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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 function and 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} 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

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involvement of PMP in the stabilization of an unpaired electron spin in an electron-transfer reduction.¹⁻⁴

Sequence alignment¹⁴ showed that E₁ 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 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 B₆-dependent enzymes is absent in E₁.^{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, E₁ 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 E₁ reflects a distinct evolutionary path for E₁, and distinguishes it from other B₆-dependent enzymes. It should be noted that apart from E₁, GABA-aminotransferase is another B₆-dependent enzyme containing a [2Fe-2S] cluster.²² However, the catalytic role of the Fe-S cluster in GABA-aminotransferase remains elusive. E₁ 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 E₁ 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 E₁ 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 E₁, 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 E₁.

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 Bradford²⁴ 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/H₂SO₄ (1:98:1) or phosphomolybdic acid (7% in EtOH).

Materials

The construction of the plasmid containing the E₁ gene (pJT18) in pUC119 have been reported elsewhere.²⁵ Enzyme E₃ used in the assay was purified from the *E. coli* JM105/pOPI cultures based on a procedure published earlier.^{12,26} Purification and reconstitution of E₁ and E₃ were carried out under semi-anaerobic conditions as previously described.^{12,14} The formate dehydrogenase used in the NADH regeneration system²⁷ 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 customly 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**CATATCAC CATGGGTG-3' (forward) and 5'-CACCCATGGTGATATGCT**TAG**CGGGATAGAAGC-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 *Escherichia coli* HB101 for overexpression of the mutant protein.

Purification of H220K-E₁ 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 (NH₄)₂Fe(SO₄)₂·6H₂O (40 mg/L). The cells were grown until OD₆₀₀ 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 5000g for 10 min to collect wet cells. The cells were re-suspended 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 27000g for 20 min and the supernatant (110 mL) was collected. To the supernatant was added (NH₄)₂SO₄ to 80% saturation (under N₂) and stirred at 4 °C for 1 h. The resulting solution was centrifuged (12000g for 15 min). The pellet was collected, re-suspended in 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 E₁ 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-E₁ 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 E₁ substrate, CDP-4-keto-6-deoxy-D-glucose (**1**), was enzymatically prepared from CDP-glucose according to a previously reported procedure.²⁸ The activity of E₁ and E₁ mutants was determined by a published continuous spectrophotometric assay that monitors the consumption of NADH in the presence of E₁ (or its mutants), E₃, and the E₁ substrate (**1**).^{9,14} The typical assay mixture consisted of 25 μM PMP, 200 μM NADH, 100 μM **1**, and 1.2 μM E₃, in 800 μL of 50 mM potassium phosphate buffer (pH 7.5). The reaction was initiated by the addition of E₁ (0.3 μM) and the E₁ activity was determined by measuring the rate of reduction of the

absorbance at 340 nm ($\epsilon = 6,220 \text{ M}^{-1}\text{cm}^{-1}$) 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 ($\epsilon = 6600 \text{ M}^{-1}\text{cm}^{-1}$) 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-Sephadex 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 M 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 earlier³² 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 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 was stirred at room temperature for 2 h and 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 was refluxed for 4 h, cooled to room temperature, and neutralized with solid NaHCO_3 . The reaction was filtered, the acetone solvent evaporated, the residue diluted with water, and 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. ^1H 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 $\text{C}_{21}\text{H}_{27}\text{O}_6$ $[\text{M}+\text{H}]^+$ 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 was stirred overnight as 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 white solid. ^1H 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) δ 137.7, 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 $\text{C}_{23}\text{H}_{29}\text{O}_{10}\text{S}_2$ $[\text{M}+\text{H}]^+$ 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. ^1H 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 $\text{C}_{22}\text{H}_{27}\text{O}_7\text{S}$ $[\text{M}+\text{H}]^+$ 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 was cooled to room temperature and 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. ^1H 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 $\text{C}_{28}\text{H}_{29}\text{O}_6$ $[\text{M}+\text{H}]^+$ 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 was stirred at 50 °C for 5 h and then cooled to room temperature. The reaction was quenched by the addition of saturated NH₄Cl (5 mL) and 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. ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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 C₂₁H₂₅O₅ [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), 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, 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). ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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 C₂₁H₂₄N₃O₄ [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 2N 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 NaHCO₃ 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). ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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 C₂₀H₂₄N₃O₄ [M+H]⁺ 370.1767, found 370.1766.

4-Azido-2,3-di-O-benzyl-4,6-dideoxy- α -D-glucopyranosyl Dibenzyldiphosphate (**16**).³⁰

To a solution of compound **15** (1.68 g, 4.54 mmol) in dry methylene chloride (50 mL) was added trichloroacetonitrile (3.68 mL, 36.7 mmol) and anhydrous K₂CO₃ (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. ¹H NMR (300 MHz, CDCl₃) 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 $\text{C}_{34}\text{H}_{37}\text{N}_3\text{O}_7\text{P}$ $[\text{M}+\text{H}]^+$ 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, 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'-monophospho-morpholidate (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 re-dissolved in pyridine (1 mL), and 1H-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). ^1H 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 $\text{C}_{15}\text{H}_{25}\text{N}_4\text{O}_{14}\text{P}_2$ $[\text{M}+\text{H}]^+$ 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. ^1H 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 $\text{C}_{21}\text{H}_{25}\text{N}_3\text{O}_4$ $[\text{M}]^+$ 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 2N HCl (15 mL). The reaction 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 was 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). ^1H 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 Dibenzyolphosphate (**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. ^1H 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 $\text{C}_{34}\text{H}_{37}\text{N}_3\text{O}_7\text{P}$ $[\text{M}+\text{H}]^+$ 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, 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 re-dissolved in pyridine (1 mL), and 1H-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. ^1H 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 $\text{C}_{15}\text{H}_{25}\text{N}_4\text{O}_{14}\text{P}_2$ $[\text{M}+\text{H}]^+$ 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 $^\circ\text{C}$ 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 $\times$ 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 E₁.^{12,14} Unlike the wild type E₁ 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-E₁ was estimated to be 0.16 equiv/monomer. It was only by reconstitution with excess PLP that a stoichiometric ratio of 1 PLP per E₁ monomer was obtained. The iron content of H220K-E₁ 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(NH₄)₂(SO₄)₂ and Na₂S)¹³ was found to be 2 Fe²⁺ per monomer. Clearly, H220K-E₁ retains its ability to bind both iron and the B₆ 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 conditions³² 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 the Catalytic Properties of H220K Mutant

The catalytic activity of H220K-E₁ was first examined for E₁ wild type activity in a coupled assay utilizing substrate **1** and E₃, where the decrease in NADH absorbance at 340 nm in the E₃-catalyzed reduction of the predicted $\Delta^{3,4}$ -glucose intermediate (**4**) was monitored.^{9,14} The mutant protein (0.3 μ M), carrying a fully reconstituted iron-sulfur core and trace amounts of PLP (<0.15 equiv), was incubated with 100 μ M **1**, 25 μ M PMP, 200 μ M NADH, and 1.2 μ M E₃ at room temperature in 50 mM potassium phosphate buffer (pH 7.5). Although the wild type E₁ was active under these assay conditions, no NADH consumption was observed with H220K-E₁. It is likely that the H220K mutation has impaired E₁'s ability to bind PMP, and thus rendered H220K-E₁ 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-E₁. 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 E₁ nor H220K-E₁ showed activity with **7**.

Two scenarios are possible: the aminosugar **7** is not a substrate for H220K-E₁ 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 E₃, and hydrolysis (**7** $\rightarrow$ **21** $\rightarrow$ **3** $\rightarrow$ **4** $\rightarrow$ **6** $\rightarrow$ **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 μ M) had no effect on turnover. Attempts to recycle the PMP coenzyme by adding α -ketoglutarate in the incubation mixture using H220K-E₁ fully reconstituted with PLP and [2Fe-2S] cluster were also

unsuccessful. These results casted doubt on H220K-E₁'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, E₃ 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-E₁ (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.

Identification of Transamination Activity for H220K-E₁ Mutant

Interestingly, an incubation mixture in which the concentration of H220K-E₁ (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 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 E₁ and E₃ in the presence of a NADH regeneration system (Figure 1, trace e).²⁷ Incubation of **7** with wild type E₁, E₃, and NADH does not produce **2** (Figure 1, trace f). These results clearly showed that compound **7** is only a substrate for H220K-E₁ and not for the wild type E₁. However, while H220K-E₁ 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-E₁ did not produce **1**. To rule out the possibility that the observed transamination activity may result from **7** reacting with a non-active site lysine-PLP imine moiety, treatment of **7** with the previously prepared H220N-E₁,¹⁴ which differs from H220K-E₁ 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-E₁ 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-E₁ must be present to facilitate the tautomerization step (**21** ↔ **3**). The catalytic residue (Enz-B₁), 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-B₁) is also expected to play a key role in abstracting the 4'-H in the dehydration step (**3** ↔ **4**). Since H220K-E₁ is capable of catalyzing the tautomerization but not the dehydration reaction, Lys220 is most likely not the catalytic residue (Enz-B₁) next to C-4'. Instead it may act as the Enz-B₂ 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 E₁, 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-B₂) facilitating the protonation and subsequent elimination of the 3-OH leaving group in the dehydration of **3** to **4** catalyzed by wild type E₁.

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 E₁ 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 E₁ 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 E₁ along with the requirement of a reductase (E₃) for turnover, allows PMP to be used as a coenzyme and makes the E₁ reaction catalytic. Together, the lysine to histidine mutation and iron-sulfur core incorporation reflects an elaborate evolutionary path for the E₁/E₃ 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}

Abbreviations

CDP	cytidine 5'-diphosphate
CMP	cytidine 5'-monophosphate
DEAE	diethylaminoethyl
E₁	CDP-6-deoxy-L- <i>threo</i> -D- <i>glycero</i> -4-hexulose-3-dehydrase
E₃	CDP-6-deoxy-L- <i>threo</i> -D- <i>glycero</i> -4-hexulose-3-dehydrase reductase
E_{od}	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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Figure 1.

HPLC analysis of several CDP-sugar standards and incubations of H220K-E₁ with compound **7**: (a) the E₁ substrate (**1**); (b) the E₁-E₃ product (**2**); (c) compound **7**; (d) incubation of **7** (32 μ M) with H220K-E₁ (180 μ M) and the NADH regeneration system;²⁷ (e) incubation of **7** (32 μ M) with H220K-E₁ (180 μ M), E₁ (0.3 μ M), E₃ (1.2 μ M), and the NADH regeneration system; (f) incubation of **7** (32 μ M) with E₁ (0.3 μ M), E₃ (1.2 μ M), 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.

Scheme 1.

Mechanism of C-3 deoxygenation catalyzed by E₁ and E₃ in the biosynthesis of 3,6-deideoxyhexoses.

Scheme 2.
Synthesis of CDP-4-amino-6-deoxy- α -D-glucose (**7**) and its 4-epimer (**20**).

Scheme 3.

Possible reaction courses upon incubation of CDP-4-amino-6-deoxy- α -D-glucose (**7**) with H220K-E₁.

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-glucose (**1**) by H220K-E₁.