J.; Mainzer, S. E.; Schmidt, B. F. *J. Bacteriol.* **1990**, *172*, 4037.
(209) Flentke, G. R.; Frey, P. A. *Biochemistry* **1990**, *29*, 2430.
(210) Swanson, B. A.; Frey, P. A. *Biochemistry* **1993**, *32*, 13231.
(211) Inspection of the abortive E·NADH·UDP-Glc complex revealed that Ser124 is hydrogen bonded to the C-4 hydroxy of UDP-Glc and is proximal to Tyr149. Lys153 has been proposed to play a role to lower the pK_a of Tyr149, which may serve as the base to deprotonate 4-OH of the substrate via Ser124 in a proton relay mechanism (Liu, Y.; Thoden, J. B.; Kim, J.; Berger, E.; Gulick, A. M.; Ruzicka, F. J.; Holden, H. M.; Frey, P. A. *Biochemistry* **1997**, *36*, 10675. Thoden, J. B.; Gulick, A. M.; Holden, H. M. *Biochemistry* **1997**, *36*, 10685.)
(212) Thoden, J. B.; Hegeman, A. D.; Wesenberg, G.; Chapeau, M. C.; Frey, P. A.; Holden, H. M. *Biochemistry* **1997**, *36*, 6294.
(213) Burma, D. P.; Horecker, B. L. *J. Biol. Chem.* **1958**, *231*, 1053.
(214) Johnson, A. E.; Tanner, M. E. *Biochemistry* **1998**, *37*, 5746.
(215) Englesberg, E.; Anderson, R. L.; Weinberg, R.; Lee, N.; Hoffee, P.; Huttenhauer, G.; Boyer, H. *J. Bacteriol.* **1962**, *84*, 137.
(216) Burma, D. P.; Horecker, B. L. *Biochim. Biophys. Acta* **1957**, *24*, 660.
(217) Lepesant, J. A.; Dedonder, R. *C. R. Hebd. Seances Acad. Sci. Paris Ser. D* **1967**, *264*, 2683.
(218) Mortlock, R. P.; Wood, W. A. *J. Bacteriol.* **1964**, *88*, 845.
(219) Fossitt, D.; Mortlock, R. P.; Anderson, R. L.; Wood, W. A. *J. Biol. Chem.* **1964**, *239*, 2110.
(220) Anderson, R. L.; Wood, W. A. *J. Biol. Chem.* **1962**, *237*, 296.
(221) Davis, L.; Lee, N.; Glaser, L. *J. Biol. Chem.* **1972**, *247*, 5862.
(222) Deupree, J. D.; Wood, W. A. *J. Biol. Chem.* **1972**, *247*, 3093.
(223) Lin, E. C. C. In *Escherichia coli and Salmonella*; Neidhart, F. C., Curtis, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Resnikoff, W. S., Riley, M., Schaechter, M., Umbarger, H. E., Eds.; American Society for Microbiology: Washington, D.C., 1996; pp 307−342.
(224) Salo, W. L.; Fossitt, D. D.; Bevill, R. D.; Kirkwood, S.; Wood, W. A. *J. Biol. Chem.* **1972**, *247*, 3098.
(225) Deupree, J. D.; Wood, W. A. *J. Biol. Chem.* **1970**, *245*, 3988.
(226) (a) Tanner, M. E. In *Comprehensive Biological Catalysis*; Sinnott, M., Ed.; Academic Press: San Diego, 1998; Vol. 3, pp 76−82. (b) Lee, L. V.; Vu, M. V.; Cleland, W. W. *Biochemistry* **2000**, *39*, 4808.

(227) McDonough, M. W.; Wood, W. A. *J. Biol. Chem*. **1961**, *236*, 1220.
(228) Dreyer, M. K.; Schulz, G. E. *Acta Crystallogr*. **1996**, *D52*, 1082.
(229) Horecker, B. L.; Tsolas, O.; Lai, C. Y. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. VII, pp 213−258.
(230) Gerlt, J. A.; Kenyon, G. L.; Kozarich, J. W.; Neidhart, D. J.; Petsko, G. A.; Powers, V. M. *Curr. Opin. Struct. Biol*. **1992**, *2*, 736.
(231) Dreyer, M. K.; Schulz, G. E. *J. Mol. Biol*. **1996**, *259*, 458.
(232) (a) Fessner, W.-D.; Schneider, A.; Held, H.; Sinerius, G.; Walter, C.; Hixon, M.; Schloss, J. V. *Angew. Chem., Int. Ed. Engl*. **1996**, *35*, 2219. (b) Lee, L. V.; Poyner, R. R.; Vu, M. V.; Cleland, W. W. *Biochemistry* **2000**, *39*, 4821.
(233) Rosso, R. G.; Adams, E. *J. Biol. Chem*. **1967**, *242*, 5524.
(234) Schirch, L.; Diller, A. *J. Biol. Chem*. **1971**, *246*, 3961.
(235) Palekar, A. G.; Tate, S. S.; Meister, A. *J. Biol. Chem*. **1973**, *248*, 1158.
(236) Ulevitch, R. J.; Kallen, R. G. *Biochemistry* **1977**, *16*, 5342.
(237) Bissett, D. L.; Anderson, R. L. *J. Biol. Chem*. **1980**, *255*, 8750.
(238) Fessner, W.-D.; Sinerius, G.; Schneider, A.; Dreyer, M.; Schulz, G. E.; Badia, J.; Aguilar, J. *Angew. Chem., Int. Ed. Engl*. **1991**, *30*, 555.
(239) Fessner, W. D.; Eyrisch, O. *Angew. Chem., Int. Ed. Engl*. **1992**, *31*, 56.
(240) Wong, C.-H.; Halcomb, R. L.; Ichikawa, Y.; Kajimoto, T. *Angew. Chem., Int. Ed. Engl*. **1995**, *34*, 412.
(241) Cardini, C. E.; Leloir, L. F. *J. Biol. Chem*. **1957**, *225*, 317.
(242) Comb, D. G.; Roseman, S. *Biochim. Biophys. Acta* **1958**, *29*, 653.
(243) Spivak, C. T.; Roseman, S. *Methods Enzymol*. **1966**, *9*, 612.
(244) Sommar, K. M.; Ellis, D. B. *Biochim. Biophys. Acta* **1972**, *268*, 590.
(245) Kawamura, T.; Ichihara, N.; Ishimoto, N.; Ito, E. *Biochem. Biophys. Res. Commun*. **1975**, *66*, 1506.
(246) Kawamura, T.; Ishimoto, N.; Ito, E. *J. Biol. Chem*. **1979**, *254*, 8457.
(247) Kawamura, T.; Ishimoto, N.; Ito, E. *Methods Enzymology* **1982**, *83*, 515.
(248) Kawamura, T.; Kimura, M.; Yamamori, S.; Ito, E. *J. Biol. Chem*. **1978**, *253*, 35957.
(249) Ghosh, S.; Roseman, S. *J. Biol. Chem*. **1965**, *240*, 1525.
(250) Lee, C.-J.; Banks, S. D.; Li, J. P. *Crit. Rev. Microbiol*. **1991**, *18*, 89.
(251) Jann; B.; Jann, K. *Curr. Top. Microbiol. Immunol*. **1990**, *150*, 19.
(252) Moxon, E. R.; Kroll, J. S. *Curr. Top. Microbiol. Immunol*. **1990**, *150*, 65.
(253) Heptinstall, S.; Archibald, A. R.; Baddiley, J. *Nature* **1970**, *225*, 519.
(254) Baddiley, J. *Acc. Chem. Res*. **1970**, *3*, 98.
(255) Morgan, P. M.; Sala, R. F.; Tanner, M. E. *J. Am. Chem. Soc*. **1997**, 10269.
(256) Salo, W. L. *Biochim. Biophys. Acta* **1976**, *452*, 625.
(257) Meiser-Dieter, U.; Barr, K.; Starman, R.; Hatch, L.; Rick, P. D. *J. Biol. Chem*. **1992**, *267*, 746.
(258) Sala, R. F.; Morgan, P. M.; Tanner, M. E. *J. Am. Chem. Soc*. **1996**, *118*, 3033.
(259) Salo, W. L.; Fletcher, H. G., Jr. *Biochemistry* **1970**, *9*, 882.
(260) Collins, P.; Ferrier, R. *Monosaccharides*; Wiley: Chichester, 1995; pp 236−237.
(261) Knapp. S.; Vocadlo, D.; Gao, Z.; Kirk, B.; Lou, J.; Withers, S. G. *J. Am. Chem. Soc*. **1996**, *118*, 6804.
(262) Verma, N.; Reeves, P. R. *J. Bacteriol*. **1989**, *171*, 5694.
(263) Hobbs, M.; Reeves, P. R. *Biochim. Biophys. Acta* **1995**, *1245*, 273.
(264) The gene was originally designated as *rfb*E[262,263] and renamed as *tyv* according to a new nomenclature for genes involved in the biosynthesis of sugars found in the LPS (Reeves, P. R.; Hobbs, M.; Valvano, M. A.; Skurnik, M.; Whitfield, C.; Coplin, D.; Kido, N.; Klena, J.; Maskell, D.; Raetz, C. R. H.; Rick, P. D. *Trends Microbiol*. **1996**, *4*, 495).
(265) (a) Hallis, T. M.; Liu, H.-w. *J. Am. Chem. Soc*. **1999**, *121*, 6765. (b) Hallis, T. M.; Zhao, Z.; Liu, H.-w. *J. Am. Chem. Soc*. **2000**, *122*, in press.
(266) Rizzo, V.; Pande, A.; Luisi, P. L. In *Pyridine Nucleotide Coenzymes*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley and Sons: New York, 1987; Vol. IIA, p 99.
(267) Glasfeld, A.; Farber, G. K.; Ringe, D.; Marcel, T.; Drocourt, D.; Tiraby, G.; Petsko, G. A. *J. Biol. Chem*. **1988**, *263*, 14612.
(268) Feather, M. S.; Deshpande, V.; Lybyer, M. J. *Biochem. Biophys. Res. Commun*. **1970**, *38*, 859.
(269) Schray, K. J.; Rose, I. A. *Biochemistry* **1971**, *10*, 1058.
(270) Chen, W.-P. *Process Biochem*. **1980**, *15*, 36.
(271) Suekane, M.; Tamura, M.; Tomimura, C. *Agric. Biol. Chem*. **1978**, *42*, 909.
(272) Viot, D. 1984, Ph.D. Thesis, L'Université des Science et Techniques de Lille, France.
(273) van Bastelaere, P. B. M.; Callens, M.; Vangrysperre, W. A. E.; Kersters-Hilderson, H. L. M. *Biochem. J*. **1992**, *286*, 729.
(274) Vangrysperre, W.; Van Damme, J.; Vandekerckhove, J.; De Bruyne, C. K.; Cornelis, R.; Kersters-Hilderson, H. *Biochem. J*. **1990**, *265*, 699.
(275) Jenkins, J.; Janin, J.; Rey, F.; Chiadmi, M.; Van Tilbeurgh, H.; Lasters, I.; De Malyer, M.; Beile, D. van; Wodak, S. J.; Lauwereys, M.; Stanssens, P.; Mrabet, N. T.; Snauwaert, J.; Mattyssens, G.; Lambeir, A. M. *Biochemistry* **1992**, *31*, 5449.
(276) Carrell, H. L.; Rubin, B. H.; Hurley, T. J.; Glusker, J. P. *J. Biol. Chem*. **1984**, *259*, 3230.
(277) Farber, G. K.; Petsko, G. A.; Ringe, D. *Protein Eng*. **1987**, *1*, 459.
(278) Henrick, K.; Blow, D. M.; Carrell, H. L.; Glusker, J. P. *Protein Eng*. **1987**, *1*, 467.
(279) Rey, F.; Jenkins, J.; Janin, J.; Lasters, I.; Alard, P.; Claessen, M.; Matthyssens, G.; Wodak, S. *Proteins*: *Struct., Funct., Genet*. **1988**, *4*, 165.
(280) Carrell, H. L.; Glusker, J. P.; Burger, V.; Manfre, F.; Tritsch, D.; Biellmann, J. F. *Proc. Natl. Acad. Sci. U.S.A*. **1989**, *86*, 4440.
(281) Henrick, K.; Collyer, C. A.; Blow, D. M. *J. Mol. Biol*. **1989**, *208*, 129.
(282) Farber, G. K.; Glasfeld, A.; Tiraby, G.; Ringe, D.; Petsko, G. A. *Biochemistry* **1989**, *28*, 7289.
(283) Dauter, Z.; Dauter, M.; Hemker, J.; Witzel, H.; Wilson, K. *FEBS Lett*. **1989**, *247*, 1.
(284) Collyer, C. A.; Henrick, K.; Blow, D. M. *J. Mol. Biol*. **1990**, *212*, 211.
(285) Whitlow, M.; Howard, A. J.; Finzel, B. C.; Poulos, T.; Winborne, E.; Gilliland, G. L. *Proteins: Struct., Funct., Genet*. **1991**, *9*, 153.
(286) Dauter, Z.; Terry, H.; Witzel, H.; Wilson, K. S. *Acta Crystallogr*. **1996**, *B46*, 833.
(287) Reider, S. V.; Rose, I. A. *J. Biol. Chem*. **1959**, *234*, 1007.
(288) Banner, D. W.; Bloomer, A. C.; Petsko, G. A.; Phillips, D. C.; Pogson, C. I.; Wilson, I. A. *Nature (London)* **1975**, *255*, 609.
(289) Albery, W. J.; Knowles, J. R. *Biochemsitry* **1976**, *15*, 5627.
(290) Schray, K. J.; Mildvan, A. S. *J. Biol. Chem*. **1972**, *247*, 2034.
(291) Young, J. M.; Schray, K. J.; Mildvan, A. S. *J. Biol. Chem*. **1975**, *250*, 9021.
(292) McKay, G. A.; Tavlarides, L. L. *J. Mol. Catal*. **1979**, *6*, 57.
(293) Bock, K.; Meldal, M.; Meyer, B.; Wiebe, L. *Acta Chem. Scand*. **1983**, *B37*, 101.
(294) Makkee, M.; Kieboom, A. P. G.; van Bekkum, H. *Recl. Trav. Chim. Pays-Bas* **1984**, *103*, 361.
(295) Collyer, C. A.; Goldberg, J. D.; Viehmann, H.; Blow, D. M.; Ramsden, N. G.; Fleet, G. W. J.; Montgomery, F. J.; Grice, P. *Biochemistry* **1992**, *31*, 12211.
(296) Collyer, C. A.; Blow, D. M. *Proc. Natl. Acad. Sci. U.S.A*. **1990**, *87*, 1362.
(297) Lee, C.; Bagdasarian, M.; Meng, M.; Zeikus, J. G. *J. Biol. Chem*. **1990**, *265*, 19082.
(298) Lambeir, A.-M.; Lauwereys, M.; Stanssens, P.; Mrabet, N. T.; Snauwaert, J.; van Tilbeurgh, H.; Matthyssens, G.; Lasters, I.; De Maeyer, M.; Wodak, S. J.; Jenkins, J.; Chiadmi, M.; Janin, J. *Biochemistry* **1992**, *31*, 5459.
(299) van Bastelaere, P. B. M.; Vangrysperre, W. A. E.; Kersters-Hilderson, H. *Biochem. J*. **1991**, *278*, 285.
(300) Allen, K. N.; Lavie, A.; Glasfeld, A.; Tanada, T. N.; Gerrity, D. P.; Carlson, S. C.; Farber, G. K.; Petsko, G. A.; Ringe, D. *Biochemistry* **1994**, *33*, 1488.
(301) Rangarajan, M.; Hartley, B. S. *Biochem. J*. **1992**, *283*, 223.
(302) Gaikwad, S. M.; More, M. W.; Vartak, H. G.; Deshpande, V. V. *Biochem. Biophys. Res. Commun*. **1988**, *155*, 270.
(303) Vangrysperre, W.; Ampe, C.; Kersters-Hilderson, H.; Tempst, P. *Biochem. J*. **1989**, *263*, 195.
(304) Gaikwad, S. M.; Pawar, H. S.; Vartak, H. G.; Deshpande, V. V. *Biochem. Biophys. Res. Commun*. **1989**, *159*, 457.
(305) Lavie, A.; Allen, K. N.; Petsko, G. A.; Ringe, D. *Biochemistry* **1994**, *33*, 5469.
(306) Bogumil, R.; Kappl, R.; Hüttermann, J.; Witzel, H. *Biochem*. **1997**, *36*, 2345.
(307) Smart, O. S.; Akins, J.; Blow, D. M. *Proteins: Struct., Funct., Genet*. **1992**, *12*, 100.
(308) Fuxreiter, M.; Farkas, Ö.; Náray-Szabó, G. *Protein Eng*. **1995**, *8*, 925.
(309) Wohl, A.; Neuberg, C. *Ber*. **1900**, *33*, 3095.
(310) Rose, I. A. *Adv. Enzymol*. **1975**, *43*, 491.
(311) Rose, I. A.; O'Connell, E. L.; Mortlock, R. P. *Biochim. Biophys. Acta* **1969**, *178*, 376.
(312) Callens, M.; Tomme, P.; Kersters-Hilderson, H.; Cornelis, R.; Vangrysperre, W.; De Bruyne, C. K. *Biochem. J*. **1988**, *250*, 285.
(313) Sudfeldt, C.; Schäffer, A.; Kägi, J. H. R.; Bogumil, R.; Schulz, H.-P.; Wulff, S.; Witzel, H. *Eur. J. Biochem*. **1990**, *193*, 863.
(314) Bogumil, R.; Kappl, R.; Hütermann, J.; Sudfeldt, C.; Witzel, H. *Eur. J. Biochem*. **1993**, *213*, 1185.
(315) Fuxreiter, M.; Böcskei, Z.; Szeibert, A.; Szabó, E.; Dallmann, G.; Náray- Szabó, G.; Asbóth, B. *Proteins: Struct., Funct., Genet*. **1997**, *28*, 183.
(316) Whitaker, R. D.; Cho, Y.; Cha, J.; Carrell, H. L.; Glusker, J. P.; Karplus, P. A.; Batt, C. A. *J. Biol. Chem*. **1995**, *270*, 22895.
(317) Allen, K. N.; Lavie, A.; Petsko, G. A.; Ringe, D. *Biochemistry* **1995**, *34*, 3742.

(318) Hu, H.; Liu, H.; Shi, Y. *Proteins: Struct., Funct., Genet.* **1997**, *27*, 545.
(319) Yamashita, M. M.; Wesson, L.; Eisenman, G.; Eisenberg, D. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 5648.
(320) Meng, M.; Lee, C.; Bagdasarian, M.; Zeikus, G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4015.
(321) Mrabet, N. T.; Van den Broeck, A.; Van den brande, I.; Stanssens, P.; Laroche, Y.; Lambier, A.-M.; Mattyssen, G.; Jenkins, J.; Chiadmi, M.; van Tilbeurgh, H.; Rey, F.; Janin, J.; Quax, W. J.; Lasters, I.; De Maeyer, M.; Wodak, S. J. *Biochemistry* **1992**, *31*, 2239.
(322) van Tilbeurgh, H.; Jenkins, J.; Chiadmi, M.; Janin, J.; Wodak, S. J.; Mrabet, N. T.; Lambeir, A.-M. *Biochemistry* **1992**, *31*, 5467.
(323) Varsani, L.; Cui, T.; Rangarajan, M.; Hartley, B. S.; Goldberg, G.; Collyer, C.; Blow, D. M. *Biochem. J.* **1993**, *291*, 575.
(324) Xiao, Y. Z.; Wu, C. J.; Long, F.; Niu, L. W.; Wang, Y. Z.; Cui, T. *Acta Biochim. Biophys. Sinica* **1995**, *27*, 469.
(325) Cooper, R. A. *Arch. Microbiol.* **1978**, *118*, 199.
(326) Babbitt, P. C.; Hasson, M. S.; Wedekind, J. E.; Palmer, D. R. J.; Barrett, W. C.; Reed, G.; Rayment, I.; Ringe, D.; Kenyon, G. L.; Gerlt, J. A. *Biochemistry* **1996**, *35*, 16489.
(327) Babbit, P. C.; Gerlt, J. A. *J. Biol. Chem.* **1997**, *272*, 30591.
(328) Babbitt, P. C.; Mrachko, G. T.; Hasson, M. S.; Huisman, G. W.; Kolter, R.; Ringe, D.; Petsko, G. A.; Kenyon, G. L.; Gerlt, J. A. *Science* **1995**, *267*, 1159.
(329) Gerlt, J. A.; Gassman, P. G. *Biochemistry* **1993**, *32*, 11943.
(330) Landro, J. A.; Gerlt, J. A.; Kozarich, J. W.; Koo, C. W.; Shah, V. J.; Kenyon, G. L.; Fujita, S.; Petsko, G. A. *Biochemistry* **1994**, *33*, 635.
(331) Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 5928.
(332) Aldrich, T. L.; Frantz, B.; Gill, J. F.; Kilbane, J. J.; Chakrabarty, A. M. *Gene* **1987**, *52*, 185.
(333) Goldman, A.; Ollis, B. L.; Steitz, T. A. *J. Mol. Biol.* **1987**, *194*, 143.
(334) Neidhart, D. J.; Kenyon, G. L.; Gerlt, J. A.; Petsko, G. A. *Nature* **1990**, *327*, 692.
(335) Palmer, D. R. J.; Wieczorek, S. J.; Hubbard, B. K.; Mrachko, G. T.; Gerlt, J. A. *J. Am. Chem. Soc.* **1997**, *119*, 9580.
(336) Palmer, D. R. J.; Gerlt, J. A. *J. Am. Chem. Soc.* **1996**, *118*, 10323.
(337) (a) Kirby, G. W.; O'Laughlin, G. J.; Robins, D. J. *J. Chem. Soc., Chem. Commun.* **1975**, 402. (b) Chari, R. V. J.; Whitman, C. P.; Kozarich, J. W.; Ngai, K.-L.; Ornston, L. N. *J. Am. Chem. Soc.* **1987**, *109*, 5514.
(338) Srinivasan, P. R.; Rothschild, J.; Sprinson, D. B. *J. Biol. Chem.* **1963**, *238*, 3176.
(339) Euverink, G. J. W.; Hessels, G. I.; Vrijbloed, J. W.; Coggins, J. R. Dijkhuizen, L. *J. Gen. Microbiol.* **1992**, *138*, 2449.
(340) Haslam, E. *The Shikimate Pathway*; Wiley: New York, 1974.
(341) Weiss, U.; Edwards, J. M. *The Biosynthesis of Aromatic Compounds*; Wiley: New York, 1980.
(342) Frost, J. W.; Bender, J. L.; Kadonaga, J. T.; Knowles, J. R. *Biochemistry* **1984**, *23*, 4470.
(343) Millar, G.; Coggins, J. R. *FEBS Lett.* **1986**, *200*, 11.
(344) Mehdi, S.; Frost, J. W.; Knowles, J. R. *Methods Enzymol.* **1987**, *142*, 306.
(345) van den Hombergh, J. P. T. W.; Moore, J. D.; Charles, I. G.; Hawkins, A. R. *Biochem. J.* **1992**, *284*, 861.
(346) Moore, J. D.; Coggins, J. R.; Virden, R.; Hawkins, A. R. *Biochem. J.* **1994**, *301*, 297.
(347) Bischoff, M.; Rosler, J.; Raesecke, H. R.; Gorlach, J.; Amrhein, N.; Schmid, J. *Plant Mol. Biol.* **1996**, *31*, 69.
(348) Bereswill, S.; Fassbinder, F.; Volzing, C.; Haas, R.; Reuter, K.; Ficner, R.; Kist, M. *Med. Microbiol. Immunol.* **1997**, *186*, 125.
(349) Barten, R.; Meyer T. F. *Mol. Gen. Genet* **1998**, *258*, 34.
(350) Kobes, R. D.; Simpson, R. T.; Vallee, B. L.; Rutter, W. J. *Biochemistry* **1969**, *8*, 585.
(351) Shen, T. Y. S.; Westhead, E. W. *Biochemistry* **1973**, *12*, 3333.
(352) Rose, I. A.; O'Connell, E. L.; Schray, K. J. *J. Biol. Chem.* **1973**, *248*, 2232.
(353) Brändén, C.-I.; Eklund, H. *Ciba Found. Symp.* **1978**, *60*, 63.
(354) Bertini, I.; Drago, R. S.; Luchinat, C. *The Coordination Chemistry of Metalloenzymes*; Reidel: Dordrecht, Holland, 1983; Chapters 8−10.
(355) Kadonaga, J. T.; Knowles, J. R. *Biochemistry* **1983**, *22*, 130.
(356) Bender, S. L.; Mehdi, S.; Knowles, J. R. *Biochemistry* **1989**, *28*, 7555.
(357) Maitra, W. I.; Sprinson, D. B. *J. Biol. Chem.* **1978**, *253*, 5426.
(358) Bender, S. L.; Widlanski, T.; Knowles, J. R. *Biochemistry* **1989**, *28*, 7560.
(359) Widlanski, T.; Bender, S. L.; Knowles, J. R. *Biochemistry* **1989**, *28*, 7572.
(360) Lambert, J. M.; Boocock, M. R.; Coggins, J. R. *Biochem. J.* **1985**, *226*, 817.
(361) Widlanski, T.; Bender, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1989**, *111*, 2299.
(362) Montchamp, J.-L.; Frost, J, W. *J. Am. Chem. Soc.* **1997**, *119*, 7645.
(363) Bartlett, P. A.; Satake, K. *J. Am. Soc. Chem.* **1988**, *110*, 1628.
(364) Parker, E. J.; Coggins, J. R.; Abell, C. *J. Org. Chem.* **1997**, *62*, 8582.
(365) Piehler, L. T.; Montchamp, J.-L.; Frost, J. W.; Manly, C. J. *Tetrahedron* **1991**, *47*, 2423.
(366) (a) Le Maréchal, P.; Froussios, C.; Level, M.; Azerad, R. *Biochem. Biophys. Res. Commun.* **1980**, *92*, 1104. (b) Le Maréchal, P.; Froussios, C.; Level, M.; Azerad, R. *Carbohydr. Res.* **1981**, *94*, 1.
(367) Montchamp, J.-L.; Frost, J. W. *J. Am. Chem. Soc.* **1991**, *113*, 6296.
(368) Myrvold, S.; Reimer, L. M.; Pompliano, D. L.; Frost, J. W. *J. Am. Chem. Soc.* **1989**, *111*, 1861.
(369) Montchamp, J.-L.; Peng, J.; Frost, J, W. *J. Org. Chem.* **1994**, *59*, 6999.
(370) Montchamp, J.-L.; Piehler, L. T.; Frost, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 4453.
(371) Montchamp, J.-L.; Frost, J, W. *J. Org. Chem.* **1994**, *59*, 7596.
(372) Montchamp, J.-L.; Piehler, L. T.; Tolbert, T. J.; Frost, J. W. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1349.
(373) Montchamp, J.-L.; Piehler, L. T.; Tolbert, T. J.; Frost, J. W. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1403.
(374) Tian, F.; Montchamp, J.-L.; Frost, J. W. *J. Org. Chem.* **1996**, 7373.
(375) Levin, D. H.; Racker, E. *J. Biol. Chem.* **1959**, *234*, 2532.
(376) Osborn, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1963**, *50*, 499.
(377) Heath, E. C.; Ghalambor, M. A. *Biochem. Biophys. Res. Commun.* **1963**, *10*, 340.
(378) Ghalambor, M. A.; Levine, E. M.; Heath, E. C. *J. Biol. Chem.* **1966**, *241*, 3207.
(379) Ray, P. H. *J. Bacteriol.* **1980**, *141*, 635.
(380) Rick, P. D.; Osborn, M. J. *J. Biol. Chem.* **1977**, *252*, 4895.
(381) Hammond, S. M.; Claesson, A.; Jansson, A. M.; Larsson, L.-G.; Pring, B. G.; Town, C. M.; Ekström, B. *Nature* **1987**, *327*, 730.
(382) Goldman, R.; Kohlbrenner, W.; Lartey, P.; Pernet, A. *Nature* **1987**, *329*, 162.
(383) Woisetschlaher, M.; Hogenauer, G. *J. Bacteriol.* **1986**, *168*, 437.
(384) Ray, P. H.; Kelsey, J. E.; Bigham, E. C.; Benedict, C. D.; Miller, T. A. In *Bacterial Lipopolysaccharides*; Anderson, L., Unger, F. M., Eds; ACS Symposium Series 231; American Chemical Society: Washington. D.C., 1983; p 141.
(385) Baasov, T.; Knowles, J. R. *J. Bacteriol.* **1989**, *171*, 6155.
(386) Kohen, A.; Jakob, A.; Baasov, T. *Eur. J. Biochem.* **1992**, *208*, 443.
(387) Pierce, J.; Serianni, A. S.; Baker, R. *J. Am. Chem. Soc.* **1981**, *107*, 2448.
(388) Liang, P. H.; Lewis, J.; Anderson, K. S.; Kohen, A.; D'Souza, F. W.; Benenson, Y.; Baasov, T. *Biochemistry* **1998**, *37*, 16390.
(389) Kohen, A.; Berkovich, R.; Belakhov, V.; Baasov, T. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1577.
(390) Rose, I. A.; O'Connell, E. L.; Noce, P.; Utter, M. F.; Wood, H. G.; Willard, J. M.; Cooper, T. G.; Benziman, M. *J. Biol. Chem.* **1969**, *244*, 6130.
(391) (a) Rose, I. A. *J. Biol. Chem.* **1970**, *245*, 6052. (b) Stubbe, J. A.; Kenyon, G. L. *Biochemistry* **1971**, *10*, 2669.
(392) Floss, H. G.; Onderka, D. K.; Carroll, M. *J. Biol. Chem.* **1972**, *247*, 736.
(393) Hedstrom, L.; Abeles, R. *Biochem. Biophys. Res. Commum.* **1988**, *157*, 816.
(394) Sheffer-Dee-Noor, S.; Belakhov, V.; Baasov, T. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1583.
(395) Baasov, T.; Sheffer-Dee-Noor, S.; Kohen, A.; Jakob, A.; Belakhov, V. *Eur. J. Biochem.* **1993**, *217*, 991.
(396) Du, S.; Plat, D.; Belakhov, V.; Baasov, T. *J. Org. Chem.* **1997**, *62*, 794.
(397) Kohen, A.; Belakhov, V.; Baasov, T. *Tetrahedron Lett.* **1994**, *35*, 3179.
(398) Baasov, T.; Kohen, A. *J. Am. Chem. Soc.* **1995**, *117*, 6165.
(399) D'Souza, F. W.; Benenson, Y.; Baasov, T. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2457.
(400) Liang, P.-H.; Kohen, A.; Baasov, T.; Anderson, K. S. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2463.
(401) Kaustov, L.; Kababya, S.; Du, S.; Baasov, T.; Gropper, S.; Shoham, Y.; Schmidt, A. *J. Am. Chem. Soc.* **2000**, *122*, 2649.
(402) Yamauchi, N.; Kakinuma, K. *J. Org. Chem.* **1995**, *60*, 5614.
(403) Kudo, F.; Tamegai, H.; Fujiwara, T.; Tagami, U.; Hirayama, K.; Kakinuma, K. *J. Antibiotics* **1999**, *52*, 559.

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