Biosynthesis of Colitose: Expression, Purification, and Mechanistic Characterization of GDP-4-keto-6-deoxy-D-mannose-3-Dehydrase (ColD) and GDP-L-colitose Synthase (ColC)†

Jenefer Alam,‡ Noelle Beyer,§ and Hung-wen Liu*

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

Received July 29, 2004; Revised Manuscript Received October 9, 2004

ABSTRACT: L-Colitose is a 3,6-dideoxyhexose found in the O-antigen of Gram-negative lipopolysaccharides. To study the biosynthesis of this unusual sugar, we have cloned and sequenced the L-colitose biosynthetic gene cluster from *Yersinia pseudotuberculosis* VI. The *colD* and *colC* genes in this cluster have been overexpressed and each gene product has been purified and characterized. Our results showed that ColD functions as GDP-4-keto-6-deoxy-D-mannose-3-dehydrase responsible for C-3 deoxygenation of GDP-4-keto-6-deoxy-D-mannose. This enzyme is coenzyme B_6-dependent and its catalysis is initiated by a transamination step in which pyridoxal 5′-phosphate (PLP) is converted to pyridoxamine 5′-phosphate (PMP) in the presene of L-glutamate. This coenzyme forms a Schiff base with the keto sugar substrate and the resulting adduct undergoes a PMP-mediated β-dehydration reaction to give a sugar enamine intermediate, which after tautomerization and hydrolysis to release ammonia yields GDP-4-keto-3,6-dideoxy-D-mannose as the product. The combined transamination−deoxygenation activity places ColD in a class by itself. Our studies also established ColC as GDP-L-colitose synthase, which is a bifunctional enzyme catalyzing the C-5 epimerization of GDP-4-keto-3,6-dideoxy-D-mannose and the subsequent C-4 keto reduction of the resulting L-epimer to give GDP-L-colitose. Reported herein are the detailed accounts of the overexpression, purification, and characterization of ColD and ColC. Our studies show that their modes of action in the biosynthesis of GDP-L-colitose represent a new deoxygenation paradigm in deoxysugar biosynthesis.

Deoxysugars are found ubiquitously in nature as constituents of plant, fungi, and bacteria. They are an important class of carbohydrates that serve as ligands for cell−cell interactions or as targets for toxins, antibodies, and microorganisms (*1*, *2*). The 3,6-dideoxyhexoses in particular have been found in the lipopolysaccharide (LPS)[1] components of a number of Gram-negative cell envelopes (*3*−*5*). These unusual dideoxyhexoses are present specifically in the O-antigen region of the LPS, where they serve as antigenic determinants and are vital for bacterial defense and survival (*6*−*9*). There are seven naturally occurring 3,6-dideoxyhexoses, six of which, paratose, abequose, tyvelose, ascarylose, and yersinioses A and B, are derived from CDP-D-glucose (see Scheme 1) (*10*), while the seventh sugar, colitose, is made from GDP-D-mannose (see Scheme 2) (*11*).

The biosynthesis of the glucose-derived 3,6-dideoxyhexose sugars is initiated by the α-D-glucose-1-phosphate cytidylyltransferase- (E_p-) catalyzed coupling of α-D-glucose-1-phosphate (**1**) and cytidine triphosphate (CTP) to form CDP-D-glucose (**2**) (Scheme 1). An irreversible intramolecular oxidation−reduction catalyzed by CDP-D-glucose 4,6-dehydratase (E_{od}), in the presence of NAD^+, converts **2** to CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose (**3**). The subsequent C-3 deoxygenation to form **4** is mediated by a pair of enzymes, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase (E_1), a pyridoxamine 5′-phosphate- (PMP-) dependent iron−sulfur-containing enzyme, and CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase reductase (E_3), a

† This work was supported in part by a National Institutes of Health Grant (GM35906). H.-w.L. also thanks the National Institute of General Medical Sciences for a Merit Award.

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

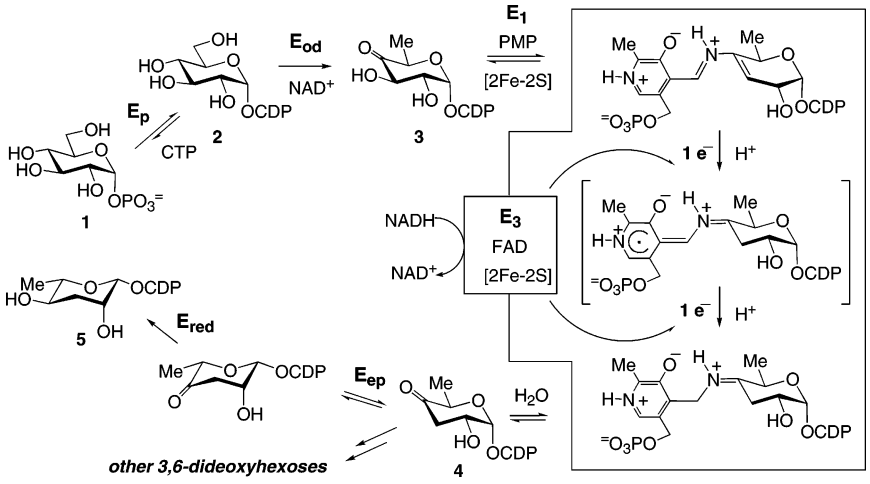
‡ Present address: Novartis Institute for Tropical Disease (NITD), Singapore.

§ Present address: Department of Chemistry, University of Wisconsin−Oshkosh, Oshkosh, WI 54901.

[1] Abbreviations: AspAT, aspartate aminotransferase; BCAT, branched chain aminotransferase; CDP, cytidine 5′-diphosphate; ColB, GDP-D-mannose 4,6-dehydratase; ColC, GDP-L-colitose synthase; ColD, GDP-4-keto-6-deoxy-D-mannose-3-dehydrase; ColE, α-D-mannose-1-phosphate guanidylyl-transferase; CTP, cytidine 5′-triphosphate; DEAE, diethylaminoethyl; DTT, dithiothreitol; E_1, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase; E_3, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase reductase; E_{od}, CDP-D-glucose 4,6-dehydratase; E_p, α-D-glucose-1-phosphate cytidylyltransferase; ESI, electrospray ionization; FAB, fast atom bombardment; FPLC, fast protein liquid chromatography; GDP, guanosine 5′-diphosphate; hFX, GDP-L-fucose synthase; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; KIE, kinetic isotope effect; LPS, lipopolysaccharide; NAD^+, β-nicotinamide adenine dinucleotide; NAD(P)H, β-nicotinamide adenine dinucleotide (phosphate), reduced form; NTA, nitrilotriacetic acid; NOESY, nuclear Overhauser exchange spectroscopy; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; SDS, sodium dodecyl sulfate; UV, ultraviolet; GCG, Genetics Computer Group, Inc.

10.1021/bi0483763 CCC: $27.50
Published on Web 12/04/2004

Scheme 1

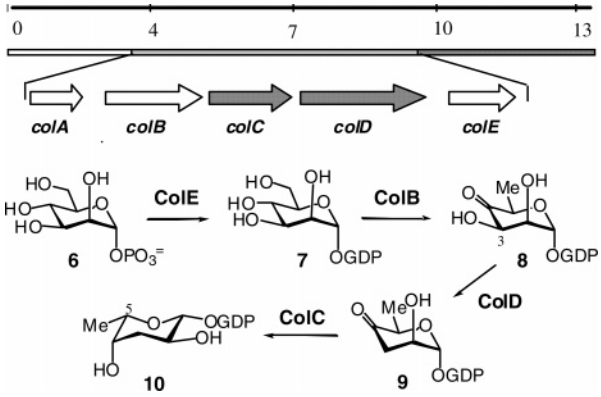


[2Fe-2S]-containing flavoprotein reductase. Intermediate **4** can then be converted to CDP-L-ascarylose (**5**) and other 3,6-dideoxyhexoses by various epimerases and ketoreductases (*5*, *9*, *10*, *12*).

In contrast, there are limited studies on the biosynthesis of the GDP-D-mannose-derived colitose. Colitose is a component in the O-antigen of *Escherichia coli* O111, *Yersinia pseudotuberculosis*, and some *Salmonella* species (*11*, *13*). In addition, a previously unknown serotype of *Vibrio cholerae* (*V. cholerae* O139 Bengal), a newly discovered second causative agent of cholera, has a short O-antigen composed of a repeating unit with two terminal colitose residues (*14*, *15*). A similar distinction has also been identified between the pathogenic and nonpathogenic *E. coli* strains: the presence of colitose in the former may enhance the lipophilic character of the endotoxin as a whole and possibly accounts for some of the different properties of the cell walls. Gram-negative bacteria of the genera *Alteromonas* and *Pseudoalteromonas*, aerobic marine heterotrophic prokaryotes that are widely distributed in the marine environment, also contain colitose in their O-specific polysaccharides (*16*). These bacteria produce a wide range of biologically active compounds, such as antibiotics, antitoxins, and antitumor and antiviral agents. The emerging importance of colitose, and its potential role in inducing properties of cells, has heightened the interest in the biosynthesis of colitose.

In an effort to elucidate the biosynthesis of this unusual sugar, we have cloned and sequenced the L-colitose biosynthetic gene cluster from *Yersinia pseudotuberculosis* VI and identified five open reading frames within this cluster (*17*). The genes responsible for colitose biosynthesis in the *E. coli* O111 O-antigen have also been reported (*18*). Functional assignment of these genes based on sequence comparison with known genes in the data bank suggested a biosynthetic pathway similar to those established for other 3,6-dideoxysugars (see Scheme 1). As depicted in Scheme 2, the proposed pathway begins with the conversion of D-mannose-1-phosphate (**6**) to GDP-D-mannose (**7**) by α-D-mannose-1-phosphate guanidylyltransferase (ColE), followed by an intramolecular oxidoreduction of **7** by GDP-D-mannose 4,6-dehydratase (ColB) to afford GDP-4-keto-6-deoxy-α-D-mannose (**8**). ColD, which is a coenzyme B_6-dependent enzyme, is believed to be responsible for the C-3 deoxygenation of **8** to give **9**. The final transformation of **9** to GDP-

Scheme 2



L-colitose (**10**) must be catalyzed by ColC, whose sequence displays characteristics for a reductase.

The proposed pathway shows two apparent disparities from those established for other 3,6-dideoxysugars: the lack of an E_3 equivalent for the C-3 deoxygenation step (**8** → **9**) and the lack of a specific epimerase to invert the configuration at C-5 (**9** → **10**). In addition, while the translated *colD* gene sequence shows moderate identity with the E_1 gene (*ddhC*/*ascC*) (*19*) of the ascarylose pathway (27% identity and 42% similarity), it lacks the putative iron−sulfur binding motif found in the E_1 sequence that mediates the E_1 radical mechanism (*20*). Thus, it is expected that the reactions catalyzed by these enzymes deviate significantly from their counterparts in the formation of other 3,6-dideoxyhexoses. To verify the assigned functions of these genes, the encoded proteins, ColD and ColC, were heterologously produced and purified. A preliminary report on ColD catalysis has already been communicated.[17] Reported herein are the detailed accounts of overexpression, purification, and characterization of ColD, which catalyzes the C-3 deoxygenation, and ColC, which catalyzes the C-5 epimerization as well as C-4 keto reduction, resulting in the conversion of **9** to GDP-L-colitose (**10**) through intermediate **12d** (see Scheme 4). Our studies clearly show that both ColD and ColC are bifunctional enzymes, and their modes of action in the biosynthesis of GDP-L-colitose represent a new paradigm in 3,6-dideoxysugar biosynthesis. On the basis of these results, ColD and ColC can be referred as GDP-4-keto-6-deoxy-D-mannose-3-dehydrase and GDP-L-colitose synthase, respectively.

EXPERIMENTAL PROCEDURES

General. The strain of *Yersinia pseudotuberculosis* VIA was a generous gift from Professor Robert Brubaker of Michigan State University (East Lansing, MI). Protein concentrations were determined according to Bradford (*21*) with bovine serum albumin as the standard. The NMR spectra were acquired on a Varian Unity 400 or 500 MHz spectrometer, and chemical shifts (δ in parts per million, ppm) are given relative to those for *t*-BuOH (the chemical shifts are 1.27 and 31.2 ppm for ^{1}H NMR and ^{13}C NMR, respectively), $CFCl_3$ (external, for ^{19}F), and aqueous 85% H_3PO_4 (external, for ^{31}P), with coupling constants reported in hertz (Hz). Fast-atom bombardment (FAB) and electrospray ionization (ESI) mass spectra were recorded by the MS facility in the Department of Chemistry and Biochemistry of the University of Texas at Austin. Cloning of the colitose gene cluster was briefly described in an earlier paper (*17*), and the details will be reported elsewhere. The two plasmids described below, pNJB2 and pNJB3, contain the *colD* and *colC* gene, respectively. These plasmids were used to transform *E. coli* HMS174 and BL21 cells, respectively, for gene expression. N-terminal sequencing of purified enzymes was performed by the Core Facilities in the Institute of Molecular and Cellular Biology of the University of Texas at Austin. The oligonucleotide primers for the polymerase chain reaction (PCR) were ordered from Gibco BRL (Gaithersburg, MD). Grafit software version 3.09b (Erithacus Software Ltd., Microsoft Corp.) was used for kinetic data analysis. Bio-Gel P2 resin was purchased from Bio-Rad Laboratories (Hercules, CA). Unless otherwise specified, all chemicals were purchased from Sigma−Aldrich Co. (St. Louis, MO) and were used without further purification.

Growth of E. coli HMS174(DE3)-pNJB2 and BL21(DE3)-pNJB3 Cells. An overnight culture of *E. coli* HMS174(DE3)-pNJB2 (*17*), grown at 37 °C with shaking at 250 rpm in LB medium supplemented with 50 μg/mL kanamycin, was diluted 200-fold with 1 L of the same medium. The culture was incubated at 37 °C until the OD_{600} reached 0.2, cooled to 25 °C, and allowed to grow at this temperature until the OD_{600} reached 0.5. It was then induced with 0.3 mM isopropyl β-D-thiogalactoside (IPTG), after which the culture was incubated for an additional 16−18 h at 25 °C. The cells were harvested by centrifugation (6000*g*, 20 min) and stored at −80 °C. A similar procedure was used to prepare the *E. coli* BL21(DE3)-pNJB3 cells. After the OD_{600} reached 0.2, the incubation temperature was lowered to 30 °C, and the cultures were incubated at this temperature until the OD_{600} reading reached 0.6, at which point the cultures were induced with IPTG to a final concentration of 0.5 mM. After 20 h further incubation at 30 °C, the cells were harvested by centrifugation at 6500*g* for 15 min at 4 °C. The pellet was stored at −80 °C until used.

Enzyme Purification of ColD. The purification was carried out at 4 °C as follows. Thawed cells were resuspended in 20 mL of lysis buffer (50 mM sodium phosphate buffer containing 15% glycerol, 300 mM NaCl, and 10 mM imidazole, pH 7.4) and disrupted by sonication in five 40-s bursts with a 1 min cooling period between each burst. Cell debris was removed by centrifugation (10000*g*, 30 min). The supernatant was placed in a clean tube and 4 mL of resuspended Ni−NTA agarose resin (Qiagen), preequilibrated with the lysis buffer, was added. Subsequently, the tube was placed on a shaker and gently shaken at 4 °C for 1 h to allow the protein to bind to the resin. The suspension was loaded onto a small glass column (Bio-Rad, 1 × 25 cm) and washed with 20 mL of the lysis buffer. Next, the resin was washed with 10 mL of the wash buffer (50 mM sodium phosphate buffer containing 15% glycerol, 300 mM NaCl, and 50 mM imidazole, pH 7.4) followed by elution with 20 mL of the elution buffer (50 mM sodium phosphate buffer containing 15% glycerol, 300 mM NaCl, and 150 mM imidazole, pH 7.4). The desired fractions (4 mL each), as determined by SDS−PAGE, were pooled and dialyzed against 1 L of 50 mM sodium phosphate buffer (pH 7.4) containing 10% glycerol. After concentration on an Amicon concentrator by use of a YM 10 membrane (Amicon), the purified enzyme was stored at −80 °C. It was stable at this temperature for at least 6 months.

Enzyme Purification of ColC. Purification of ColC followed the same procedure used to purify ColD except for the buffers, which did not contain NaCl. After binding of the sample to the Ni−NTA resin, the resin was washed with 10 mL of the wash buffer (50 mM sodium phosphate buffer, pH 7.4, containing 15% glycerol and 30 mM imidazole) and the protein was eluted with 20 mL of elution buffer (50 mM sodium phosphate buffer, pH 7.4, containing 15% glycerol and 150 mM imidazole). The desired fractions were identified by SDS−PAGE, pooled, concentrated (YM 10 membrane), and desalted by dialysis against 2 L of 50 mM sodium phosphate buffer containing 15% glycerol, pH 7.4. The purified enzyme was stored at −80 °C. At this temperature, the enzyme was stable for at least 6 months.

Molecular Mass Determination. The native molecular mass of ColD and ColC proteins were estimated by gel filtration performed on a Pharmacia FPLC equipped with a Superdex 200 HR 10/30 column. The proteins were eluted with 50 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl at a flow rate of 0.7 mL/min. The system was calibrated with protein standards (Sigma) and the data were analyzed by the method of Andrews (*22*). The subunit molecular mass was estimated by SDS−PAGE as described by Laemmli (*23*).

Enzyme Assay. The activity of ColD was determined by a discontinuous HPLC assay on a C18 column (Microsorb-MV, Varian, 4.6 × 250 mm) eluted isocratically with 2% acetonitrile in 50 mM triethylammonium acetate buffer, pH 6.8. The flow rate was 1 mL/min and the elution was monitored at 254 nm. The substrate for ColD, **8**, which is the product of GDP-D-mannose 4,6-dehydratase (ColB) reaction, was generated from GDP-D-mannose (**7**) in situ by ColB. A typical assay mixture contained 1.8 mM **7**, 40 μM PLP, 2 mM L-glutamate, 1.5 μM ColB, and 0.65 μM ColD in 60 μL of 50 mM sodium phosphate buffer, pH 7.0. The solution was incubated at 37 °C for 30 min and frozen with liquid nitrogen to terminate the reaction. The frozen sample was quickly thawed by mixing it with the elution buffer. Subsequently, it was filtered through a membrane (Acrodisc13, 0.2 μm, Gelman) to remove the protein and immediately analyzed by HPLC as described above.

In a typical assay to determine ColC activity, 0.5 mM GDP-mannose (**7**), 0.42 μM ColB, 0.011 mM PLP, 0.55 mM L-glutamate, and 0.18 μM ColD in 50 mM sodium phosphate buffer, pH 7.5, were incubated at room temperature for 2 h.

To this mixture were added 1.15 μM ColC and 0.75 mM NADH. The total volume was 60 μL. The incubation was continued for 30 min and then quenched by freezing with liquid nitrogen. The analysis of the incubation products was conducted by HPLC under the same conditions described above for the ColD assay.

Preparation and Characterization of ColD Reaction Product. The ColD product was prepared on a large scale by incubating 47.5 mM GDP-D-mannose (**7**), 1.5 mM PLP, and 33.9 mM L-glutamate with 54.8 μM ColB and 9.5 μM ColD in 250 μL of 50 mM sodium phosphate buffer (pH 7.0), overnight at 37 °C. The protein was removed by a Centricon-10 microconcentrator (Amicon) and the filtrate was purified by HPLC on an Econosil C18 column (10 × 250 mm) eluted isocratically with 2% acetonitrile in 50 mM triethylammonium acetate buffer (pH 6.8). The fractions containing product **9** were pooled and desalted on an Econo-Pac 10 DG (Bio-Rad) column. Product elution was achieved with water. This desalting procedure was repeated after lyophilization. The purified **9** was stored at −80 °C. ^{1}H NMR (^2H_2O) δ 7.99 (1H, s, H-6″ base), 5.82 (1H, d, J = 5.9 Hz, H-1′ ribose), 5.39 (1H, br d, J = 7.5 Hz, H-1), 4.50 (1H, q, J = 6.6 Hz, H-5), 4.41 (1H, m), 4.24 (2H, m, including H-2), 4.10 (2H, m), 2.93 (1H, dd, J = 15.5 and 4.0 Hz, 3-H_{eq}), 2.34 (1H, dd, J = 15.5 and 3.0 Hz, 3-H_{ax}), 1.14 (3H, d, J = 6.6 Hz, 5-Me). ^{13}C NMR (^2H_2O) δ 211.2 (C-4), 159.2, 152.0, 137.9, 95.1 (d, J = 6.0 Hz, C-1), 87.1, 84.0 (d, J = 9.1 Hz), 73.8, 72.7 (C-5), 70.6, 70.1 (d, J = 10.1 Hz, C-2), 65.6 (d, J = 5.0 Hz), 42.4 (C-3), 13.8 (C-6). High-resolution negative ion FABMS calcd for $C_{16}H_{22}N_5O_{14}P_2$ $[M-H]^-$ 570.0639, found m/z 570.0636.

Preparation and Characterization of ColC Reaction Product. The substrate for the ColC reaction was prepared by a large scale ColB/ColD reaction in which 24.8 mM GDP-D-mannose (**7**), 2.1 mM PLP, 30 mM L-glutamate, 64.7 μM ColB, and 10.4 μM ColD in 725 μL of 50 mM sodium phosphate buffer (pH 7.0), were incubated at 37 °C overnight. The proteins were removed by a Centricon 10 microconcentrator. To the filtrate were added 35 mM NADH and 53.9 μM ColC to a final volume of 1 mL. The pH of the mixture was adjusted to 7.5 and the reaction was allowed to run at room temperature overnight. The protein was removed by a Centricon 10 microconcentrator (Amicon) and the filtrate purified by FPLC on a MonoQ HR (10/10) column. The product was eluted by a gradient of water as solvent A and 400 mM NH_4HCO_3 as solvent B where the gradient ran from 0% B for 4 min, 0−60% B over 20 min, 60−100% B over 5 min, followed by a 5 min wash by 100% B. The flow rate was 3 mL/min. The ColC product **10** eluted at 23.5 min. ^{1}H NMR (^2H_2O) δ 8.05 (1H, s, H-6″ base), 5.84 (1H, d, J = 6.0 Hz, H-1′ ribose), 4.83 (1H, t, J = 7.5 Hz, H-1), 4.44 (1H, t, J = 4.5 Hz), 4.26 (1H, m), 4.12 (2H, m), 3.64 (3H, m, H-2, 4, 5), 2.10 (1H, ddd, J = 14.6, 4.6, and 3.1 Hz, 3-H_{eq}), 1.61 (1H, ddd, J = 14.6, 14.6, and 3.0 Hz, 3-H_{ax}), 1.08 (3H, d, J = 6.5 Hz, 5-Me). ^{13}C NMR (^2H_2O) δ 159.1, 154.2, 137.9, 116.4, 100.6 (d, J = 6.0 Hz, C-1), 87.1, 84.0 (d, J = 9 Hz), 74.9 (C-4), 73.8, 70.7, 68.2 (C-5), 66.0 (d, J = 10 Hz, C-2), 65.6 (d, J = 5.0 Hz), 36.6 (C-3), 15.8 (C-6). High-resolution negative ion FABMS calcd for $C_{16}H_{24}N_5O_{14}P_2$ $[M-H]^-$ 572.0795, found m/z 572.0813.

Determination of Kinetic Parameters of ColD-Catalyzed Reaction. A series of samples containing the purified ColD enzyme (90 nM), GDP-4-keto-6-deoxy-D-mannose (**8**) (10, 15, 30, 45, and 60 μM), PLP (0.5 μM), and various fixed concentrations of L-glutamate (4, 8, 12, 16, 24 mM) in a total volume of 60 μL of 50 mM sodium phosphate buffer (pH 7.0) was prepared. Each sample was incubated at 37 °C and the extent of conversion was controlled to be within 30% by properly adjusting the incubation time. The reactions were terminated and analyzed by HPLC as described above, and the ratio of integration of product and substrate peak was used to determine the activity. The kinetic parameters were deduced by plotting these data on the basis of the rate equation of a ping-pong mechanism

$$v = \frac{V_{max}[A][B]}{K_{mA}[A] + K_{mB}[B] + [A][B]} \quad (1)$$

in which K_{mA} is the Michaelis−Menten constant for substrate A, K_{mB} is the Michaelis−Menten constant for substrate B, and V_{max} is the maximum rate. Initial rates were measured in triplicate with each individual measurement plotted.

Determination of Kinetic Parameters of ColC-Catalyzed Reaction. The rate of the ColC-catalyzed reaction was determined spectrophotometrically by following the consumption of NADH (ϵ_{340} = 6220 M^{-1} cm^{-1}) at 30 °C. A series of samples containing 100 μM NADH and varying amounts of GDP-4-keto-3,6-dideoxy-D-mannose (**9**) (10, 30, 40, 50, and 80 μM) in 200 μL of 50 mM sodium phosphate buffer, pH 7.5, was prepared. The reaction was initiated by the addition of ColC (1.5 μM). The labeled GDP-[3,5-$^2H_2$]-4-keto-3,6-dideoxymannose, which was prepared as described below, was used for studying the kinetic isotope effects of the epimerization step. Samples containing 75 μM stereospecifically deuterated NADH were used for studying the kinetic isotope effects of the hydride transfer step. To determine the Michaelis constant of NADH, the assay was repeated by incubating 300 μM GDP-4-keto-3,6-dideoxy-D-mannose (**9**) and varying concentrations of NADH (15, 30, 45, 75, and 150 μM) in 200 μL of 50 mM sodium phosphate buffer, pH 7.5. A similar study was also performed with NADPH as the coenzyme. The kinetic parameters were deduced by fitting the data to the Michaelis−Menten equation.

Incubation of ColD with Its Substrate in Buffer Made with ^2H_2O. A 250 μL reaction mixture containing 47.5 mM GDP-D-mannose (**7**), 1.5 mM PLP, 33.9 mM L-glutamate, 54.8 μM ColB, and 9.5 μM ColD in 50 mM sodium phosphate buffer (pD 7.4) made from >90% deuterated water (Cambridge Isotope Laboratories) was incubated overnight at 37 °C. The proteins were removed by a Centricon-10 microconcentrator (YM-10 membrane) and the filtrate was purified on an HPLC Econosil C18 column (10 × 250 mm) as described above. The pooled fractions containing product **9** were subjected to two consecutive elutions via an Econo-Pac 10 DG (Bio-Rad) column in order to desalt the fractions. Product elution was achieved with ^2H_2O. After lyophilization, the purified product, GDP-[3,5-$^2H_2$]-4-keto-3,6-dideoxy-mannose, was analyzed by ^{1}H NMR spectroscopy to determine the regio- and stereochemistry of deuterium incorporation. ^{1}H NMR (^2H_2O) δ 8.02 (1H, s, H-6″ base), 5.83 (1H, d, J = 5.9 Hz, H-1′ ribose), 5.62 (1H, d, J = 7.5 Hz, H-1 of the hydrate form), 5.38 (1H, d, J = 8.0 Hz, H-1 of the ketone form), 4.67 (1H, m, H-2′), 4.42 (1H, m, H-3′),

4.26 (2H, m, H-4′ and H-2), 4.12 (2H, m, H-5′), 2.36 (1H, d, $J = 3.0$ Hz, 3-H_{ax}), 1.12 (3H, s, 5-Me). High-resolution negative ion FABMS calcd for $C_{16}H_{19}{}^2H_2N_5O_{14}P_2$ $[M-H]^-$ 571.0685, found *m/z* 571.0678.

Incubation of ColC with Substrate in Buffer Made with ^2H_2O. The 400 μL reaction mixture contained 30.7 mM GDP-4-keto-3,6-dideoxy-D-mannose (**9**), 40.3 mM NADH, and 65.7 μM ColC in 50 mM sodium phosphate buffer (pD 7.9) made from >90% deuterated water. The reaction was incubated overnight at room temperature and stopped by removing the protein with a Centricon-10 microconcentrator. The product was purified by FPLC as described above and analyzed for deuterium incorporation by ^{1}H NMR spectroscopy. ^{1}H NMR (^2H_2O) δ 7.89 (1H, s, H-8″ base), 5.72 (1H, d, $J = 6.0$ Hz, H-1′ ribose), 4.8 (overlapped with H_2O, H-1), 4.32 (1H, t, $J = 5.2$ Hz), 4.13 (1H, m), 3.99 (2H, m), 3.54 (2H, m, H-2, 4), 1.97 (1H, ddd, $J = 14.0$, 4.0, and 3.1 Hz, 3-H_{eq}), 1.47 (1H, ddd, $J = 14.0$, 12.8, and 4.0 Hz, 3-H_{ax}), 0.95 (3H, s, 5-Me).

Preparation of [4′-^{3}H]PMP. Racemically labeled [4′-^{3}H]-PMP was prepared according to a published method with minor modifications (*24*). Briefly, the reaction mixture (pH 4∼5) containing 16.5 μmol of PMP and 5.7 μmol of PLP in 600 μL of ^3H_2O (15 mCi, Sigma) was incubated in the dark at room temperature for 4 days. The reaction mixture was then loaded onto a Dowex 1X2-100 column (acetate form, 1.5 × 15 cm, Sigma) equilibrated with 0.1 N NaOH. After the column was washed with 0.1 N NaOH and water, PMP was eluted with 0.1 N HCl. PMP eluted in this manner was sufficiently pure as assessed by ^{1}H NMR spectroscopy of a control run with ^2H_2O, making further purification unnecessary. Radioactivity was determined with a Beckman LS6500 multipurpose scintillation counter with biodegradable counting scintillant (Amersham). The specific radioactivity of tritiated PMP was 1.3 μCi/μmol.

Preparation of (4′S)- and (4′R)-[4′-^{3}H]PMP. The *pro-R* labeled [4′-^{3}H]PMP was prepared by incubating the racemic [4′-^{3}H]PMP with apo-aspartate aminotransferase (apo-AspAT), while the *pro-S* labeled [4′-^{3}H]PMP was made by incubating the racemic [4′-^{3}H]PMP with apo-branched chain aminotransferase (apo-BCAT). Preparation of the apo forms of AspAT and BCAT from the corresponding holoenzymes followed literature protocols (*24*). Briefly, AspAT (glutamic−oxalacetic transaminase type II-A from porcine heart, 6 mg) or BCAT (from *E. coli*, 10 mg) was incubated with 30 mM L-cysteinesulfinic acid in 500 μL of 100 mM Tris-HCl buffer (pH 8.5) at 30 °C for 90 min. The reaction mixture was dialyzed against 0.5 M sodium phosphate buffer (pH 5.0 for AspAT and pH 6.5 for BCAT) for 24 h, with three changes of the buffer. This was followed by dialysis against 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 8.0) for 24 h with three buffer changes. The loss of PLP from each enzyme was confirmed by UV spectral analysis. These apoenzymes were then used for the conversion of the racemic [4′-^{3}H]PMP to (4′*S*)- and (4′*R*)-[4′-^{3}H]PMP. The reaction mixture contained 10.5 μmol of [4′-^{3}H]PMP and 18 nmol of apo-AspAT or 21 nmol of apo-BCAT in 500 μL of 50 mM Tris-HCl buffer (pH 8.0), along with 1 μmol of unlabeled PMP as a carrier. The reaction was incubated at 30 °C for 15 h, quenched by the addition of 15 μL of 12 N HCl, incubated at 30 °C for another 3 h, and then incubated at 75 °C for 15 min. After removal of the protein precipitates by a microconcentrator with a YM-30 membrane, the filtrate was dried in a rotary evaporator under reduced pressure and the residue was resuspended in H_2O (1 mL). The resulting (4′*R*)-[4′-^{3}H]PMP (0.37 μCi/μmol) and (4′*S*)-[4′-^{3}H]PMP (0.32 μCi/μmol) were stored at −20 °C.

Stereochemistry of ColD-Catalyzed C-4′ Deprotonation of the PMP−Substrate Complex. In this study, 40 μL of the substrate solution, which contained GDP-D-mannose (**7**) (0.44 mg, 0.72 μmol) and ColB (6.5 nmol) in 400 μL of 50 mM sodium phosphate buffer (pH 7.0), was added to an assay solution containing the stereospecifically labeled [4′-^{3}H]PMP coenzyme (3 μmol) and ColD (40 μg) in the same sodium phosphate buffer, resulting in a total volume of 200 μL. The reaction was run at 37 °C for 3 h, followed by the addition of activated charcoal (10% solution, 200 μL) to bind the cofactor. The resulting solution was mixed vigorously on a vortex mixer for 1 min and centrifuged for 10 min at 11000*g* to precipitate the charcoal. The supernatant (150 μL) was removed and analyzed by scintillation counting. A control reaction was carried out in parallel without substrate.

Preparation of Stereospecifically Deuterated NADH. (4*R*)-[4-^{2}H]NADH was prepared with NAD^+ (185 μmol), [$^2H_6$]-ethanol (19.2 μmol), and yeast alcohol dehydrogenase (2.5 mg, Sigma) in 5 mL of 100 mM ammonium bicarbonate buffer made from >90% deuterated water, pD 8.5, at 37 °C by a literature procedure (*25*, *26*). After removal of the protein by a Centricon-10 microconcentrator, the labeled coenzyme was purified by FPLC equipped with a MonoQ (10/10) column. A linear gradient of 0−50% B over 20 min, followed by 50−100% B over 4 min, and ending with 100% B for 8 min was used to elute the (4*R*)-[4-^{2}H]NADH, where solvent A was water and solvent B was 0.5 M NH_4HCO_3 buffer. The flow rate was 2 mL/min, and NADH was eluted at 19 min. The (4*S*)-[4-^{2}H]NADH sample was prepared with 150 μmol of NAD^+, 300 μmol of dithiothreitol (DTT), 5 μmol of lipoamide, and 10 units of lipoamide dehydrogenase (Sigma) in 25 mL of 50 mM ammonium bicarbonate buffer made from >90% deuterated water, pD 8.5 (*27*). Small aliquots (50 μL) of NaO^2H were added to the incubation at room temperature to keep the pD between 8 and 9, while production of [4-^{2}H]NADH was monitored at 340 nm. The protein was removed by use of a Centricon-10 microconcentrator and the filtrate was purified on a DEAE-Sepharose column (4 × 18 cm). The labeled coenzyme was eluted with a 0−0.2 M ammonium bicarbonate gradient. Fractions (6 mL) were collected, and the desired fractions were combined and lyophilized to give a pale yellow solid. NMR analysis of both (4*R*) and (4*S*) products validated the chirality and confirmed the deuterium incorporation.

Determination of Stereospecificity of Hydride Transfer from NADH in the ColC-Catalyzed Reaction. The 475 μL incubation mixture contained 6.2 mM stereospecifically labeled NADH, 4.13 mM sugar substrate **9**, and 0.4 mM ColC in 50 mM sodium phosphate buffer, pH 7.5. After overnight incubation at room temperature, the enzyme was removed by a Centricon-10 microconcentrator, and the resulting sugar product was purified by FPLC as described above for the purification of GDP-L-colitose (**10**). The desired fractions were combined, lyophilized, redissolved in ^2H_2O, and analyzed by ^{1}H NMR spectroscopy.

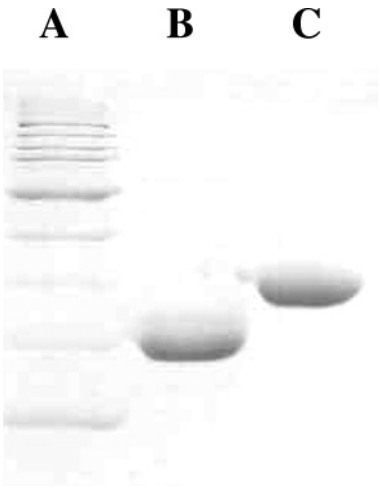


FIGURE 1: SDS−PAGE gel of ColC and ColD after Ni−NTA purification. Lane A, molecular weight markers: triosephosphate isomerase (lower darker band, 27 kDa), thioredoxin reductase (34.6 kDa), MBP2 (42.7 kDa), glutamic dehydrogenase (55.6 kDa), and bovine serum albumin (higher darker band, 66 kDa). Lane B, ColC; lane C, ColD.

Incubation of ColC with Substrate in the Absence of NADH. After the ^{1}H NMR spectrum of a reaction mixture containing 14.6 mM GDP-4-keto-3,6-dideoxy-D-mannose (**9**) in 600 μL of 50 mM sodium phosphate buffer made from 10% deuterated water (pH 7.5) was recorded, 71 μM ColC was added to the reaction mixture. The progress of the reaction was monitored at 10-min intervals over 3 h by ^{1}H NMR spectroscopy.

Incubation of ColC with Substrate and NAD$^+$. A ^{1}H NMR spectrum of a reaction mixture containing 14.6 mM GDP-4-keto-3,6-dideoxy-D-mannose (**9**) and 14.6 mM NAD$^+$ in 600 μL of 50 mM sodium phosphate buffer made from 10% deuterated water (pH 7.5) was recorded. Subsequently, 71 μM ColC was added to the NMR tube to initiate the reaction. The progress of the reaction was monitored by ^{1}H NMR spectroscopy at 10-min intervals over 3 h.

Incubation of ColC with Substrate and NAD$^+$ in Buffer Made with ^2H_2O. A 400 μL reaction mixture containing 13.3 mM GDP-4-keto-3,6-dideoxy-D-mannose (**9**), 20 mM NAD$^+$, and 284 μM ColC in 50 mM sodium phosphate buffer made from >90% deuterated water (pD 7.9) was prepared. The reaction was incubated overnight at room temperature and the protein was removed by use of a Centricon-10 microconcentrator. The filtrate was purified by FPLC under the same conditions described for the purification of GDP-L-colitose (**10**). The desired fractions were combined, lyophilized, and analyzed for deuterium incorporation in the product by ^{1}H NMR spectroscopy.

RESULTS

Enzyme Purification and Characterization. The N-terminal His_6-tagged ColC and ColD enzymes were purified to near homogeneity on a Ni−NTA column as illustrated in Figure 1. The yield of ColC was about 8 mg/L of culture, and that of ColD was typically 9 mg/L homogeneous protein. The identity of each purified protein was confirmed by N-terminal sequence analysis in which the first 10 amino acid residues matched those of the corresponding translated gene sequence. A subunit molecular mass of 36 kDa for ColC and 46 kDa for ColD, as estimated by SDS−PAGE, correlates well to the predicted value of 35 234 Da and 46 127 Da calculated from the translated *colC* and *colD* sequence plus the His-tag. The molecular masses, estimated by gel filtration, suggest that both ColC and ColD exist as homodimers in the native state. No absorbance band was apparent above 300 nm for either ColC or ColD.

Calatytic Properties of ColD. The translated *colD* gene sequence shows moderate sequence identity with that of the E_1 gene (*ddhC*/*ascC*) (*19*) of the ascarylose pathway (see Scheme 1), suggesting that ColD might also utilize PMP as a coenzyme for its catalysis. Experimental confirmation was obtained from the observation that turnover occurred when ColD was incubated with substrate (**8**) in the presence of excess PMP but not with PLP. However, the rate of product formation increased by more than 40-fold when L-glutamate was included in the PLP incubation mixture. Since α-ketoglutarate was found as a byproduct in the reaction mixture, it became clear that ColD can act as a transaminase, recognizing both PMP and PLP. Further experimentation revealed that L-glutamate is a better amino donor than either L-glutamine (~50% activity) or L-alanine (<5% activity), while D-glutamate and L-aspartate are inactive as cosubstrates. On the basis of these findings, the first step of ColD catalysis is likely the conversion of PLP to PMP via an aminotransferase reaction.

Isolation and Characterization of the ColD Reaction Product. The ability of ColD to function as the C-3 dehydrase was established by an HPLC-based assay in which GDP-D-mannose (**7**), PLP, and L-glutamate were incubated with appropriate amounts of ColB and ColD at 37 °C for 30 min. In addition to the unreacted **7** and GDP-4-keto-6-deoxy-D-mannose (**8**), the product of ColB reaction, a new compound with a retention time of 10.5 min, was found in the reaction mixture. The retention times for **7** and **8** in the HPLC chromatogram are 6.6 and 8.9 min, respectively. A large-scale incubation reaction allowed the isolation and identification of this compound as GDP-4-keto-3,6-dideoxy-D-mannose (**9**). The coupling patterns as well as the chemical shifts of the 3-H_{eq} and 3-H_{ax} signals, each appearing as a doublet of doublets at δ 2.93 and 2.34, respectively, are characteristic of the assigned structure **9**. This structural assignment is further supported by the high-resolution MS data.

Isolation and Characterization of the ColC Reaction Product. The ability of ColC to function as the GDP-L-colitose synthase was verified by the same HPLC-based assay described above, in which **9**, generated in situ, was reacted with ColC in the presence of NADH. A new peak with a retention time of 10 min was observed under the HPLC conditions described above. This peak partially overlapped with that of the ColD product (**9**) and was absent when ColC was omitted from the incubation mixture. As expected, the 3-H_{eq} and 3-H_{ax} signals of the isolated product appear as a doublet of doublets of doublets at δ 2.10 and 1.61, respectively, revealing the presence of a new hydrogen at the adjacent C-4. This product was identified as an L-pyranose because NOESY analysis showed proximity relationships between H-3$_{eq}$ and H-2, between H-5 and H-1, and among H-6, H-4, and H-3$_{ax}$. These results clearly indicate that ColC is a bifunctional enzyme, catalyzing C-5 epimer-

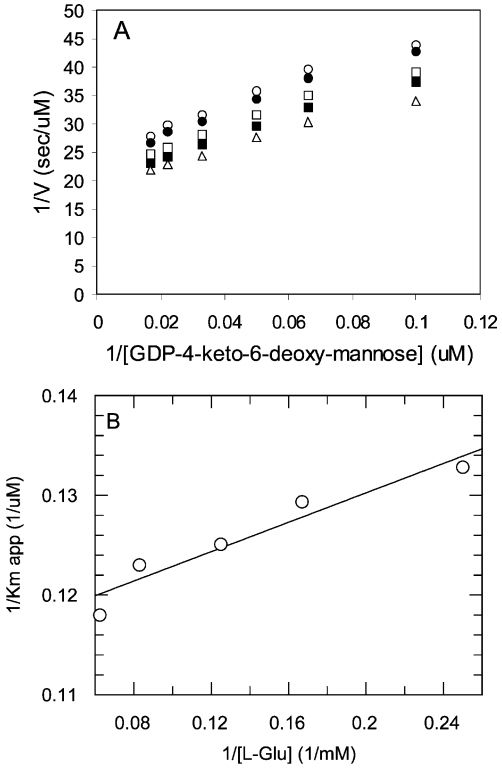


FIGURE 2: Determination of steady-state kinetic parameters for the reaction catalyzed by ColD. Rates of individual reactions were determined by incubating varying amounts of GDP-4-keto-6-deoxy-D-mannose (**8**) (10−60 μM) with 90 nM ColD and various fixed concentrations of L-glutamate (4 mM, ○; 8 mM, ●; 12 mM, □; 16 mM, ■; 24 mM, △) as described under Experimental Procedures. (A) Double-reciprocal plot of the initial rates against the sugar substrate (**8**) concentrations. (B) Replot of the intercepts on the *y*-axis in panel A ($1/K_{m(8),app}$) against the reciprocal concentrations of L-glutamate.

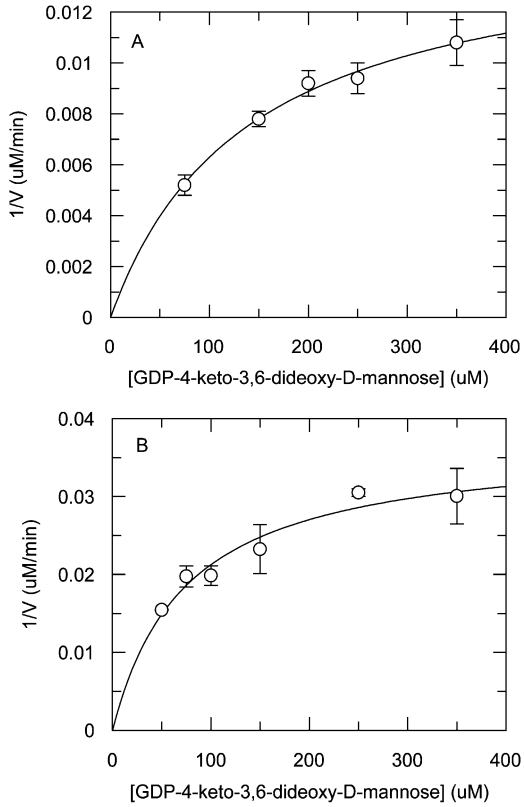


FIGURE 3: Determination of steady-state kinetic parameters for the reaction catalyzed by ColC. Rates of individual reactions were determined by incubating varying amounts of GDP-4-keto-3,6-dideoxy-D-mannose (**9**) (10−80 μM) with 1.5 μM ColC and a fixed concentration of (A) NADH (100 μM) or (B) NADPH (100 μM) as described under Experimental Procedures. The initial rates were plotted against the sugar substrate (**9**) concentrations to obtain the data shown above.

ization as well as C-4 reduction of the ColD product (**9**; see Scheme 2).

Kinetic Properties of ColD and ColC. The kinetic parameters for the ColD and ColC reactions were determined under steady-state conditions. At low substrate concentrations, reciprocal plots of the ColD reaction rates versus the concentration of **8** at various but fixed levels of L-glutamate yielded a set of parallel lines (Figure 2A). The intercepts along the *x*-axis of which are reciprocals of the apparent maximum rates, $1/V_{app}$, and the intercepts on the *y*-axis are negative reciprocals of the apparent Michaelis constants $1/K_{m(8)app}$. Replots of these intercepts against reciprocal concentrations of L-glutamate were also linear. The plot of $1/K_{m(8)app}$ versus 1/[L-Glu] yielded $K_{m(8)}$ (Figure 2B), and that of $1/V_{app}$ against 1/[L-Glu] gave V and $K_{m(L-Glu)}$ (not shown). The analyses gave a k_{cat} of 0.6 s^{-1} for the overall reaction, a K_m of 8.5 μM for GDP-4-keto-6-deoxy-D-mannose (**8**), and a K_m of 1.4 mM for L-glutamate.

The kinetic parameters for the ColC reaction were determined spectrophotometrically by following the consumption of NADH at 340 nm ($\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) and fitting the data to the Michaelis−Menten equation (Figure 3). The K_m for **9** was determined to be 139 ± 18 μM with a V_{max} of 0.015 ± 0.008 μM min^{-1} in the presence of NADH (Figure 3A). The corresponding parameters determined when the assay was carried out in the presence of NADPH are K_m for **9** = 75 ± 15 μM and V_{max} = 0.037 ± 0.003 μM min^{-1} (Figure 3B).

Stereochemistry of ColD-Catalyzed Deprotonation of the PMP−Substrate Complex. According to the proposed mechanism for ColD, loss of the OH group at C-3 of the substrate is initiated by deprotonation at C-4′ of the cofactor−substrate complex (**11a**; see Scheme 3). To test whether this deprotonation is stereospecific as it is for other coenzyme B_6-dependent enzymes, (4′*R*)-[4′-^{3}H]PMP and (4′*S*)-[4′-^{3}H]-PMP were individually incubated with ColD and the substrate (**8**) generated in situ from GDP-D-mannose (**7**) by ColB. Since the C-4′ hydrogen of the enzyme-bound PMP may be labile, an otherwise identical control reaction was carried out in the absence of substrate. As shown in Table 1, PMP alone and the PMP−substrate complex are quite stable under the assay conditions. However, incubation of (4′*R*)-[4′-^{3}H]-PMP with ColD released 10 times more radioactivity than did the incubation of (4′*S*)-[4′-^{3}H]PMP with ColD [Table 1, (445−168)/(114−93) ∼ 13]. Hence, the deprotonation is clearly a highly selective process that preferentially removes the *pro-R* hydrogen from C-4′ of PMP.

Stereospecificity of C-3 Hydrogen Incorporation in the ColD Reaction. In the ColD-catalyzed deoxygenation, the hydroxyl group at C-3 of **8** is replaced by a solvent hydrogen to generate the product. To assess whether the deoxygenation reaction is entirely enzyme-catalyzed, the stereospecificity of hydrogen incorporation at C-3 was determined by carrying out the ColD reaction in ^2H_2O. NMR analysis of the ColD product (**9**) isolated from this experiment showed a dramatic decrease in the intensity of the signal at δ 2.93 (3-H_{eq})

Scheme 3

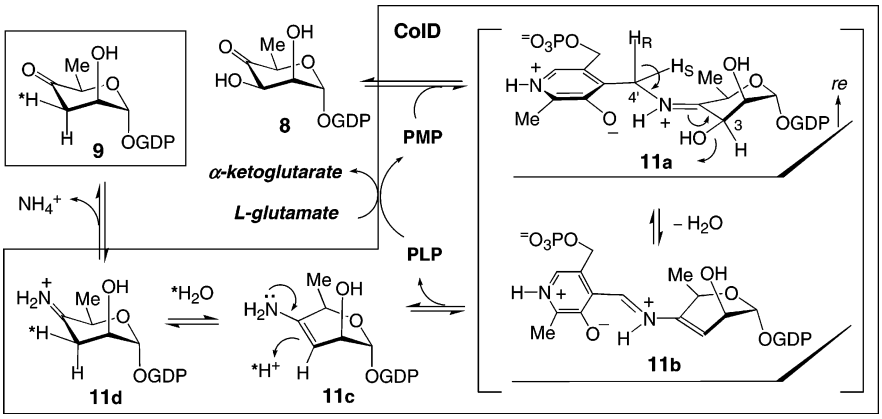
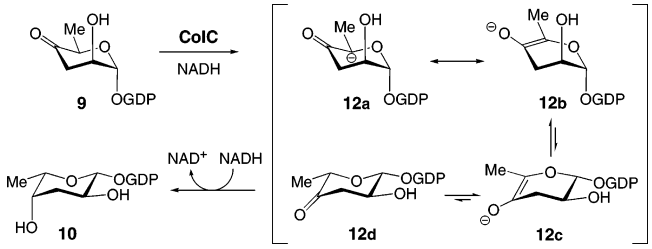


Table 1: Stereospecificity of ColD-Catalyzed Deprotonation of the C-4′ Proton of the PMP−Substrate Complex (**11a**)

incubation[a]	tritium released (dpm)	
	(4′*R*)-[4′-^{3}H]PMP	(4′*S*)-[4′-^{3}H]PMP
PMP	120 ± 6	95 ± 14
PMP + sugar substrate (**8**)	127 ± 9	94 ± 2
PMP + ColD	168 ± 7	93 ± 2
PMP + sugar substrate (**8**) + ColD	445 ± 20	114 ± 7

[a] The (4′*R*)- and (4′*S*)-[4′-^{3}H]PMP used in each experiment have comparable amounts of radioactivity (2240 dpm).

Scheme 4



coupled with a change in the splitting pattern of the signal at δ 2.34 (3-H_{ax}). These data reflect the incorporation of a deuterium at the equatorial position at C-3 of **9**. Since the solvent hydrogen incorporation at C-3 is stereospecific, this process must be enzyme-mediated, and the overall displacement proceeds by a retention mechanism.

Solvent Hydrogen Exchange in the ColC-Catalyzed C-5 Epimerization Step. As indicated above, the product of the ColC reaction is an L-pyranose. Thus, inversion of configuration at C-5 must be part of the ColC-catalyzed reaction, converting its substrate **9**, which is a D-sugar, to GDP-L-colitose (**10**), which is an L-sugar. This ColC-catalyzed epimerization is likely a result of deprotonation of the proton at C-5 of **9** followed by reprotonation from the opposite side to afford the L-isomer of **9** (**12d** in Scheme 4). In this mechanism, a solvent hydrogen is expected to be incorporated at C-5 after epimerization. To verify this proposal, the ColC reaction was conducted in ^2H_2O. ^{1}H NMR analysis of the colitose product isolated from this incubation indeed revealed the incorporation of a deuterium at the C-5 position. This observation is consistent with the proposed deprotonation/reprotonation mechanism. Interestingly, no reduction in the intensity of the signal corresponding to the proton at C-3 of the colitose product is discernible, despite the fact that this proton, which is adjacent to the 4-keto group (in **9**), can potentially be exchanged with solvent hydrogen. Apparently, the initial hydrogen activation for epimerization is regiospecifically controlled by the chiral environment of the active site.

Stereospecificity of Hydride Transfer from NADH in the ColC Reaction. The stereospecificity of nicotinamide oxidation is a highly conserved characteristic of a given class of oxidoreductases and has been correlated with the orientation of binding of the pyridine nucleotide to the enzyme. To establish the stereospecificity of the ColC-catalyzed NADH oxidation, stereospecifically labeled NADH samples were incubated with the enzyme, and the colitose product (**10**) was purified by FPLC as described. On the basis of ^{1}H NMR analysis, it was found that incubation with (4*S*)-[4-^{2}H]NADH led to deuterium incorporation at C-4 of **10**, because the peak integral at δ 3.64 (4-H) decreases from three to two hydrogens (4-H overlapped with 2- and 5-H) and the C-3 proton signals at δ 2.10 and 1.61 split into a doublet of doublets. In contrast, colitose isolated from the incubation with (4*R*)-[4-^{2}H]NADH showed no change in the integration and splitting patterns of its NMR signals. Thus, the stereochemical preference of ColC with regard to the transfer of one of the diastereotopic methylene hydrogens at C-4 of the dihydronicotinamide ring is established to be *pro-S* stereospecific.

Kinetic Isotope Effects with Stereospecifically Labeled NADH in the ColC Reaction. To validate the *pro-S* stereospecificity of NADH oxidation and to determine whether the hydride transfer from NADH is the rate-limiting step in the ColC reaction, the kinetic isotope effect (KIE) on k_{cat} was determined by use of stereospecifically labeled NADH. When (4*S*)-[4-^{2}H]NADH was used in the reaction mixture, a KIE of 1.4 ± 0.5 was measured, while no isotope effect (1.0 ± 0.2) was detected with (4*R*)-[4-^{2}H]NADH. The kinetic isotope effect observed with the 4*S*- but not the 4*R*-labeled NADH is consistent with the *pro-S* stereospecificity of hydride transfer from NADH. However, such a small isotope effect suggests that the hydride transfer step is not a significant rate-limiting step in the ColC reaction.

Incubation of ColC with Substrate in the Absence of NADH. In an attempt to separate the C-5 epimerization from the C-4 reduction step in the ColC reaction, a set of ColC incubations was carried out in which NADH was omitted, thereby preventing the reduction of the putative 4-keto-L-sugar intermediate **12d**, the nascent product of the epimerization reaction. Since epimerization is expected to be a

reversible process, an equilibrium of the D- and L-sugar epimers with distinct NMR spectral features should be established during the course of the ColC reaction. The doublet at δ 1.08 assigned to the methyl group at C-5 of **9** was chosen as the reference signal for monitoring the progress of the reaction. The reaction was followed by ^{1}H NMR spectroscopy at 10-min intervals for 3 h. No reduction of the reference signal and no new set of doublets for the methyl group at C-5 of the L-epimer (**12d**) were observed. Hence, this experiment failed to provide insight into the epimerization process.

Incubation of ColC with NAD$^+$ in Buffer Made with ^2H_2O. Since binding the nicotinamide coenzyme to the active site may be a prerequisite for the ColC reaction, a second NMR experiment was attempted under the same conditions as described above, except for the inclusion of an excess of NAD$^+$. Unfortunately, no spectral changes were observed during the 3-h incubation period. This result may be explained by an equilibrium greatly favoring the formation of **9** over that of the L-epimer (**12d**). Thus, an overnight reaction was carried out in which ColC was incubated with **9** and NAD$^+$ in buffer made from ^2H_2O. If the epimerization proceeds in favor of the reverse direction (**12d** → **9**), a deuterium label is predicted to be incorporated into C-5 of the recovered substrate due to the reversal of the deprotonation/reprotonation step. While no new product was found in the incubation mixture, NMR analysis of the recovered substrate (**9**) revealed the incorporation of deuterium at the C-5 position. Control reactions without enzyme or NAD$^+$ showed no deuterium exchange. These results are consistent with a scenario in which deprotonation and inversion of configuration at C-5 occurs, but the accumulation of the epimerized product (**12d**) is prevented by an unfavorable equilibrium. It is the subsequent reduction by NADH that provides the driving force to push the equilibrium to completion (**12d** → **10**).

Kinetic Isotope Effects for 3,5-Dideuterated Substrate in the ColC Reaction. To determine whether deprotonation of 5-H of **9**, which triggers C-5 epimerization, constitutes a rate-determining step in the ColC reaction, the kinetic isotope effect on k_{cat} with GDP-[3,5-$^2H_2$]-4-keto-3,6-dideoxy-D-mannose as substrate was determined. This doubly labeled compound was isolated from an incubation mixture containing GDP-D-mannose (**7**), ColB, ColD, and all the necessary components in deuterated buffer. The fact that a KIE of 2.6 ± 0.4 was measured clearly indicated that the C−H bond cleavage at C-5 is at least partially rate-limiting in the ColC reaction.

DISCUSSION

Deoxygenation reactions in biological systems are pivotal transformations and encompass a diverse range of mechanisms. A novel deoxygenation reaction has been found in the biosynthesis of CDP-L-ascarylose (**5**) and related 3,6-dideoxyhexoses (*5*, *9*, *10*, *12*). This C-3 deoxygenation step is catalyzed by a pair of enzymes, E_1 and E_3, and the reaction is initiated by the E_1-catalyzed dehydration to release the 3-OH group followed by an E_3-mediated stepwise $1e^-/1e^-$ reduction (Scheme 1). The lack of an E_3 equivalent in the colitose pathway indicates that the mechanism of C-3 deoxygenation in colitose biosynthesis must be significantly different from the E_1/E_3 paradigm established for the formation of other 3,6-dideoxyhexoses. Since the translated *colD* gene shows moderate sequence identity with that of the E_1 gene of the ascarylose pathway and shares a unique feature with E_1 containing a histidine at position 192 (like H220 in E_1) instead of a highly conserved lysine found in other B_6-dependent enzymes (*20*), its encoded protein ColD may act as an E_1 equivalent in the colitose pathway (*17*, *18*). However, unlike E_1, ColD lacks the iron−sulfur binding motif, implicating a unique function.

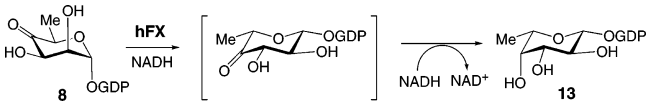
Indeed, our experiments show that ColD is solely responsible for C-3 deoxygenation as opposed to the strategy found in the biosynthesis of other 3,6-dideoxyhexoses, which require a separate dehydrase (E_1) and reductase (E_3). As depicted in Scheme 3, the first half of the ColD reaction is a transamination step in which PLP is converted to PMP in the presence of L-glutamate. The PMP coenzyme then forms a Schiff base with the sugar substrate (**8**) in the second half reaction, and the resulting adduct (**11a**) undergoes a 1,4-dehydration to eliminate the 3-OH group. Hydrolysis of the nascent $\Delta^{3,4}$-aminomannoseen intermediate (**11b**) leads to an enamine sugar intermediate **11c**, which after tautomerization and hydrolysis to release ammonia yields GDP-4-keto-3,6-dideoxy-D-mannose (**9**) as the product. The stereospecific incorporation of a deuterium at 3-H_{eq} of **9** when the reaction is conducted in deuterated buffer indicates that tautomerization of **11c** is an enzyme-mediated event.

The second half of the ColD reaction is mechanistically reminiscent of the PLP-dependent serine/threonine dehydratases (*28*). In the case of serine/threonine dehydratases, the nitrogen atom in the nascent enamine product after dehydration is derived from the amino group of the substrate. In **11c**, the nitrogen atom in the nascent enamine is derived from PMP coenzyme. Hence, ColD catalysis is not a simple dehydration reaction because a transamination step is required to reload the amino group on the coenzyme after each catalytic cycle. Nevertheless, the ColD reaction resembles enzymes in the transaminase family because the Michaelis constant for the keto acid substrate is lower than that of the donor amino acid (*29*). A similar trend has also been reported for the transaminase from *E. coli* strain B, which catalyzes the conversion of TDP-4-keto-6-deoxy-D-glucose (*30*) to the corresponding 4-amino-4,6-dideoxy sugar (*31*).

The *pro-R* stereospecificity exhibited by ColD in the deprotonation from C-4′ of the PMP−substrate Schiff base (**11a**) is another unusual property rarely found among coenzyme B_6-dependent enzymes. For most enzymes in this family, including E_1, this process is *pro-S* specific (*32*, *33*). Only a few exceptions to this highly conserved stereochemical preference are known (*34*). These include branched-chain L-amino acid aminotransferase of *E. coli* (*35*) and the D-amino acid aminotransferase of *Bacillus* sp. YM-1 (*36*). These two enzymes exhibit significant sequence identity with each other but none with the translated sequence of *colD*.

The fact that the C-4′ deprotonation step is *pro-R* stereospecific suggests that ColD has the *re* face of the complex **11b** (at C-4′) accessible to active-site catalytic groups. The retention of configuration at C-3 deduced from the solvent incorporation study shows that the departure of the hydroxyl group and the addition of a solvent hydrogen at C-3 occur suprafacially. In fact, the bond cleavage and formation at C-3 and C-4′ are expected to occur on the same (*re*) face of

Scheme 5



the Schiff base complex, since such a single reactive face has been established as a general trait for coenzyme B_6-dependent reactions (*37*, *38*). As a result, the dehydration catalyzed by ColD is likely a *syn* elimination. Overall, the utilization of PMP in a dehydration reaction is unique, but the combined deoxygenation−transamination activity truly places ColD in a class by itself. The chemistry elucidated by this study represents a new mechanistic strategy used by nature to perform sugar deoxygenation.

The final stage of GDP-L-colitose formation is catalyzed by ColC, which functions as a C-5 epimerase and also a C-4 reductase. So far only one other example of such a transformation has been biochemically characterized in the biosynthesis of unusual sugars. This occurs in the biosynthesis of GDP-L-fucose (**13**) in *E. coli*, where the conversion of GDP-4-keto-6-deoxy-D-mannose (**8**) to GDP-L-fucose (**13**), involving inversion at C-3/C-5 followed by reduction at C-4, is catalyzed by a single enzyme, hFX (GDP-L-fucose synthase, Scheme 5) (*39*). While ColC and hFX share good sequence identity (38% identity and 57% similarity) and play similar roles in their respective pathways, their reactions are distinct by substrate specificity, a 3,6-dideoxyhexose for ColC versus a 6-deoxyhexose for hFX. However, binding of the nicotinamide cofactor is a prerequisite for epimerization in both cases.

It is worth mentioning that ColC is a dimeric protein with a small subunit size of 308 amino acids. Analysis of its sequence revealed the presence of the conserved ^{132}**Y**XXX^{136}**K** motif and a NAD(P) binding fold, ^{7}**G**XX**G**XX^{13}**G**, at its N-terminus. All of these features are characteristic for short-chain alcohol dehydrogenases (ADHs) (*40*, *41*). The stereochemical preference for the transfer of the *pro-S* hydrogen of NADH during catalysis further corroborates ColC as a member of the short-chain ADH family. Similar characteristics have also been noted for TDP-L-rhamnose synthetase (*42*), CDP-D-paratose synthase (*43*), and UDP-D-galactose epimerase (*44*), which all involve the reduction of a 4-keto group of a hexose. Thus, the available information suggests that all 4-ketosugar reductases may be related to the short-chain ADH family.

ColC is a rare bifunctional enzyme, illustrating nature's diversity in making unusual sugars. A possible mechanism for ColC catalysis can be summarized as shown in Scheme 4. After substrate binding, deprotonation at C-5 yields **12a** (in resonance with **12b**). Subsequent inversion of configuration followed by reprotonation at C-5 give the epimerized product **12d**. An equilibrium favoring **9** over **12d** is implicated by the fact that no epimerization product is accumulated prior to reduction, after deprotonation at C-5 has occurred. The kinetic isotope effect of 2.6 ± 0.4, displayed for reaction with [3,5-$^2H_2$]-**9** as the substrate, indicates that deprotonation at C-5 is a rate-determining step in the ColC reaction. The final stage of ColC catalysis involves the transfer of the *pro-S* hydrogen of NADH to reduce the 4-keto group of **12d** to give GDP-L-colitose (**10**). The *pro-S* specificity of the hydride transfer step reflects the fact that substrate is bound to the *si* face of the nicotinamide ring in the active site of ColC. Since the kinetic isotope effect of this step is relatively small (KIE = 1.4 ± 0.5), this hydride transfer is not a significant rate-limiting step. However, such a reduction is essential to drive the equilibrium to completion.

In summary, nature has devised many elegant methods to construct deoxyhexoses (*45*). The transformations catalyzed by ColD and ColC represent a new paradigm of deoxygenation where the C−O bond to be ruptured is at the α-position to a keto group. This mechanistic variation is a testimony to the evolutionary diversity of biological C−O bond cleavage events.

ACKNOWLEDGMENT

We thank Professor Robert Brubaker of Michigan State University for providing the strain of *Yersinia pseudotuberculosis* VIA and Dr. Yung-nan Liu for her technical help in this research.

REFERENCES

1. Allen, H. J., and Kisailus, E. C. *Glycoconjugates: Composition, Structure, and Function*, Marcel Dekker, New York, 1992.
2. Weymouth-Wilson, A. C. (1997) The role of carbohydrates in biologically active natural products, *Nat. Prod. Rep. 14*, 99−110.
3. Hanessian, S. (1996) Deoxy sugars, *Adv. Carbohydr. Chem. Biochem. 21*, 143−207.
4. Williams, N. R., and Wander, J. D. (1980) Deoxy and branched-chain sugars, in *The Carbohydrates: Chemistry and Biochemistry* (Pigman, W., and Horton, D., Eds.) Vol. 1B, pp 761−798, Academic Press, New York.
5. Johnson, D. A., and Liu, H.-w. (1999) Deoxysugars: occurrence, genetics, and mechanism of biosynthesis, in *Comprehensive Chemistry of Natural Product Chemistry* (Barton, D., Nakanishi, K., and Meth-Cohn, O. Eds.) pp 311−365, Pergamon, New York.
6. Lüderitz, O., Staub, A. M., and Westphal, O. (1996) Immunochemistry of O and R antigens of *Salmonella* and related Enterobacteriaceae, *Bacteriol. Rev. 30*, 192−248.
7. Bishop, C. T., and Jennings, H. J. (1982) Immunology of polysaccharides, in *The Polysaccharides* (Aspinall, G. O., Ed.) Vol. 1, pp 291−330, Academic Press, Orlando, FL.
8. Raetz, C. R. H. (1990) Biochemistry of endotoxins, *Annu. Rev. Biochem. 59*, 129−170.
9. Liu, H.-w., and Thorson, J. S. (1994) Pathways and mechanisms in the biogenesis of novel deoxy sugars by bacteria, *Annu. Rev. Microbiol. 48*, 223−256.
10. He, X., Agnihotri, G., and Liu, H.-w. (2000) Novel enzymatic mechanisms in carbohydrate metabolism, *Chem. Rev. 100*, 4615−4661.
11. Heath, E. C., and Elbein, A. D. (1962) The enzymatic synthesis of guanosine diphosphate colitose by a mutant strain of *Escherichia coli*, *Proc. Natl. Acad. Sci. U.S.A. 48*, 1209−1216.
12. Hallis, T. M., and Liu, H.-w. (1999) Learning nature's strategies for making deoxy sugars: pathways, mechanisms and combinatorial applications, *Acc. Chem. Res. 32*, 579−588.
13. Kenne, L., Lindberg, B., Soderholm, E., Bundle, D. R., and Griffith, W. (1983) Structural studies of the *O*-antigens from *Salmonella greenside* and *Salmonella adelaide*, *Carbohydr. Res. 111*, 289−296.
14. Hisatsune, K., Kondo, S., Isshiki, Y., Iguchi, T., Kawamata, Y., and Shimada, T. (1993) *O*-Antigenic lipopolysaccharide of *Vibrio cholerae* O139 Bengal, a new epidemic strain for recent cholera in the Indian subcontinent, *Biochem. Biophys. Res. Commun. 196* (3), 1309−1315.
15. Oscarson, S., Tedebark, U., and Turek, D. (1997) Synthesis of colitose-containing oligosaccharide structures found in polysaccharides from *Vibrio cholerae* O139 synonym bengal using thioglycoside donors, *Carbohydr. Res. 299*, 159−164.
16. Muldoon, J., Perepelov, A. V., Shashkov, A. S., Gorshkova, R. P., Nazarenko, E. L., Zobkov, V. A., Ivanova, E. P., Knirel, Y. A., and Savage, E. L. (2001) Structure of a colitose-containing *O*-specific polysaccharide of the marine bacterium *Pseudomonas tetradontis* IAM 14160, *Carbohydr. Res. 222*, 41−46.

17. Beyer, N., Alam, J., Hallis, T. M., Guo, Z., and Liu, H.-w. (2003) The biosynthesis of GDP-L-colitose: C-3 deoxygentation is catalyzed by a unique coenzyme B_6-dependent enzyme, *J. Am. Chem. Soc. 125*, 5584−5585.
18. Bastin, D. A., and Reeves, P. R. (1995) Sequence and analysis of the O antigen gene (*rfb*) cluster of *Escherichia coli* 0111, *Gene 164*, 17−23.
19. Thorson, J. S., Lo, S. F., Ploux, O., He, X., and Liu, H.-w. (1994) Studies of the biosynthesis of 3,6-dideoxyhexoses: molecular cloning and characterization of the *asc* (ascarylose) region from *Yersinia pseudotuberculosis* serogroup VA, *J. Bacteriol. 176*, 5483−5493.
20. Agnihotri, G., Liu, Y. N., Paschal, B., and Liu, H.-w. (2004) Identification of an unusual [2Fe-2S]-binding motif in CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase: implication on C-3 deoxygenation in the biosynthesis of 3,6-dideoxyhexoses, *Biochemistry 43*, 14265−14274.
21. Bradford, M. N. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding, *Anal. Biochem. 72*, 248−254.
22. Andrews, P. (1964) Estimation of the molecular weights of proteins by Sephadex gel-filtration, *Biochem. J. 91*, 222−233.
23. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
24. Matsuhashi, M., and Strominger, J. L. (1966) Thymidine diphosphate 4-acetamido-4,6-dideoxyhexoses. III. purification and properties of thymidine diphosphate 4-keto-6-deoxy-D-glucose transaminase from *Escherichia coli* strain B, *J. Biol. Chem. 241*, 4738−4744.
25. Oppenheimer, N. J., Arnold, L. J., and Kaplan, N. O (1971) A structure of pyridine nucleotides in solution, *Proc. Natl. Acad. Sci. U.S.A. 68*, 3200−3205.
26. Gassner, G., Wang, L., Batie, C., and Ballou, D. P. (1994) Reaction of phthalate dioxygenase reductase with NADH and NAD: kinetic and spectral characterization of intermediates, *Biochemistry 33*, 12184−12193.
27. Arnold, L. J., and You, K. (1978) The hydride transfer stereospecificity of nicotinamide adenine dinucleotide linked enzymes: a proton magnetic resonance technique, *Methods Enzymol. 54*, 223−232.
28. Miles, E. W. (1986) Pyridoxal phosphate enzymes catalyzing β-elimination and β-replacement reactions, in *Vitamin B_6 Pyridoxal Phosphate, Chemical, Biochemical, and Medical Aspects* (Dolphin, D., Poulson, R., and Avramovic, O., Eds.) Part B, pp 253−310, Wiley-Interscience: New York.
29. Salerno, C., Giartosio, A., and Fasella, P. (1986) Transaminases, in *Vitamin B_6 Pyridoxal Phosphate, Chemical, Biochemical, and Medical Aspects* (Dolphin, D., Poulson, R., and Avramovic, O., Eds.) Part B, pp 118−167, Wiley-Interscience: New York.
30. Okazaki, R., Okazaki, T., Strominger, J. L., and Michelson, A. M. (1962) Thymidine diphosphate 4-keto-6-deoxy-D-glucose, an intermediate in thymidine diphosphate L-rhamnose synthesis in *Escherichia coli* strains, *J. Biol. Chem. 237* (10), 3014−3026.
31. Matsuhashi, M., and Strominger, J. L. (1966) Thymidine diphosphate 4-acetamido-4,6-dideoxyhexoses. III. purification and properties of thymidine diphosphate 4-keto-6-deoxy-D-glucose transaminase from *Escherichia coli* strain B, *J. Biol. Chem. 241*, 20, 4738−4744.
32. Vederas, J. C., and Floss, H. G. (1980) Stereochemistry of pyridoxal phosphate catalyzed enzyme reactions, *Acc. Chem. Res. 13*, 455−463.
33. Rètey, J., and Robinson, J. A. (1982) Pyridoxal phosphate-dependent enzymic reactions, in *Stereospecificity in Organic Chemistry and Enzymology*, Chapt. 7, pp 151−184, Verlag Chemie: Weinheim, Germany.
34. Yoshimura, T., Nishimura, K., Ito, J., Esaki, N., Kagamiyama, H., Manning, J. M., and Soda, K. (1993) Unique stereospecificity of D-amino acid aminotransferase and branched-chain L-amino acid aminotransferase for C-4′ hydrogen transfer of the coenzyme, *J. Am. Chem. Soc. 115*, 3897−3900.
35. Kuramitsu, S., Ogawa, T., Ogawa, H., and Kagamiyama, H. (1985) Branched-chain amino acid aminotransferase of *Escherichia coli*: nucleotide sequence of the *ilvE* gene and the deduced amino acid sequence, *J. Biochem. (Tokyo) 97*, 993−999.
36. Tanizawa, K., Masu, Y., Asano, S., Tanaka, H., and Soda, K. (1989) Thermostable D-amino acid aminotransferase from a thermophilic *Bacillus* species. Purification, characterization, and active site sequence determination, *J. Biol. Chem. 264*, 2445−2449.
37. Dunathan, H. C. (1971) Stereochemical aspects of pyridoxal phosphate catalysis, *Adv. Enzymol. Relat. Areas Mol. Biol. 35*, 79−134.
38. Palcic, M. M., and Floss, H. G. (1986) Conformation and stereochemistry of free and bound pyridoxal phosphate and its derivatives, in *Vitamin B_6 Pyridoxal Phosphate, Chemical, Biochemical, and Medical Aspects* (Dolphin, D., Poulson, R., and Avramovic, O., Eds.) Part A, pp 25−68, Wiley-Interscience, New York.
39. Chang, S., Duerr, B., and Serif, G. (1988) An epimerase-reductase in L-fucose synthesis, *J. Biol. Chem. 263*, 1963−1967.
40. Jörnvall, H., Persson, B., and Jeffery, J. (1987) Characteristics of alcohol/polyol dehydrogenases. The zinc-containing long-chain alcohol dehydrogenases, *Eur. J. Biochem. 167*, 195−201.
41. Reid, M. F., and Fewson, C. A. (1994) Molecular characterization of microbial alcohol dehydrogenases, *Crit. Rev. Microbiol. 20*, 13−56.
42. Leriche, C., He, X., Chang, C.-W. T., and Liu, H.-w. (2003) Reversal of the apparent regiospecificity of NAD(P)H-dependent hydride transfer: the properties of the difluoromethylene group, a carbonyl mimic, *J. Am. Chem. Soc. 125*, 6348−6349.
43. Hallis, T. M., Lei, Y., Que, N. L. S., and Liu, H.-w. (1998) Mechanistic studies of the biosynthesis of paratose: purification and characterization of CDP-paratose synthase, *Biochemistry 37*, 4935−4945.
44. Thoden, J. B., Frey, P. A., and Holden, H. M. (1996) Molecular structure of the NADH/UDP-glucose abortive complex of UDP-galactose 4-epimerase from *Escherichia coli*: implications for the catalytic mechanism, *Biochemistry 35*, 5137−5144.
45. He, X., and Liu, H.-w. (2002) Mechanisms of enzymatic C−O bond cleavages in deoxyhexose biosynthesis, *Curr. Opin. Chem. Biol. 6*, 590−597.

BI0483763