A Retro-Evolution Study of CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase (E_1) from *Yersinia pseudotuberculosis*: Implications for C-3 Deoxygenation in the Biosynthesis of 3,6-Dideoxyhexoses†

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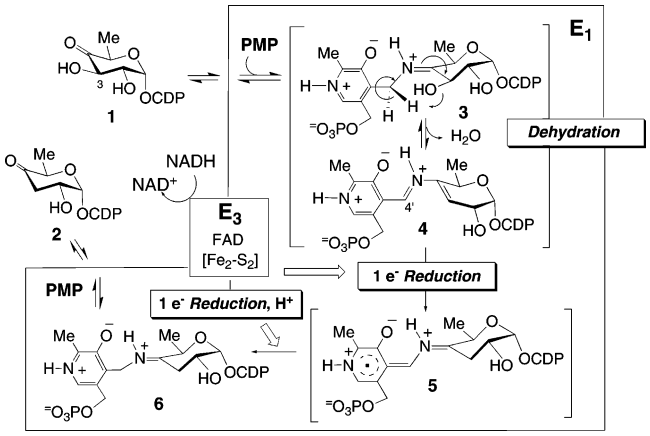
ABSTRACT: CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1), which catalyzes C-3 deoxygenation of CDP-4-keto-6-deoxyglucose in the biosynthesis of 3,6-dideoxyhexoses, shares a modest sequence identity with other B_6-dependent enzymes, albeit with two important distinctions. It is a rare example of a B_6-dependent enzyme that harbors a [2Fe-2S] cluster, and a highly conserved lysine that serves as an anchor for PLP in most B_6-dependent enzymes is replaced by histidine at position 220 in E_1. Since alteration of His220 to a lysine residue may produce a putative progenitor of E_1, the H220K mutant was constructed and tested for the ability to process the predicted substrate, CDP-4-amino-4,6-dideoxyglucose, using PLP as the coenzyme. Our data showed that H220K-E_1 has no dehydrase activity, but can act as a PLP-dependent transaminase. However, the reaction is not catalytic since PLP cannot be regenerated during turnover. Reported herein are the results of this investigation and the implications for the role of His220 in the catalytic mechanism of E_1.

The C-3 deoxygenation step in the biosynthesis of 3,6-dideoxyhexoses, in which CDP-4-keto-6-deoxy-D-glucose (**1**) is converted to CDP-4-keto-3,6-dideoxy-D-glucose (**2**), is catalyzed by CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase (E_1) and a reductase (E_3) (*1*−*4*).[1] E_1 is a dimeric protein containing one pyridoxamine 5′-phosphate (PMP) and a [2Fe-2S] cluster per subunit (*5*−*7*). E_3 belongs to the flavodoxin-$NADP^+$ reductase family and contains FAD and a plant-type ferredoxin [2Fe-2S] center in the active site (*8*, *9*). The reaction catalyzed by E_1 and E_3 is initiated by the formation of a Schiff base between PMP and the 4-keto group of **1** (Scheme 1) (*5*, *6*). Subsequent abstraction of the *pro-S* 4′-H of the external Schiff base (**3**) results in the loss of the 3-OH group and leads to the conjugated intermediate (**4**) (*1*−*7*). A sequential two-electron reduction relayed from NADH via E_3-bound FAD and the [2Fe-2S] centers of E_1 and E_3 drives the reaction to completion and regenerates the PMP coenzyme (*10*, *11*). A radical intermediate represented by **5** has been established by EPR analysis during turnover (*12*, *13*). The participation of PMP in deoxygenation is unusual, as is the direct involvement of PMP in the stabilization of an unpaired electron spin in an electron-transfer reduction (*1*−*4*).

Scheme 1: Mechanism of C-3 Deoxygenation Catalyzed by E_1 and E_3 in the Biosynthesis of 3,6-Dideoxyhexoses



Sequence alignment (*14*) showed that E_1 belongs to a group of structurally well characterized PLP-dependent enzymes with a topology typical for the aspartate aminotransferase family (also known as fold-type I or α-family) (*15*, *16*), which includes 3-amino-5-hydroxybenzoic acid

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[1] Abbreviations: CDP, cytidine 5′-diphosphate; CMP, cytidine 5′-monophosphate; DEAE, diethylaminoethyl; E1, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase; E3, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase; Eod, CDP-D-glucose 4,6-dehydratase; DIPAD, diisopropyl azodicarboxylate; DTT, dithiothreitol; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; LB, Luria−Bertani; NAD^+, β-nicotinamide adenine dinucleotide; NADH, β-nicotinamide adenine dinucleotide, reduced form; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; ORF, open reading frames; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; SDS, sodium dodecyl sulfate.

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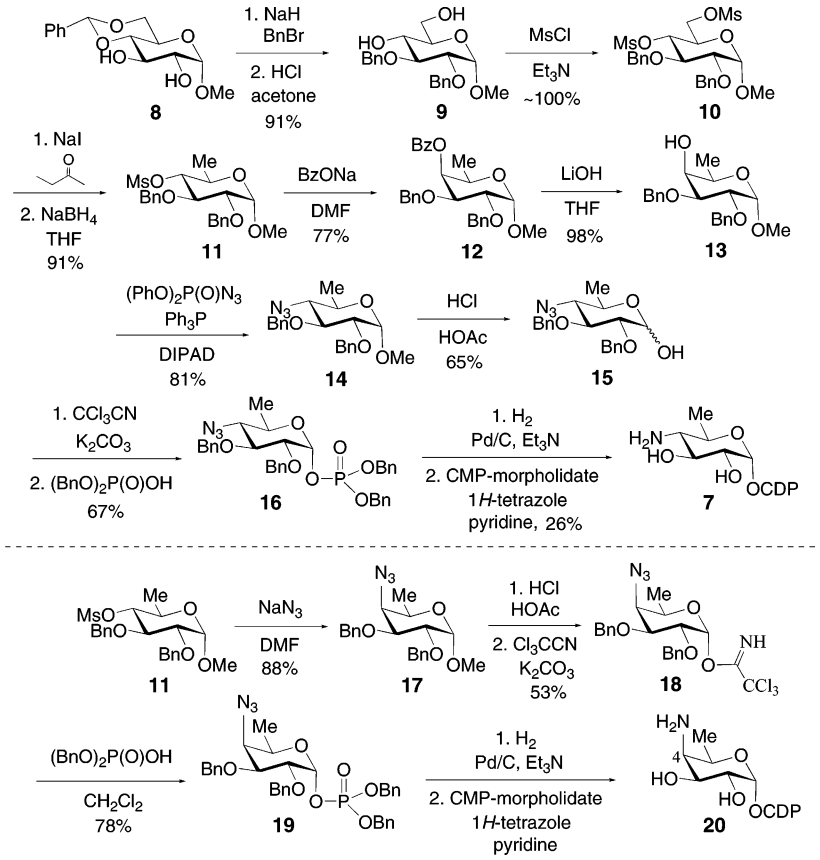
Scheme 2: Synthesis of CDP-4-amino-6-deoxy-α-D-glucose (**7**) and Its 4-Epimer (**20**)



synthase (*17*), arnb aminotransferase (*18*), and *Thermus thermophilus* aspartate aminotransferase (*19*). There are, however, two important distinctions. First, the highly conserved Schiff-base forming lysine that serves as an anchor for pyridoxal 5′-phosphate (PLP) in other B_6-dependent enzymes is absent in E_1 (*14*, *20*, *21*). Instead, a histidine residue is present in its place at position 220, which has been proposed to be the active site base responsible for the 4′-H abstraction based on early site-directed mutagenesis studies (*14*). Second, E_1 contains an unusual [2Fe-2S] binding motif (*20*, *21*). The replacement of the active site lysine by a histidine residue along with the presence of an iron−sulfur cluster in E_1 reflects a distinct evolutionary path for E_1, and distinguishes it from other B_6-dependent enzymes. It should be noted that, apart from E_1, GABA-aminotransferase is another B_6-dependent enzyme containing a [2Fe-2S] cluster (*22*). However, the catalytic role of the Fe−S cluster in GABA-aminotransferase remains elusive. E_1 is thus the only example of a PMP-dependent enzyme that contains a mechanistically defined and relevant [2Fe-2S] cluster. Since mutations leading to these two unique sites in E_1 may have arisen from independent evolutionary events, a "retro-evolution" in which His220 is changed to a lysine residue may produce a putative progenitor of E_1 which binds PLP, holds a [2Fe-2S] center, and possesses a distinct catalytic activity. A study of the catalytic ability of this "halfway" mutant may shed light on the evolutionary path of E_1, especially on how it becomes a unique PMP-dependent dehydrase. Accordingly, the H220K mutant was constructed and tested for the ability to process the predicted substrate, CDP-4-amino-4,6-dideoxyglucose (**7**, Scheme 2). Reported herein are the results of this investigation and the implications for the role of His220 in the catalytic function and mechanism of E_1.

EXPERIMENTAL PROCEDURES

General. The general methods and protocols for recombinant DNA manipulations were as described by Sambrook et al. (*23*). DNA sequencing was performed by the Core Facilities in the Institute of Molecular and Cellular Biology at the University of Texas at Austin. Protein concentrations were determined according to Bradford (*24*) using bovine serum albumin as the standard. The NMR spectra were acquired on a Varian Unity 300 or 500 MHz spectrometer, and chemical shifts (δ in ppm) are given relative to those for the corresponding solvent peak with coupling constants reported in hertz (Hz). Mass spectra were recorded by the MS facility at the Department of Chemistry and Biochemistry of the University of Texas at Austin. Flash chromatographic separations were performed on silica gel 60 (230−400 mesh). Analytical thin layer chromatography was carried out on Merck silica gel 60 G-254 plates, and the spots were visualized either under UV light or by heating plates previously stained with solutions of vanillin/ethanol/H_2SO_4 (1:98:1) or phosphomolybdic acid (7% in EtOH).

Materials. The construction of the plasmid containing the E_1 gene (pJT18) in pUC119 has been reported elsewhere (*25*). Enzyme E_3 used in the assay was purified from the *Escherichia coli* JM105/pOPI cultures based on a procedure published earlier (*12*, *26*). Purification and reconstitution of E_1 and E_3 were carried out under semianaerobic conditions as previously described (*12*, *14*). The formate dehydrogenase used in the NADH regeneration system (*27*) is from *Candida*

boidinii, ordered from Sigma. All culture media were products of Difco (Detroit, MI), and the Bradford reagent for protein quantitation was purchased from BioRad (Hercules, CA). All electrophoresis materials were purchased from Gibco BRL or Bio-Rad (Hercules, CA). DEAE Sepharose fast flow resin and MonoQ HR 10/10 FPLC columns were acquired from Amersham Pharmacia Biotech (Piscataway, NJ). All chemicals were analytical grade or the highest quality commercially available. The oligonucleotide primers for the polymerase chain reaction (PCR) were custom-prepared by IDT DNA Technologies (Coralville, IA). All enzymes used for DNA manipulations were obtained from Invitrogen (Carlsband, CA).

Construction of H220K Mutant. The H220K mutant was prepared using the Sculptor in vitro mutagenesis kit. The primers used to introduce the point mutations, where the sequence in bold denotes the codon change for the mutation, were 5′-GCTTCTATCCCGCT**AAG**CATATCACCATGGGTG-3′ (forward) and 5′-CACCCATGGTGATATG**CTT**AGCGG-GATAGAAGC-3′ (reverse). The sequence of the mutated gene was determined to ensure that only the desired base change was present. The resulting mutant gene was excised from the pUC119 vector and cloned into the *Eco*RI and the *Bam*HI site of the expression vector pTrc99A. This plasmid, designated as pHC8, was used to transform *E. coli* HB101 for overexpression of the mutant protein.

Purification of H220K-E_1 Mutant Protein. An overnight culture of the H220K mutant strain (*E. coli* HB101/pHC8) was used to inoculate (1:200 dilution) 6 L of Luria−Bertani (LB) broth containing ampicillin (0.1 mg/mL) and $(NH_4)_2$-$Fe(SO_4)_2 \cdot 6H_2O$ (40 mg/L). The cells were grown until OD_{600} reached 0.3−0.6, and the cells were cooled on ice before induction with isopropyl β-D-thiogalactoside (IPTG, 0.3 mM final). After induction, the cells were grown at 18 °C for 16−18 h. The cells were harvested by centrifugation at 5000*g* for 10 min to collect wet cells. The cells were resuspended in 200 mL of buffer A (20 mM Tris·HCl, pH 7.5, degassed) and sonicated for 1 min (repeated five times) on ice. The cell debris was removed by centrifugation at 27000*g* for 20 min, and the supernatant (110 mL) was collected. To the supernatant was added $(NH_4)_2SO_4$ to 80% saturation (under N_2), and the mixture was stirred at 4 °C for 1 h. The resulting solution was centrifuged (12000*g* for 15 min). The pellet was collected, resuspended in a minimal amount of buffer A, and dialyzed for 3 h (4 °C, 1 L, 4 changes of buffer). The resulting liquid (100 mL) was loaded onto a DEAE-Sepharose column (2.5 × 34 cm) equilibrated with buffer A. The proteins were eluted first with 100 mL of buffer A, then a linear gradient of 250 mL of buffer A and 250 mL of buffer A containing 0.4 M NaCl. Fractions of 3 mL were collected. The fractions containing E_1 mutant protein were identified by their brown color (fractions 140−157). These fractions were combined, concentrated to 10 mL, and desalted using an ultrafiltration cell (YM10 membrane, Amicon). The concentrated proteins were further purified by FPLC, equipped with a MonoQ 10/10 column, using buffer A and 0.5 M NaCl in buffer A (buffer B) at a rate of 3 mL/min to elute the proteins (at $t = 0$, 0% B; 4 min, 50% B; 20 min, 95% B; 21 min, 100% B; 25 min, 100% B; 26 min, 0% B; and 32 min, 0% B). The desired protein was eluted between 10 and 12 min. The combined H220K-E_1 fractions were desalted and concentrated to 8 mL, aliquoted in 1 mL portions, and stored at −80 °C for future use. Protein concentration was 10 mg/mL for a yield of 80 mg from 6 L of culture.

Enzyme Characterization and Activity Assay. The E_1 substrate, CDP-4-keto-6-deoxy-D-glcuose (**1**), was enzymatically prepared from CDP-glucose according to a previously reported procedure (*28*). The activity of E_1 and E_1 mutants was determined by a published continuous spectrophotometric assay that monitors the consumption of NADH in the presence of E_1 (or its mutants), E_3, and the E_1 substrate (**1**) (*9*, *14*). The typical assay mixture consisted of 25 μM PMP, 200 μM NADH, 100 μM **1**, and 1.2 μM E_3, in 800 μL of 50 mM potassium phosphate buffer (pH 7.5). The reaction was initiated by the addition of E_1 (0.3 μM), and the E_1 activity was determined by measuring the rate of reduction of the absorbance at 340 nm ($\epsilon = 6220\ M^{-1}\ cm^{-1}$) within the initial 1 min. The amount of iron associated with wild type E_1 and the E_1 mutants was quantitated using the method of Fish (*29*). The stoichiometry of bound PMP per E_1 subunit was determined by a fluorimetric measurement of the quantity of released PMP from a denatured enzyme sample of known concentration (*14*). The amount of PLP bound to H220K-E_1 was determined by denaturing the enzyme (10 mM dithiothreitol (DTT), 3 M urea, and 10 mM hydroxylamine phosphate, 4 °C, overnight) and quantitatively measuring the released PLP at 388 nm ($\epsilon = 6600\ M^{-1}\ cm^{-1}$) under alkaline conditions (0.1 N NaOH) (*30*, *31*).

Reconstitution of Iron−Sulfur Center. The reconstitution of the iron−sulfur center of E_1 and H220K-E_1 was carried out according to a published procedure (*13*). Briefly, the protein sample was denatured in the presence of DTT by the addition of urea to a final concentration of 3 M. This was followed by the addition of 6-fold excess Fe^{2+} in the form of ferrous ammonium sulfate and S^{2-} in the form of Na_2S. The resulting mixture was incubated at room temperature for 2 h, and the reconstituted enzyme was purified through a DEAE-Sepharose column (1 × 10 cm) that had been pre-equilibrated with 25 mM Tris·HCl buffer, pH 7.5. Elution was conducted using a linear gradient of 0.16−0.36 mM NaCl in the same buffer.

Reconstitution of H220K-E_1 with PLP. The as-isolated H220K-E_1 was incubated with excess PLP (6-fold) in 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM DTT at 4 °C for 16 h. The reconstituted enzyme was purified through a BioRad G-10 column. This step could also be combined with the iron−sulfur reconstitution step as described above for a fully reconstituted enzyme.

*Synthesis of CDP-4-amino-4,6-dideoxy-D-glucose (**7**) and CDP-4-amino-4,6-dideoxy-D-galactose (**20**).* The preparation of **7** was based on a similar sequence of reactions reported earlier (*32*) with modifications. All manipulations were conducted under a dry argon atmosphere. All solvents were dried before use. The drying agent used in workup was anhydrous sodium sulfate unless specified otherwise.

*Methyl 2,3-Di-O-benzyl-α-D-glucopyranoside (**9**).* To a slurry of 60% NaH (3.8 g, 95 mmol) in DMF (100 mL) cooled to 0 °C was added methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**8**, 10 g, 35.4 mmol). The reaction mixture was stirred for 10 min, and benzyl bromide (10.7 mL, 89.5 mmol) was slowly added followed by tetrabutylammonium iodide (2.88 g, 7.8 mmol). The reaction mixture was stirred at room temperature for 2 h, and the reaction was

quenched by addition of 10% HCl (2 mL). Most of the solvent was evaporated under reduced pressure, and the residue was diluted with water. The mixture was extracted with ether, and the combined extracts were dried and concentrated to give a yellow oil. The crude oil was dissolved in a solution of acetone (90 mL), water (20 mL), and concentrated HCl (4 mL). The reaction mixture was refluxed for 4 h, cooled to room temperature, and neutralized with solid $NaHCO_3$. The reaction mixture was filtered, the acetone solvent evaporated, the residue diluted with water, and the mixture extracted with ethyl acetate. The combined extracts were washed with water and brine, dried, and concentrated. Purification by flash column chromatography (2:3 to 1:1 EtOAc−hexanes) yielded 12.1 g (91.3%) of **9** as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41−7.30 (m, 10 H), 5.05 (d, 1H, J = 11.7 Hz), 4.79 (d, 1H, J = 12.3 Hz), 4.73 (d, 1H, J = 11.7 Hz), 4.68 (d, 1H, J = 12.3 Hz), 4.62 (d, 1H, 3.3 Hz), 3.84−3.78 (m, 2H), 3.67−3.61 (m, 2H), 3.56−3.49 (m, 2H), 3.40 (s, 3H), 2.40 (brs, 1H), 2.01 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.6, 137.9, 128.6, 128.5, 128.1, 128.0, 127.9, 98.1, 81.3, 79.8, 75.4, 73.1, 70.6, 70.4, 62.4, 55.2; CI-HRMS calcd for $C_{21}H_{27}O_6$ $[M + H]^+$ 375.1808, found 375.1807.

*Methyl 2,3-Di-O-benzyl-4,6-di-O-methylsulfonyl-α-D-glucopyranoside (**10**).* To a solution of **9** (10.5 g, 28.0 mmol) in methylene chloride (150 mL) cooled to 0 °C was added triethylamine (16.0 mL, 115 mmol), followed by methylsulfonyl chloride (8.7 mL, 112 mmol). The reaction mixture was stirred overnight as it warmed to room temperature. Water was added to quench the reaction, and the mixture was extracted with ethyl acetate. The combined organic extracts were washed with water and brine, dried, and concentrated. Purification by flash column chromatography (1:3 EtOAc in hexanes) yielded 14.8 g (quantitative) of **10** as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.31 (m, 10H), 5.06 (d, 1H, J = 11.1 Hz), 4.73 (d, 1H, J = 12.0 Hz), 4.66 (d, 1H, J = 11.1 Hz), 4.61 (d, 1H, 3.3 Hz), 4.60 (d, 1H, J = 12.0 Hz), 4.50−4.43 (m, 2H), 4.33(dd, 1H, J = 4.8, 10.8 Hz), 4.04−3.95 (m, 2H), 3.58 (dd, 1H, J = 3.3, 9.6 Hz), 3.38 (s, 3H), 3.01 (s, 3H), 2.79 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ137.7, 137.3, 128.6, 128.5, 128.2, 128.1, 127.9, 127.6, 97.6, 79.9, 78.3, 77.1, 75.5, 73.4, 67.5, 67.1, 55.8, 38.5, 37.4; CI-HRMS calcd for $C_{23}H_{29}O_{10}S_2$ $[M + H]^+$ 529.1202, found 529.1202.

*Methyl 2,3-Di-O-benzyl-6-deoxy-4-O-methylsulfonyl-α-D-glucopyranoside (**11**).* To a solution of compound **10** (5.6 g, 10.6 mmol) in 2-butanone (150 mL) was added NaI (15.8 g, 105 mmol) in one portion. The reaction was refluxed under nitrogen atmosphere for 8 h. The solvent was removed under reduced pressure, and the residue was dissolved in 100 mL each of water and ethyl acetate. The organic layer was separated, and the aqueous phase was extracted with more ethyl acetate. The combined organic extracts were washed with saturated Na_2SO_3 solution and brine, dried, and concentrated. The 6-iodo product was crystallized from a solution of EtOAc/hexanes. The 6-iodo compound was dissolved in THF (100 mL). To this solution was added $NaBH_4$ (1.60 g, 42.2 mmol) followed by methanol (2 mL). The reaction was refluxed under nitrogen for 22 h till the substrate was completely consumed. The mixture was cooled to room temperature and was quenched by careful addition of water. The mixture was extracted with ethyl acetate, and the combined extracts were washed with water and brine, dried with $MgSO_4$, and concentrated. Purification by flash column chromatography (1:3 EtOAc in hexanes) yielded 4.19 g (91%) of **11** as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32−7.26 (m, 10H), 5.06 (d, 1H, J = 11.4 Hz), 4.71 (d, 1H, J = 12.3 Hz), 4.64 (d, 1H, J = 12.3 Hz), 4.60 (d, 1H, J = 11.4 Hz), 4.52 (d, 1H, J = 3.3 Hz), 4.17 (t, 1H, J = 9.6 Hz), 3.94 (t, 1H, J = 9.6 Hz), 3.82 (qd, 1H, J = 6.3, 9.6 Hz), 3.55 (dd, 1H, J = 3.3, 9.6 Hz), 3.36 (s, 3H), 2.77 (s, 3H), 1.29 (d, 3H, 6.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.9, 137.5, 128.6, 128.5, 128.2, 127.8, 127.6, 97.4, 83.2, 80.5, 78.5, 75.4, 73.3, 65.3, 55.4, 38.6, 17.5; CI-HRMS calcd for $C_{22}H_{27}O_7S$ $[M + H]^+$ 435.1478, found 435.1477.

*Methyl 4-O-Benzoyl-2,3-di-O-benzyl-6-deoxy-α-D-galactopyranoside (**12**).* A mixture of **11** (2.11 g, 4.86 mmol), sodium benzoate (2.09 g, 14.5 mmol), and 15-crown-5 (50 μL) in DMF (100 mL) was refluxed under argon for 20 h. The reaction mixture was cooled to room temperature, and the reaction was quenched by the addition of water. Most of the DMF was removed under reduced pressure, and the residue was dissolved in water and extracted with ethyl acetate. The combined organic extracts were washed with water and brine, dried, and concentrated. Purification by flash column chromatography (1:3 EtOAc in hexanes) yielded 1.72 g (77%) of **12** as a clear oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.10−8.06 (m, 2H), 7.60−7.55 (m, 1H), 7.48−7.43 (m, 2H), 7.38−7.24 (m, 10H), 5.64 (dd, 1H, J = 0.9, 3.3 Hz), 4.85 (t, 2H), 4.75 (d, 1H, J = 3.3 Hz), 4.72 (d, 1H, J = 12.3 Hz), 4.64 (d, 1H, J = 11.4 Hz), 4.11 (m, 2H), 3.95 (dd, 1H, J = 3.3, 9.9 Hz), 3.43 (s, 3H), 1.20 (d, 3H, J = 6.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.1, 138.3, 138.1, 132.9, 129.9, 129.8,128.3, 128.2, 128.1, 127.7, 127.6, 127.3, 99.0, 76.2, 74.7, 73.5, 71.7, 71.4, 64.6, 55.3, 16.1; CI-HRMS calcd for $C_{28}H_{29}O_6$ $[M + H]^+$ 461.1964, found 461.1963.

*Methyl 2,3-Di-O-benzyl-6-deoxy-α-D-galactopyranoside (**13**).* To a solution of **12** (1.60 g, 3.48 mmol) in THF (10 mL), MeOH (2 mL), and water (2 mL) was added LiOH (1.0 g, 23.8 mmol) in one portion. The reaction mixture was stirred at 50 °C for 5 h and then cooled to room temperature. The reaction was quenched by the addition of saturated NH_4-Cl (5 mL), and the mixture was extracted with ethyl acetate. The combined organic extracts were washed with brine, dried, and concentrated. Purification by flash column chromatography (1:3 to 1:2 EtOAc in hexanes) yielded 1.21 g (98%) of **13** as white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36−7.25 (m, 10H), 4.79 (d, 2H, J = 11.4 Hz), 4.69 (d, 1H, J = 11.4 Hz), 4.65 (d, 1H, J = 11.7 Hz), 4.61 (d, 1H, J = 3.3 Hz), 3.89−3.77 (m, 4H), 3.35 (s, 3H), 2.43 (br s, 1H), 1.25 (d, 3H, J = 6.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.4, 138.2, 128.5, 128.4, 128.0, 127.9, 127.8, 127.8, 98.5, 77.9, 75.5, 73.4, 72.8, 70.3, 65.0, 55.3, 16.1; CI-HRMS calcd for $C_{21}H_{25}O_5$ $[M + H]^+$ 357.1702, found 357.1706.

*Methyl 4-Azido-2,3-di-O-benzyl-4,6-dideoxy-α-D-glucopyranoside (**14**).* To a solution of compound **13** (2.70 g, 7.50 mmol) and triphenylphosphine (2.37 g, 9.0 mmol) in dry THF (10 mL) cooled to 0 °C was added diisopropyl azodicarboxylate (DIPAD, 1.84 mL, 9.30 mmol). The mixture was stirred at 0 °C for 5 min, and then diphenylphosphoryl azide (2.44 mL, 11.3 mmol) was added (*33*). After stirring at room temperature overnight, the solvent was

removed and the residue was purified by flash column chromatography (5% EtOAc in hexanes) to give **14** in 96% yield (2.77 g): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40−7.26 (m, 10H), 4.94 (d, 1H, J = 10.5 Hz), 4.80 (d, 1H, J = 10.5 Hz), 4.76 (d, 1H, J = 11.7 Hz), 4.63 (d, 1H, J = 11.7 Hz), 4.51 (d, 1H, J = 3.6 Hz), 3.82 (t, 1H, J = 9.3 Hz), 3.56−3.49 (m, 2H), 3.34 (s, 3H), 3.06 (dd, 1H, J = 9.3, 9.9 Hz), 1.25 (d, 3H, J = 6.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.0, 137.9, 128.5, 128.4, 128.3, 128.1, 128.0, 127.8, 98.0, 79.9, 79.8, 75.7, 73.2, 68.0, 65.8, 55.3, 18.4; CI HRMS calcd for $C_{21}H_{24}N_3O_4$ $[M - H]^+$ 382.1767, found 382.1762.

*4-Azido-2,3-di-O-benzyl-4,6-dideoxy-D-glucose (**15**).* A solution of compound **14** (3.10 g, 8.09 mmol) in acetic acid (40 mL) and 2 N HCl (1 mL) was heated to 100 °C for 2 h. The mixture was cooled to room temperature and then extracted with ethyl acetate (100 mL). The combined organic portions were washed with saturated $NaHCO_3$ and brine, dried, and concentrated. Purification by flash column chromatography (1:2 EtOAc in hexanes) afforded 1.68 g (56% yield) of **15** as an off-white solid containing a mixture of α and β anomers. About 0.42 g of unreacted reactant **14** was also recovered (overall yield 65%, based on recovered material): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39−7.27 (m, 10H), 5.11 (t, 0.6H, J = 3.0 Hz), 4.95−4.64 (m, 4H), 4.62 (dd, 0.4H, J = 5.4, 7.5 Hz), 3.84−3.77 (m, 1.2H), 3.55−2.99 (m, 2.8H), 1.33 (d, 1.2H, J = 6.0 Hz), 1.26 (d, 1.8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.1, 137.9, 137.7, 137.5, 128.6, 128.5, 128.4, 128.4, 128.3, 128.2, 128.1, 128.1, 128.0, 127.9, 97.2, 91.1, 83.2, 82.6, 80.0, 79.5, 75.7, 75.6, 74.8, 73.2, 70.6, 67.9, 67.6, 66.2, 18.5, 18.4; ES-HRMS calcd for $C_{20}H_{24}N_3O_4$ $[M + H]^+$ 370.1767, found 370.1766.

*4-Azido-2,3-di-O-benzyl-4,6-dideoxy-α-D-glucopyranosyl Dibenzylphosphate (**16**) (**30**).* To a solution of compound **15** (1.68 g, 4.54 mmol) in dry methylene chloride (50 mL) were added trichloroacetonitrile (3.68 mL, 36.7 mmol) and anhydrous K_2CO_3 (2.51 g, 18.2 mmol). The mixture was stirred at room temperature overnight and then filtered through a pad of Celite. The filtrate was evaporated to give the crude product. Without further purification, the crude compound was dissolved in dry methylene chloride (30 mL) and mixed with dibenzyl phosphate (1.35 g, 4.78 mmol). The mixture was stirred at room temperature for 2 h, the solvent was evaporated, and the residue was purified by flash column chromatography (1:4 to 1:2 EtOAc in hexanes) to give **16** (1.91 g) in 67% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35−7.24 (m, 20H), 5.85 (dd, 1H, J = 3.3, 6.9 Hz), 5.09−5.02 (m, 4H), 4.89−4.85 (d, 1H, J = 10.8 Hz), 4.75 (d, 2H, J = 10.8 Hz), 4.62 (d, 1H, J = 11.1 Hz), 3.74−3.54 (m, 3H), 3.09 (t, 1H, J = 9.6 Hz), 1.18 (d, 3H, J = 6.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.7, 137.3, 135.6, 128.5, 128.5, 128.4, 128.4, 128.3, 128.1, 128.0, 127.9, 127.69, 127.68, 95.2, 79.2, 78.8, 75.6, 72.9, 69.3, 69.2, 68.3, 67.2, 18.3; ^{31}P NMR (121 MHz, $CDCl_3$) δ −0.99; CI-HRMS calcd for $C_{34}H_{37}N_3O_7P$ $[M + H]^+$ 630.2369, found 630.2377.

*CDP-4-amino-4,6-dideoxy-α-D-glucopyranose (**7**).* Compound **16** (115 mg, 0.183 mmol) and triethylamine (19 mg, 0.188 mmol) were dissolved in a solution of 1:1 dioxane−H_2O (4 mL), and to this solution 10% Pd/C (100 mg) was added. The mixture was stirred for 24 h at room temperature, and the resulting suspension was filtered through a Celite pad and concentrated to give the crude product. Without purification, the phosphoric salt was dissolved in dry pyridine (2 mL) together with 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium cytidine 5′-monophosphomorpholidate (190 mg, 0.26 mmol). The resulting solution was concentrated to dryness. After repeating this process for 3 times, the residue was dried over P_2O_5 at room temperature under vacuum overnight. The dry residue was redissolved in pyridine (1 mL), and 1*H*-tetrazole (40 mg, 0.57 mmol) was added (*34*). The solution was stirred at room temperature for 3 days. The solvent was removed under vacuum. The residue was dissolved in 1 mL of water and purified by Bio-Gel P2 column eluted with 80 mM NH_4HCO_3 solution to give the final product **7** (26 mg, 26% yield): ^{1}H NMR (300 MHz, D_2O) δ 7.81 (d, 1H, J = 7.8 Hz), 5.98 (d, 1H, J = 7.8 Hz), 5.85 (d, 1H, J = 3.9 Hz), 5.45 (dd, 1H, J = 3.6, 7.2 Hz), 4.23−4.08 (m, 6H), 3.76 (t, 1H, J = 9.9 Hz), 3.49 (m, 1H), 2.85 (d, 1H, J = 10.2 Hz), 1.20 (d, 3H, J = 6.6 Hz); ^{13}C NMR (125 MHz, D_2O) δ 165.2, 156.4, 142.1, 96.6, 95.43 (d, C-1, J = 6.6 Hz), 89.6, 83.0 (d, J = 9.0 Hz), 74.4, 72.0 (d, J = 8.6 Hz), 69.5, 68.9, 65.9, 64.9 (d, J = 5.0 Hz), 57.2, 17.0; ^{31}P NMR (121 MHz, D_2O) δ −10.20 (d, J = 19.9 Hz), −12.08 (d, J = 21.3 Hz); ESI-HRMS calcd for $C_{15}H_{25}N_4O_{14}P_2$ $[M + H]^+$ 547.0843, found 547.0835.

*Methyl 4-Azido-2,3-di-O-benzyl-4,6-dideoxy-α-D-galactopyranoside (**17**).* A suspension of **11** (3.11 g, 7.12 mmol) and sodium azide (2.32 g, 35.6 mmol) in DMF (60 mL) was heated to 100 °C overnight. The solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate and washed with water. The combined organic fractions were washed with water and brine, dried, and concentrated. Purification by flash column chromatography (1:3 EtOAc in hexanes) yielded 2.41 g (88%) of **17** as a clear oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41−7.23 (m, 10H), 4.83 (d, 1H, J = 12.0 Hz), 4.81 (d, 1H, J = 12.0 Hz), 4.73 (d, 1H, J = 11.7 Hz), 4.64 (d, 1H, J = 11.7 Hz), 4.55 (d, 1H, J = 3.6 Hz), 4.01 (dd, 1H, J = 3.6, 9.9 Hz), 3.88 (dq, 1H, J = 1.5, 6.6 Hz), 3.82 (dd, 1H, J = 3.6, 9.9 Hz), 3.68 (dd, 1H, J = 1.5, 3.6 Hz), 3.32 (s, 3H), 1.20 (d, 3H, J = 6.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.2, 138.1, 128.4, 128.3, 128.0, 127.7, 127.7, 127.6, 98.5, 77.9, 75.8, 73.6, 73.1, 64.9, 64.1, 55.3, 17.2; EI-HRMS calcd for $C_{21}H_{25}N_3O_4$ $[M]^+$ 383.1845, found 383.1834.

*4-Azido-2,3-di-O-benzyl-4,6-dideoxy-β-D-galactopyranosyl Trichloroacetimidate (**18**).* Compound **17** (2.4 g, 6.25 mmol) was dissolved in acetic acid (50 mL) and 2 N HCl (15 mL). The reaction mixture was heated to 90 °C for 2 h. The mixture was cooled to room temperature and then extracted with ethyl acetate (100 mL). The combined organic portions were washed with saturated $NaHCO_3$, brine, dried, and concentrated. The residue was dissolved in methylene chloride (30 mL), to which were added trichloroacetonitrile (3.1 mL, 31.3 mmol) and anhydrous K_2CO_3 (2.16 g, 15.6 mmol). The mixture was stirred at room temperature overnight and then filtered through a pad of Celite. The filtrate was evaporated and purified by flash chromatography (1:3 EtOAc in hexanes) to give **18** in 53% yield (1.71 g): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.64 (s, 1H), 7.37−7.28 (m, 10H), 5.68 (d, 1H, J = 8.4 Hz), 4.90 (d, 1H, J = 11.1 Hz), 4.79 (d, 1H, J = 11.1 Hz), 4.77 (s, 2H), 3.94 (dd, 1H, J = 8.4, 9.3 Hz), 3.88 (dq, 1H, J = 1.5, 6.6 Hz), 3.80−3.68 (m, 2H), 1.34 (d, 3H, J = 6.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.4, 138.0, 137.6, 128.3, 128.32,

128.0, 127.84, 127.75, 98.2, 81.2, 77.7, 75.5, 73.1, 70.0, 63.5, 17.4.

*4-Azido-2,3-di-O-benzyl-4,6-dideoxy-α-D-galactopyranosyl Dibenzylphosphate (**19**).* To a solution of compound **18** (1.71 g, 3.33 mmol) in dry methylene chloride (50 mL) was added dibenzyl phosphate (1.13 g, 4.0 mmol). The mixture was stirred at room temperature for 2 h, and the solvent was evaporated. The residue was purified by flash column chromatography (1:2 EtOAc in hexanes) to give **19** (1.64 g) in 78% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36−7.20 (m, 20H), 5.82 (dd, 1H, J = 2.7, 6.3 Hz), 5.06−4.96 (m, 4H), 4.78 (d, 1H, J = 11.7 Hz), 4.72 (s, 2H), 4.70 (d, 1H, J = 12.0 Hz), 3.98 (dq, 1H, J = 1.5, 6.6 Hz), 3.92−3.87 (m, 2H), 3.68 (m, 1H), 1.14 (d, 3H, J = 6.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.8, 137.7, 135.8, 135.7, 128.5, 128.43, 128.40, 128.3, 128.0, 127.8, 127.74, 127.69, 127.66, 127.60, 96.2 (d, 1C, J = 6.1 Hz), 75.2 (d, 1C, J = 7.1 Hz), 73.4, 73.0, 69.2 (d, 1C, J = 5.5 Hz), 69.1 (d, 1C, J = 5.4 Hz), 66.8, 64.2, 17.1; ^{31}P NMR (121 MHz, $CDCl_3$) δ −1.10; CI-HRMS calcd for $C_{34}H_{37}N_3O_7P$ $[M + H]^+$ 630.2369, found 630.2384.

*CDP-4-amino-4,6-dideoxy-α-D-galactopyranose (**20**).* Compound **19** (120 mg, 0.192 mmol) and triethylamine (21 mg, 0.21 mmol) were dissolved in a solution of 1:1 dioxane−H_2O (10 mL), and to this solution 10% Pd/C (100 mg) was added. The mixture was stirred at room temperature under a hydrogen atmosphere for 2 days, and the resulting suspension was filtered through a Celite pad and concentrated to give the crude products as phosphate triethylammounium salts. Without purification, the crude products were dissolved in dry pyridine (2 mL) together with 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium cytidine 5′-monophosphomorpholidate (190 mg, 0.26 mmol). The resulting solution was concentrated to dryness. After repeating this process for 3 times, the residue was dried over P_2O_5 at room temperature under vacuum overnight. The dry residue was redissolved in pyridine (1 mL), and 1*H*-tetrazole (40 mg, 0.57 mmol) was added (*34*). The solution was stirred at room temperature for 3 days. The solvent was removed under vacuum. The residue was dissolved in 1 mL of water and purified by Bio-Gel P2 column with 80 mM NH_4HCO_3 solution as eluent to give the final product **7** (6 mg) in 5.7% yield: ^{1}H NMR (300 MHz, D_2O) δ 7.90 (d, 1H, J = 7.8 Hz), 6.05 (d, 1H, J = 8.1 Hz), 5.86 (d, 1H, J = 4.2 Hz), 5.48 (dd, 1H, J = 4.2, 7.5 Hz), 4.42 (m, 1H), 4.25−4.06 (m, 6H), 3.53 (t, 1H, J = 3.3 Hz), 3.49 (m, 1H), 1.16 (d, 3H, J = 6.6 Hz); ^{13}C NMR (125 MHz, D_2O) δ 165.0, 156.0, 142.2, 96.5, 95.3 (d, 1C, J = 7.1 Hz), 89.6, 83.0 (d, 1C, J = 9.2 Hz), 74.5, 69.5, 67.9, 66.2, 64.8, 64.1, 55.8, 15.8; ^{31}P NMR (121 MHz, D_2O) δ −10.2 (d, J = 21.4 Hz), −12.0 (d, J = 24.4 Hz); ESI-HRMS calcd for $C_{15}H_{25}N_4O_{14}P_2$ $[M + H]^+$ 547.0843, found 547.0859.

HPLC Analysis of E_1 Catalyzed Reactions. A sample containing 32 μM CDP-4-amino-4,6-dideoxy-D-glucose (**7**) and 180 μM H220K-E_1, which was fully reconstituted with PLP and the [2Fe-2S] cluster, in 50 mM potassium phosphate buffer (pH 7.5) was incubated at 37 °C for 1 h. In parallel, an incubation containing 32 μM **7**, 180 μM H220K-E_1, 0.3 μM E_1, 1.2 μM E_3, and a NADH regenerating system consisting of 10 μM NADH, 50 mM sodium formate, and 0.05 unit of formate dehydrogenase (*Candida boidinii*) (*27*) in 50 mM potassium phosphate buffer (pH 7.5) was also carried out under the same conditions. Each sample was analyzed by HPLC on a Dionex CarboPac PA1 column (4 × 250 mm), and the reaction mixture was eluted with a linear gradient from 10 to 500 mM ammonium acetate buffer (pH 7.0). The flow rate was 1 mL/min, and the detector was set at 260 nm. The retention times were 29.2 min for the E_1 substrate (**1**), 32.0 min for the E_1-E_3 product (**2**), and 17.1 min for **7**.

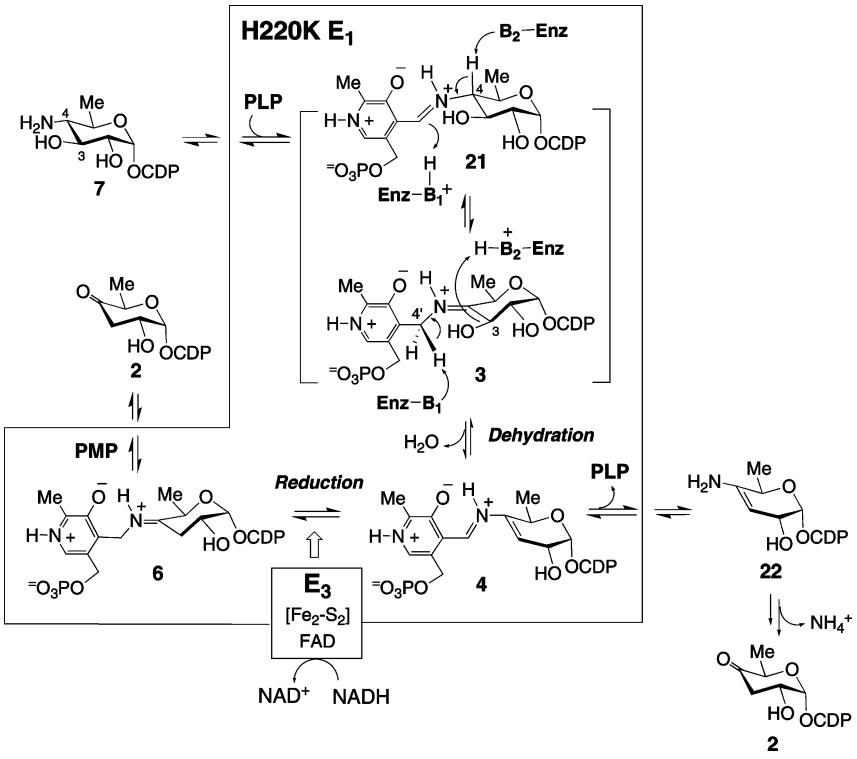
RESULTS AND DISCUSSION

Properties of H220K Mutant. The H220K mutant was prepared by using the Sculptor *in vitro* mutagenesis kit. The mutant protein was purified to nearly homogeneity, as determined by SDS−PAGE, based on the published procedure developed for the wild type E_1 (*12*, *14*). Unlike the wild type E_1 which displays a prominent absorbance maximum near 330 nm, the mutant protein exhibits a λ_{max} at 420 nm, an indicator of covalently bound PLP forming an internal aldimine with lysine (*35*−*37*). The amount of PLP bound was determined by denaturing the enzyme and quantitatively measuring the released PLP under alkaline conditions (*30*, *31*). The ratio of bound PLP to H220K-E_1 was estimated to be 0.16 equiv/monomer. It was only by reconstitution with excess PLP that a stoichiometric ratio of 1 PLP per E_1 monomer was obtained. The iron content of H220K-E_1 was determined by measuring the concentration of a colored complex formed between the iron released from the enzyme with ferrozine and neocuprine (*29*). The amount of iron associated with this enzyme after reconstitution (6-fold excess of $Fe(NH_4)_2(SO_4)_2$ and Na_2S) (*13*) was found to be 2 Fe^{2+} per monomer. Clearly, H220K-E_1 retains its ability to bind both iron and the B_6 coenzyme.

*Synthesis of Predicted Substrate, CDP-4-amino-4,6-deoxy-D-glucose (**7**), and Its Galactose Analogue (**20**).* The proposed aminosugar substrate **7** was synthesized from methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**8**) as shown in Scheme 2. The C-6 deoxygenation to make **11** was accomplished by iodination of **10** followed by hydride reduction of the 6-iodo group. The configuration at C-4 of **11** was inverted by the S_N2 displacement reaction with a benzoyl group. After hydrolysis, the amino group, masked as an azide moiety, was introduced at C-4 of **13** under Mitsunobu conditions (*32*) to give **14**. Demethylation at C-1 of **14** was achieved by acid hydrolysis, and the product **15** was benzylphosphorylated to give **16**. The final steps involved hydrogenation of **16** (*33*), and coupling of the resulting product with CMP-morpholidate (*34*). The corresponding 4-epimer, **20**, was prepared in a similar manner (see Scheme 2).

Studies on the Catalytic Properties of H220K Mutant. The catalytic activity of H220K-E_1 was first examined for E_1 wild type activity in a coupled assay utilizing substrate **1** and E_3, where the decrease in NADH absorbance at 340 nm in the E_3-catalyzed reduction of the predicted $\Delta^{3,4}$-glucoseen intermediate (**4**) was monitored (*9*, *14*). The mutant protein (0.3 μM), carrying a fully reconstituted iron−sulfur core and trace amounts of PLP (<0.15 equiv), was incubated with 100 μM **1**, 25 μM PMP, 200 μM NADH, and 1.2 μM E_3 at room temperature in 50 mM potassium phosphate buffer (pH 7.5). Although the wild type E_1 was active under these assay conditions, no NADH consumption was observed with

Scheme 3: Possible Reaction Courses upon Incubation of CDP-4-amino-6-deoxy-α-D-glucose (**7**) with H220K-E_1



$H220K-E_1$. It is likely that the H220K mutation has impaired E_1's ability to bind PMP, and thus rendered $H220K-E_1$ inactive. As expected, the 4-ketosugar substrate (**1**) was unable to react with the trace amount of PLP coenzyme bound as an internal aldimine to Lys220 in $H220K-E_1$. Surprisingly, similar results were also observed when the aminosugar **7** and PLP replaced **1** and PMP, respectively, in the above assay mixture. Neither wild type E_1 nor $H220K-E_1$ showed activity with **7**.

Two scenarios are possible: the aminosugar **7** is not a substrate for $H220K-E_1$ or the reaction is not catalytic due to a problem with coenzyme regeneration. As shown in Scheme 3, a sequence of external aldimine formation, dehydration, reduction by E_3, and hydrolysis (**7** → **21** → **3** → **4** → **6** → **2**) would result in the formation of PMP, but not the catalytically active PLP, at the end of the reaction. However, the addition of excess PLP (25 μM) had no effect on turnover. Attempts to recycle the PMP coenzyme by adding α-ketoglutarate in the incubation mixture using $H220K-E_1$ fully reconstituted with PLP and [2Fe-2S] cluster were also unsuccessful. These results cast doubt on $H220K-E_1$'s ability to catalyze the dehydration−reduction sequence.

A mechanism resembling those of PLP-dependent serine/threonine dehydratases (*38*, *39*), while less likely, was also considered. In this scenario, E_3 and NADH are not required, and regeneration of PLP would be achieved by hydrolysis of the predicted dehydration product **4**. Meanwhile, the enamine sugar (**22**) would undergo tautomerization followed by hydrolysis of the ensuing imine to give **2** and ammonia (Scheme 3). A similar mechanism has been found for the ColD enzyme, which catalyzes the C-3 deoxygenation in the biosynthesis of GDP-L-colitose (*40*). To test this hypothesis (**7** → **21** → **3** → **4** → **22** → **2**, Scheme 3), an incubation of fully reconstituted $H220K-E_1$ (1 μM) with **7** (50 μM) in 1 mL of 50 mM potassium phosphate buffer (pH 7.5) was conducted at 37 °C for 3 h. Unfortunately, HPLC analysis again failed to detect **2** as the product.

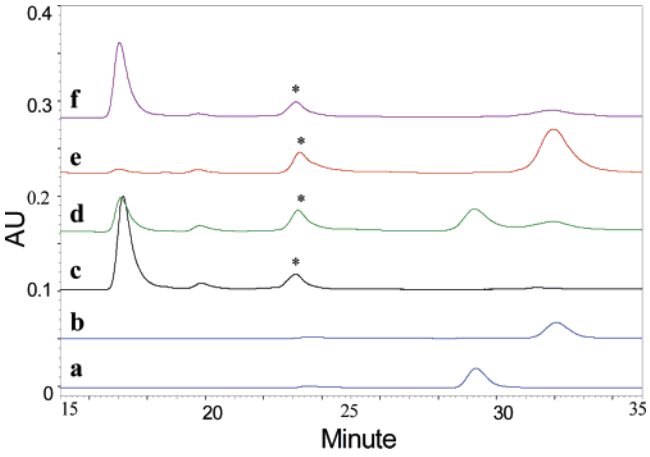
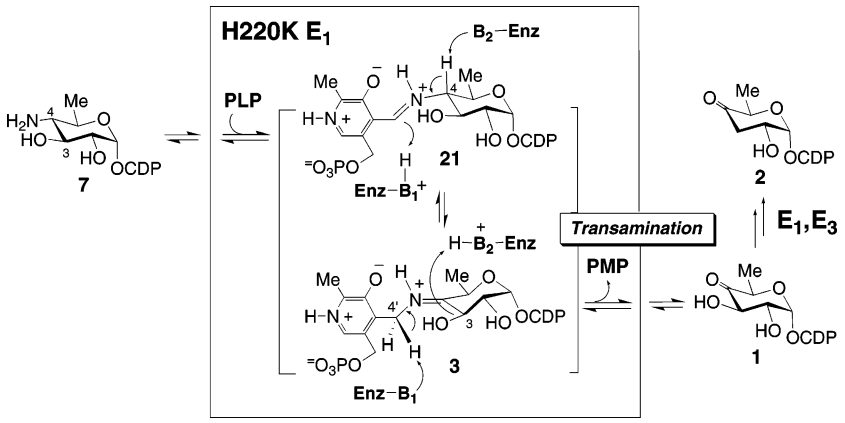


FIGURE 1: HPLC analysis of several CDP-sugar standards and incubations of $H220K-E_1$ with compound **7**: (a) the E_1 substrate (**1**); (b) the E_1-E_3 product (**2**); (c) compound **7**; (d) incubation of **7** (32 μM) with $H220K-E_1$ (180 μM) and the NADH regeneration system (*27*); (e) incubation of **7** (32 μM) with $H220K-E_1$ (180 μM), E_1 (0.3 μM), E_3 (1.2 μM), and the NADH regeneration system; (f) incubation of **7** (32 μM) with E_1 (0.3 μM), E_3 (1.2 μM), and the NADH regeneration system. The peak labeled with ∗ is a decomposition product derived from compound **7** during HPLC analysis. The small bump eluted at around 32 min in traces d and f is due to an impurity associated with the NADH regeneration system.

Identification of Transamination Activity for $H220K-E_1$ Mutant. Interestingly, an incubation mixture in which the concentration of $H220K-E_1$ (180 μM) was nearly 6-fold that of **7** (32 μM) revealed that a significant fraction of **7**, which has a retention time of 17.1 min (see Figure 1, trace c), was consumed and a new peak appeared at 29.2 min (trace d, in the presence or in the absence of NADH regeneration system). The retention time for this new peak is identical to

Scheme 4: The Proposed Reaction Mechanism for the Conversion of CDP-4-amino-6-deoxy-α-D-glucose (**7**) to CDP-4-keto-6-deoxy-α-D-glcuose (**1**) by H220K-E_1



that of **1** (trace a). High resolution MS characterization (calculated 546.0605, observed 546.0625) confirmed that the product is indeed CDP-4-keto-6-deoxyglucose (**1**), which could be converted to **2** *in situ* by the wild type E_1 and E_3 in the presence of a NADH regeneration system (Figure 1, trace e) (*27*). Incubation of **7** with wild type E_1, E_3, and NADH does not produce **2** (Figure 1, trace f). These results clearly showed that compound **7** is only a substrate for H220K-E_1 and not for the wild type E_1. However, while H220K-E_1 can be considered a *de facto* transaminase, the reaction is not catalytic since PLP cannot be regenerated during turnover. Efforts to achieve multiple turnovers by adding excess PLP or α-ketoacids were not successful. Yet, the reaction is stereospecific, because incubation of the C-4 epimer **20** with H220K-E_1 did not produce **1**. To rule out the possibility that the observed transamination activity may result from **7** reacting with a nonactive site lysine-PLP imine moiety, treatment of **7** with the previously prepared H220N-E_1 (*14*), which differs from H220K-E_1 only for lacking the active-site lysine, was also carried out. The fact that no product formation was detected in the incubation strongly suggested that the conversion of **7** to **1** by H220K-E_1 occurs in the enzyme active site.

Mechanistic Implications. The conversion of **7** to **1** likely proceeds via Schiff base formation, tautomerization, and hydrolysis (**7** → **21** → **3** → **1**, Scheme 4). An acid−base pair in the active site of H220K-E_1 must be present to facilitate the tautomerization step (**21** ⇆ **3**). The catalytic residue (Enz-B_1), located near C-4′, which acts as the general acid in the forward direction should serve as the base in the reverse reaction. This residue (Enz-B_1) is also expected to play a key role in abstracting the 4′-H in the dehydration step (**3** ⇆ **4**). Since H220K-E_1 is capable of catalyzing the tautomerization but not the dehydration reaction, Lys220 is most likely not the catalytic residue (Enz-B_1) next to C-4′. Instead it may act as the Enz-B_2 residue mediating the protonation/deprotonation at C-4 during the interconversion between **21** and **3**. Likewise, His220, which is expected to occupy a similar location in the active site of wild type E_1, should also be distant from C-4′. Thus, His220 may not be responsible for its previously proposed role in the 4′-H abstraction of **3** (*14*). Because His220 is a crucial residue involved in the dehydration step as indicated by early mutagenesis studies (*14*), it more likely assumes a general acid role (acting as Enz-B_2) facilitating the protonation and subsequent elimination of the 3-OH leaving group in the dehydration of **3** to **4** catalyzed by wild type E_1.

Summary. Overall, this work is significant for two reasons. First, the fact that mutation of histidine to lysine at position 220 specifically impedes the dehydration step has allowed us to propose a new role for His220 in E_1 reaction. Instead of being the active site residue in the vicinity of C-4′ of **3**, it may function as a general acid in the dehydration reaction making 3-OH as a better leaving group. The failure to transform the H220K mutant into a PLP-dependent dehydrase may simply be due to the inability of Lys220 to assist protonation of the 3-OH of **3** in the dehydration step. Second, our investigation offers an interesting possibility: that the replacement of the active site lysine by a histidine residue in E_1 might be a critical evolutionary step that converts a PLP-dependent transaminase into a unique PMP-dependent dehydrase. The recruitment of an iron−sulfur cluster in E_1, along with the requirement of a reductase (E_3) for turnover, allows PMP to be used as a coenzyme and makes the E_1 reaction catalytic. Together, the lysine to histidine mutation and iron−sulfur core incorporation reflects an elaborate evolutionary path for the E_1/E_3 catalyzed reaction. Hence, this study represents an interesting example that reveals Nature's sophistication in devising a novel strategy to catalyze a simple, albeit mechanistically challenging reaction (*4*, *41*).

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