Mechanistic and Stereochemical Studies of a Unique Dehydration Catalyzed by CDP–4-Keto-6-deoxy-D-glucose-3-dehydrase: A Pyridoxamine 5′-Phosphate Dependent Enzyme Isolated from *Yersinia pseudotuberculosis*†

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ABSTRACT: CDP–4-keto-6-deoxy-D-glucose-3-dehydrase (E_1) purified from *Yersinia pseudotuberculosis* is a pyridoxamine 5′-phosphate (PMP) dependent enzyme which catalyzes the C–O bond cleavage at C-3 of a CDP–4-keto-6-deoxy-D-glucose substrate, a key step in the formation of 3,6-dideoxyhexoses. Since enzyme E_1 utilizes the PMP cofactor in a unique manner, it is essential to establish its role in E_1 catalysis. When an incubation was conducted in $[^{18}O]H_2O$, incorporation of ^{18}O into positions C-3 and C-4 of the recovered substrate was observed. This result not only provided the evidence necessary to reveal the reversibility of E_1 catalysis but also lent credence to the formation of a $\Delta^{3,4}$-glucoseen intermediate. In view of E_1 catalysis being initiated by a C-4′ deprotonation of the PMP–substrate complex, the stereochemical course of this step was examined using chemically synthesized (4′*S*)- and (4′*R*)-[4′-^{3}H]PMP as probes. Our results clearly demonstrated that the stereochemistry of this deprotonation is *pro-S* specific, which is in agreement with the stereochemical consistency found with other vitamin B_6 phosphate dependent enzymes. The fact that reprotonation at C-4′ of the PMP–$\Delta^{3,4}$-glucoseen complex in the reverse direction of E_1 catalysis was also found to be *pro-S* stereospecific strongly suggested that enzyme E_1, like most of its counterparts, has the *si* face of its cofactor–substrate complex exposed to solvent and accessible to active-site catalytic groups as well. These stereochemical studies have given support to the role postulated for the PMP cofactor in the proposed mechanism, and they also suggest that the active site of E_1 may share features similar to other PLP/PMP-linked enzymes which control the orientation of the cofactor–substrate complex. It is worth noting that enzyme E_1 cannot finish C-3 deoxygenation without CDP–6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3) which reduces the nascent E_1 product, driving the equilibrium to completion. Although chemical reducing reagents failed to trap the transient E_1 product, two well-known electron shuttle proteins were able to generate a small amount of the dideoxyhexose product. The fact that other electron-transfer reductases can act as substitutes for E_3 provided compelling evidence supporting our earlier notion that the E_1 product is reduced by a stepwise $1e^-/1e^-$ transfer mechanism. Thus, E_1, despite its having evolved an unusual role for the PMP cofactor, has retained all the essential elements of catalysis common to other vitamin B_6 phosphate dependent enzymes. These results also support Dunathan's hypothesis that this class of enzymes, regardless of its catalytic diversity, evolved from a common progenitor.

The vitamin B_6 phosphate dependent enzymes are an important class of catalysts that mediate a wide variety of biological transformations involved in the synthesis, degradation, and interconversion of amino acids, such as transamination, decarboxylation, racemization, β- and γ-elimination, and substitution (Adams, 1976; Akhtar et al., 1984; Evangelopoulos, 1984; Dolphin et al., 1986). Although the catalytic roles of vitamin B_6 phosphate are amazingly versatile, the common theme to the various catalytic functions of its dependent enzymes relies primarily on the ability of this cofactor to act as an electron sink, temporarily storing the electrons that are later used for the cleavage and/or formation of covalent bonds. While the aldehyde form of this cofactor, pyridoxal 5′-phosphate (PLP), is the most common form for vitamin B_6 phosphate dependent enzymes, the amine form of this coenzyme, pyridoxamine 5′-phosphate (PMP), has also been shown to play an important role in the reactions mediated by transaminases (Braunstein, 1973; Christen & Metzler, 1985; Salerno et al., 1986; Kirsch et al., 1984). Since a great number of vitamin B_6 phosphate linked enzymes have been isolated and characterized, chemistry of this important cofactor has been well established. In fact, both PLP and PMP are now perceived as the standard coenzymes for the metabolism of amino acids.

Interestingly, investigations done on the biosynthesis of 3,6-dideoxyhexoses, the dominant antigenic determinants found in the O-specific side chains of cell wall lipopolysaccharides of a number of Gram-negative bacteria (Lüderitz et al., 1966; Hanessian, 1966; Williams & Wander, 1980; Bishop & Jennings, 1982), paved the way to the discovery of a unique PMP-dependent enzyme that participates in a dehydration reaction (Gonzalez-Porque & Strominger, 1972a; Rubenstein & Strominger, 1974a; Gonzalez-Porque, 1986). As depicted in Scheme I, this enzyme is CDP-4-keto-6-deoxy-D-glucose-3-dehydrase (E_1)[1] which catalyzes the C–O bond cleavage at C-3 during the biosynthesis of CDP–ascarylose (**1**), a 3,6-dideoxy-L-*arabino*-hexopyranose derived from CDP–4-keto-6-deoxy-D-glucose (**2**). Since significant losses of activity occurred when the crude enzyme extract was subjected to

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[1] Abbreviations: E_1, CDP–4-keto-6-deoxy-D-glucose-3-dehydrase; E_3, CDP–6-deoxy-$\Delta^{3,4}$-glucoseen reductase; E_{od}, CDP-D-glucose oxidoreductase; AAT, aspartate aminotransferase; TBA, 2-thiobarbituric acid; ee, enantiomeric excess; BSA, bovine serum albumin; MMO, methane monooxygenase; GABA, γ-aminobutyric acid; DCPIP, dichlorophenolindophenol.

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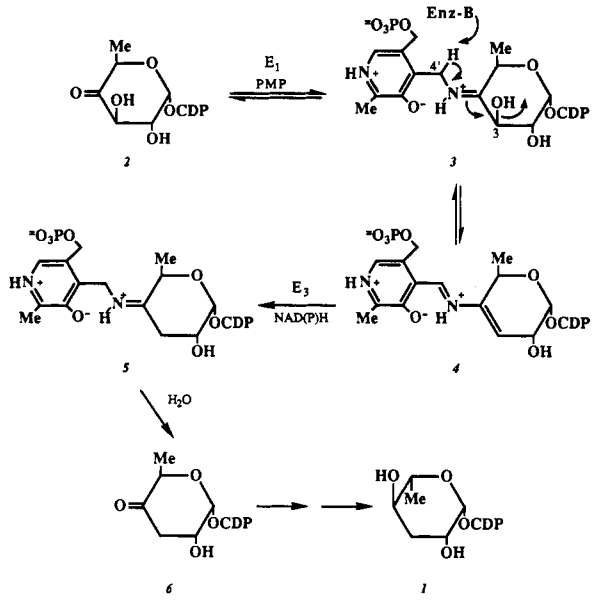
Scheme I



anion-exchange chromatography, it was eventually discovered that the purified protein was in fact an apoenzyme. The PMP dependence of this enzymatic step was later established by the isolation and characterization of PMP from the early fractions of the anion-exchange chromatography which could reconstitute E_1 activity (Gonzalez-Porque & Strominger, 1972a,b). The need for PMP was further confirmed because authentic PMP could serve as a substitute in the restoration of enzyme activity.

The proposed mechanism of E_1-mediated deoxygenation involves the coupling of the coenzyme with the C-4 keto group of the substrate (**2**) to form a Schiff base (**3**) followed by a C-4′ proton abstraction from the resulting adduct (**3**) that triggers the expulsion of the C-3 hydroxy group (Scheme I) (Rubenstein & Strominger, 1974a; Gonzalez-Porque, 1986). This enzymatic process is unique since it represents the only PMP-dependent catalysis that is not a transamination reaction. Although a reductive step catalyzed by an NAD(P)H-dependent enzyme, CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3), has been shown to constitute the second phase of C-3 deoxygenation (Scheme I) (Rubenstein & Strominger, 1974b; Gonzalez-Porque, 1986; Han & Liu, 1988; Han et al., 1990), the putative $\Delta^{3,4}$-glucoseen intermediate (**4**) has never been isolated or characterized. Hence, the intimate mechanism of this PMP-dependent sugar deoxygenation remains uncertain. In an attempt to explore the mechanism of this unique enzymatic process, we have recently purified an "E_1 equivalent" to homogeneity from *Yersinia pseudotuberculosis* (Weigel et al., 1992) and carried out detailed stereochemical analysis of the reactions it mediates. Since studies of the catalysis of vitamin B_6 phosphate linked enzymes have shown a remarkable stereochemical uniformity in which the bond cleavage and/or formation, with few exceptions, always take place at the *si* face of C-4′ in the substrate–cofactor complex (Vederas & Floss, 1980; Rétey & Robinson, 1982; Palcic & Floss, 1986), such stereochemical consistency is expected to be preserved by an enzyme that adheres to the well-established vitamin B_6 phosphate cofactor chemistry. Thus, a study directed at elucidating the stereochemical course of the reaction mediated by E_1, whose catalytic function is beyond the scope entailed by other vitamin B_6 phosphate dependent enzymes, may provide unique mechanistic insights that are not available from other experimental approaches. Summarized in this paper are the results of these stereochemical analyses and their implication on the mechanism of the key step initiating C-3 deoxygenation.

Experimental Procedures

Materials. Commercially available enzymes used in the assays and most other biochemicals were purchased from Sigma (St. Louis, MO). $[^3H]H_2O$ and $[^{18}O]H_2O$ were from Amersham (Arlington Heights, IL), and $[^3H]NaBH_4$ was from New England Nuclear (Boston, MA). Scintillation counting was done using Ecoscint A biodegradable scintillation solution from National Diagnostics (Manville, NJ). The bacterial strain *Y. pseudotuberculosis* was kindly provided by Dr. Otto Lüderitz of the Max Planck Institute for Immunobiology, West Germany. The enzyme CDP-4-keto-6-deoxy-D-glucose-3-dehydrase (E_1) was purified to homogeneity as described in the preceding paper (Weigel et al., 1992). CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3) was purified by a modified procedure (Miller and Liu, unpublished results) of Han et al. (1990). CDP-D-glucose oxidoreductase (E_{od}) was isolated from the same *Y. pseudotuberculosis* strain by members of this laboratory (Liu et al., unpublished results). Racemic [4′-^{3}H]PMP was prepared by a published procedure (Voet et al., 1973; Rubenstein & Strominger, 1974a) with minor modification. The stereospecifically labeled (4′*R*)- and (4′*S*)-[4′-^{3}H]PMP were synthesized as described by Yang et al. (1991). The GC/MS standards, abequitol tetraacetate, glucitol tetraacetate, and fucitol pentaacetate, were prepared from the corresponding hexoses as described in the preceding paper (Weigel et al., 1992). Since the substrate of E_1 is not readily available, it has to be prepared in situ from CDP-glucose prior to each experiment. A general procedure for the preparation of E_1 substrate involves the incubation of CDP-glucose, NAD^+, and E_{od} (40–60 μg) in potassium phosphate buffer (10 mM, pH 7.5) at 37 °C for 30 min. An aliquot of this solution, designated as the substrate solution, was then added to a mixture of PMP and E_1, or PMP, NADH, E_1, and E_3, to study the deoxygenation reaction.

Incubation of E_1 and Its Substrate in $[^{18}O]H_2O$. To an aliquot of the substrate solution [CDP-D-glucose (0.88 mg, 1.5 μmol) and NAD^+ (0.36 mg, 0.55 μmol) in 15 μL of buffer] was added PMP (2.8 ng, 0.01 nmol) and E_1 (75 μg), along with Tris-HCl (1.8 mg, 11.4 μmol) and Tris base (0.34 mg, 2.8 μmol), dissolved in 300 μL of $[^{18}O]H_2O$. The reaction was kept at 27 °C for 4 h. Sodium borohydride (1 mg, 26 μmol) was added and allowed to react for 30 min at room temperature. The solution was centrifuged, and the supernatant was subjected to HPLC purification. The products were isolated using an analytical Partisil SAX 10 anion-exchange column (4.6 × 250 mm) with a 20-min linear gradient from 0.03 to 0.12 M potassium phosphate buffer (pH 6.6) and a flow rate of 1 mL/min. Since all of the cytidine diphosphate containing hexose derivatives were eluted together with approximately the same retention time (5.4 min), they were collected in a single fraction. The fraction containing these compounds was acidified to pH 2.0 with concentrated HCl, boiled for 10 min, and then neutralized with concentrated KOH to pH 7.0. After lyophilization, the solid residue was redissolved in 0.6 mL of 0.5 M NH_4OH and then reduced and acetylated according to a procedure reported by Blakeney et al. (1983). Specifically, the above sample was mixed with 1.0 mL of DMSO, treated with $NaBH_4$ (21 mg, 0.55 mmol), stirred at 40 °C for 90 min, and quenched with 0.1 mL of glacial acetic acid. To this solution was added 1-methylimidazole (0.2 mg, 2.5 μmol) in 6.0 mL of acetic anhydride, and the resulting mixture was stirred at room temperature for 1 h. The reaction was then

quenched with 5.0 mL of methanol at 0 °C, and the peracetylated hexitols were extracted into chloroform. The combined organic extracts were washed with brine, dried over anhydrous sodium sulfate, filtered, and concentrated. The sample was then subjected to GC/MS analysis.

E_1-Catalyzed Deprotonation from C-4′ of the PMP–Substrate Complex. An aliquot (40 μL) of the substrate solution [CDP–D-glucose (0.24 mg, 0.4 μmol), NAD^+ (0.26 mg, 0.4 μmol) in 220 μL of buffer] was added to an assay solution containing the stereospecifically labeled [4′-^{3}H]PMP coenzyme (4.2 nmol) and E_1 (130 μg) in the same potassium phosphate buffer (total volume 200 μL). The reaction was allowed to proceed at 27 °C for 1 h. Activated charcoal (10% solution, 200 μL) was added to the reaction mixture at the end of the incubation. The resulting solution was mixed vigorously on a vortex mixer for 1 min followed by centrifugation to precipitate the charcoal. The supernatant (150 μL) was then removed and analyzed by scintillation counting. A control was run in parallel without substrate.

E_1-Catalyzed Reprotonation at C-4′ of the PMP–$\Delta^{3,4}$-Glucoseen Complex. To a substrate solution containing CDP–D-glucose (0.3 mg, 0.5 μmol) and NAD^+ (0.1 mg, 0.15 μmol) in 50 μL of buffer was added PMP (28 μg, 0.1 μmol), NADH (0.35 mg, 0.5 μmol), enzyme E_1 (475 μg) and 300 μL of [^{3}H]H_2O (58 mCi/mL). The reaction was kept at 27 °C for 8 h and then heated at 100 °C for 2 min to denature the enzyme. The released PMP cofactor was isolated by applying the solution to a Dowex 1X8 (OAc^-) column (1 × 6.5 cm) which had been preequilibrated with 0.1 M ammonium acetate buffer, pH 10.6 (Sevenson et al., 1990). The column was first washed with 40 mL of the same buffer followed by elution of the PMP with 40 mL of 0.1 M ammonium acetate, pH 7.0. Fractions containing the PMP coenzyme were combined and lyophilized to dryness. The radioactive powder was then redissolved in a minimal amount of water and further purified by HPLC using a Partisil SAX 10 analytical anion-exchange column (4.6 × 250 mm). A linear 20-min gradient from 0.03 to 0.12 M potassium phosphate buffer (pH 6.6) was used with a flow rate of 1 mL/min to elute the compound. Under these conditions, the peak containing the majority of radioactivity was eluted at the same time as standard PMP (11.7 min). This fraction was collected and lyophilized.

The lyophilized [^{3}H]PMP powder was redissolved in water and part of the solution (20 μL, 220000 dpm) was treated with bovine intestine alkaline phosphatase (40 μL, 5 units) in 90 μL of 50 mM Tris-HCl buffer (pH 9.0) containing 1.0 mM $MgCl_2$ and 0.1 mM $ZnCl_2$. The resulting mixture was allowed to react at 30 °C for 7 h to hydrolyze the 5′-phosphate group. The pyridoxamine product was purified by applying the incubation solution to a Dowex 1X8 (OAc^-) column (1 × 6.5 cm) which had been preequilibrated with 0.1 M ammonium acetate, pH 10.6. The column was first washed with 40 mL of the same buffer and subsequently by 40 mL of 0.1 M ammonium acetate, pH 8.0. The radioactive material which eluted as a single peak was collected and lyophilized. The recovery yield of pyridoxamine based on its radioactivity was 92%.

Meanwhile, the apoenzyme of porcine heart aspartate aminotransferase (AAT) was prepared by incubating the holoenzyme (12 units in 400 μL of 0.1 M Tris-HCl buffer, pH 8.5) with 4.0 mM cysteine sulfinate at 30 °C for 1 h (Pfister et al., 1985). The treated transaminase was then dialyzed against 0.5 M sodium phosphate buffer, pH 5.1, for 8 h with two buffer changes (each 400 mL) followed by dialyzing back into 0.1 M Tris-HCl buffer, pH 8.5. Part of the apoenzyme (150 μL, 4.6 units) was used to incubate with α-ketoglutarate (0.25 mM) and the aforementioned [^{3}H]pyridoxamine (40 μL, 90000 dpm) in 50 mM Tris-HCl buffer, pH 8.5 (total volume 500 μL). After 12 h at 30 °C, the reaction mixture was boiled for 2 min followed by centrifugation to remove the denatured protein. The precipitate was repetitively washed with a minimal amount of buffer and recentrifuged. The combined supernatant and washings were checked for radioactivity and lyophilized. This procedure was repeated until the radioactivity of the solid residue remained unchanged. In order to calibrate the contribution arising from background radioactivity to the observed reading, activated charcoal (10% solution, 0.5 mL) was added to the sample solution to absorb pyridoxamine and/or unreacted PMP while only impurities would stay in solution. Thus, the final ratios of tritium release have been corrected for by subtracting the background radioactivity which may be ascribed to some radioactive contaminant present in the original pyridoxamine sample. It is noteworthy that the chemically synthesized and highly purified racemic [4′-^{3}H]pyridoxamine was completely absorbed by the charcoal under these conditions and did not show any radioactivity in the supernatant.

E_1–E_3-Catalyzed Reprotonation at C-4′ of the PMP–$\Delta^{3,4}$-Glucoseen Complex. The stereochemical course of the reprotonation in the presence of E_3 was determined by an experiment analogous to the one described above except for the addition of 100 μg of E_3 and NADH (0.35 mg, 0.5 μmol), together with E_1, to the initial incubation mixture.

Reduction of the E_1–$\Delta^{3,4}$-Glucoseen Complex by Hydride Reagents. To an aliquot (40 μL) of the substrate solution [CDP–D-glucose (0.59 mg, 1.0 μmol), NAD^+ (0.26 mg, 0.4 μmol) in 100 μL of buffer] was added PMP (0.1 mg, 0.4 μmol) and E_1 (100 μg). The resulting mixture was kept at 27 °C for 4 h. Sodium borohydride (1 mg, 26 μmol) or sodium cyanoborohydride (1.6 mg, 26 μmol) was then added, and the reduction was allowed to proceed for 30 min at room temperature. The solutions were boiled for 1 min and centrifuged to remove the enzyme precipitate. The supernatants were then subjected to product analysis by the TBA method or the GC/MS procedure as outlined in the preceding paper (Weigel et al., 1992).

Reduction of the E_1–$\Delta^{3,4}$-Glucoseen Complex by Other Reductases. Diaphorase and methane monooxygenase reductase were tested for their competence as E_3 substitutes. Diaphorase (50 μL, 0.8 nmol), purchased from Sigma, was in 0.2 M Tris-HCl buffer (pH 7.5) containing 0.3 M KCl, 0.55 mM FMN, and 4.0 mM BSA. Methane monooxygenase reductase (50 μL, 1.4 nmol), a generous gift from Professor John Lipscomb, was in 50 mM potassium phosphate buffer, pH 7.5. The experiment was started by the incubation of an aliquot (40 μL) of the substrate solution [CDP–D-glucose (0.59 mg, 1.0 μmol), NAD^+ (0.26 mg, 0.4 μmol) in 100 μL of buffer] with a mixture of PMP (0.1 mg, 0.4 μmol), NADH (0.28 mg, 0.4 μmol), E_1 (100 μg), and the appropriate reductase in 230 μL of 10 mM potassium phosphate buffer (pH 7.5). The reaction was kept at 27 °C for 4 h. Sodium borohydride (0.15 mg, 4.0 μmol) was then added and allowed to react for 30 min at room temperature. The solution was boiled, centrifuged, and analyzed for product formation by the TBA method or the GC/MS procedure as outlined in the preceding paper (Weigel et al., 1992). The yield of product was estimated by comparison of the areas underneath the GC peaks for the product derivative abequitol/paratitol tetraacetate with those of the glucitol and fucitol/quinovitol peracetates.

Scheme II

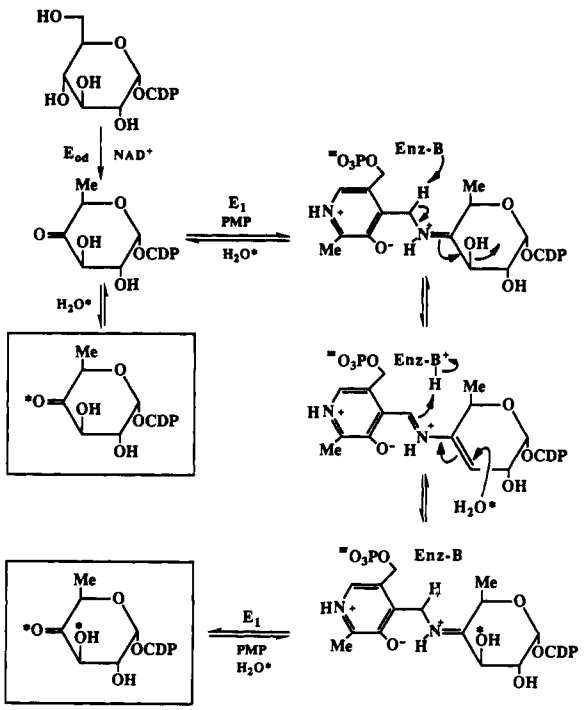


Table I: Pertinent Mass Fragments of the Fucitol/Quinovitol Pentaacetate Sample Derived from Recovered Substrate

	pertinent mass fragments (m/z)				
incubation[a]	C_1–C_4	C_3–C_6	C_1–C_3	C_2–C_6	C_1–C_4
standard	289	231	217	201	187
PMP + substrate	291	233	217	203	189
PMP + substrate + E_1	291, 293	233, 235	217, 219	203, 205	189, 191

[a] The experimental details are described in Experimental Procedures. All incubations were performed in [^{18}O]H_2O.

Results

Reversibility of the E_1-Catalyzed Reaction. To test the likelihood that E_1 catalysis is reversible, incubation of enzyme, cofactor, and substrate in buffers prepared from [^{18}O]H_2O was carried out according to a procedure developed by Rubenstein and Strominger (1974a). If the catalysis proceeds in both directions, an ^{18}O label is predicted to be incorporated into C-3 of the recovered substrate due to the reversal of the dehydration step. An ^{18}O incorporation at C-4 of the recovered substrate is also expected because of the nonspecific hydration of the keto moiety (Scheme II). In order to determine the presence of ^{18}O isotopes, the recovered substrate was treated with $NaBH_4$ to reduce the C-4 keto group and the cytidine diphosphate substituent at C-1 was removed by acid hydrolysis. The resulting alditols were further reduced and acetylated, and the peracetylated sugar alditols were then analyzed by GC/MS to determine the extent of ^{18}O incorporation. As can be seen in Table I, those fragments containing C-4 were always increased by two mass units, while those containing C-3 were increased by two mass units only when E_1 was added. These results unequivocally demonstrated that the E_1-catalyzed reaction is reversible.

Stereochemistry of E_1-Catalyzed Deprotonation of the PMP–Substrate Complex. Since enzyme E_1 catalyzed deoxygenation utilizing PMP as a cofactor is not a typical vitamin B_6 dependent reaction, it remains uncertain whether the stereochemical uniformity found in many enzymes of this class is still maintained by E_1. According to the postulated mechanism for E_1, expulsion of the OH group at C-3 of the substrate is initiated by deprotonation at C-4′ of the cofactor–substrate complex (Scheme I). In order to test whether this deprotonation is stereospecific as it is for other vitamin B_6 dependent enzymes, (4′*R*)-[4′-^{3}H]PMP (89% ee) and (4′*S*)-[4′-^{3}H]PMP (88% ee) (Yang et al., 1991) were separately incubated with E_1 and the CDP-4-keto-6-deoxy-D-glucose substrate generated in situ from CDP-D-glucose by E_{ox}. Since the C-4′ hydrogen of the enzyme-bound PMP may be labile, an identical incubation which left out the substrate was run as the control. As shown in Table II, PMP alone and the PMP–substrate complex are quite stable under the assay conditions. However, incubation in the presence of E_1 with (4′*S*)-[4′-^{3}H]PMP released 8.9 times as much radioactivity as (4′*R*)-[4′-^{3}H]PMP. Hence, the deprotonation is clearly a stereospecific process that preferentially removes the *pro-S* hydrogen from C-4′ of PMP.

Table II: Stereospecificity of E_1-Catalyzed Deprotonation of the PMP–Substrate Complex

	tritium released (dpm)	
incubation[a]	(4′*R*)-[4′-^{3}H]PMP	(4′*S*)-[4′-^{3}H]PMP
PMP	308	633
PMP + substrate	350	685
PMP + E_1	2331	1946
PMP + substrate + E_1	3288[b]	10497

[a] Incubation conditions are described in Experimental Procedures. Approximately equal amounts of radioactivity (28 000 dpm) for both (4′*R*)- and (4′*S*)-[4′-^{3}H]PMP were used in these experiments. [b] The (4′*R*)-[4′-^{3}H]PMP used in these experiments contains 5.5% of the (4′*S*)-isomer. Since the deprotonation is *pro-S* specific, contamination by the latter isomer may contribute to part of the observed activity.

Stereochemistry of E_1-Catalyzed Reprotonation at C-4′ of the PMP Coenzyme. In order to completely define the stereochemical course of the E_1-catalyzed reaction, the stereochemistry of reprotonation at C-4′ of the cofactor should also be established. In view of the ^{18}O incorporation experiment, the E_1-catalyzed dehydration–rehydration is clearly a reversible process. Therefore, if the reaction is run in tritiated buffer, tritium is expected to be incorporated into C-4′ of the PMP–substrate complex during rehydration. The stereospecificity of such a tritium incorporation into PMP can be deduced on the basis of the amount of radioactivity released from the dephosphorylated coenzyme upon incubation with apoaspartate aminotransferase, a process which is known to preferentially remove the *pro-S* hydrogen from C-4′ of pyridoxamine (Dunathan et al., 1968, 1974; Besmer & Arigoni, 1969; Gehring, 1984; Tobler et al., 1986).

As detailed in the Experimental Procedures, enzyme E_1, unlabeled PMP, and the CDP-4-keto-6-deoxy-D-glucose substrate were incubated in buffer prepared with [^{3}H]H_2O. As anticipated, the isolated PMP cofactor was found to be tritium labeled. Since the apoaminotransferase binds pyridoxamine much less tightly than PMP, the purified coenzyme was dephosphorylated prior to stereochemical analysis. This treatment was necessary to facilitate more hydrogen exchange as the pyridoxamine goes in and out of the active site of the apoaminotransferase more readily than that of PMP (Wada & Snell, 1962). Following the incubation of the labeled pyridoxamine with apoaspartate aminotransferase in the presence of α-ketoglutarate, the solution was lyophilized twice and checked for the loss of radioactivity. The control was run in parallel following an identical reaction sequence except for the deletion of the apoaminotransferase treatment step. Nearly quantitative retention of radioactivity in pyridoxamine was found in the control experiment. A second control aimed at checking the activity of the apoaspartate aminotransferase was

Table III: Stereospecificity of E_1-Catalyzed Reprotonation at C-4′ of the PMP–$\Delta^{3,4}$-Glucoseen Complex

incubation[a]	tritium retention[b] in pyridoxamine (%)	tritium released[b] from pyridoxamine (%)
pyridoxamine[c]	95	5
racemic pyridoxamine[d] + AAT	52	48
pyridoxamine[c] + AAT	18	82

[a] The iterative procedures used are described in Experimental Procedures. [b] Ratios are calculated on the basis of a number derived from subtracting the background radioactivities of impurities from the reading of the original pyridoxamine sample. [c] Derived from PMP recovered from the incubation with E_1 along with the subtrate and necessary cofactors in $[^3H]H_2O$. [d] Derived from chemically synthesized racemic [4′-^{3}H]PMP which was dephosphorylated by alkaline phosphatase.

Table IV: Stereospecificity of E_1–E_3-Catalyzed Reprotonation at C-4′ of the PMP–$\Delta^{3,4}$-Glucoseen Complex

incubation[a]	tritium retention[b] in pyridoxamine (%)	tritium released[b] from pyridoxamine (%)
pyridoxamine[c]	94	6
racemic pyridoxamine[d] + AAT	47	53
pyridoxamine[c] + AAT	17	83

[a] Iterative procedures used are described in Experimental Procedures. [b] Ratios are calculated on the basis of a number derived from subtracting the background radioactivities of impurities from the reading of the original pyridoxamine sample. [c] Derived from PMP recovered from the incubation with E_1–E_3 along with the substrate and necessary cofactors in $[^3H]H_2O$. [d] Derived from chemically synthesized racemic [4′-^{3}H]PMP, which was dephosphorylated by alkaline phosphatase.

also performed by incubating the apotransaminase directly with chemically synthesized racemic [4′-^{3}H]PMP. As expected, approximately 50% of the tritium was released, since only half of the sample was *pro-S* labeled. As summarized in Table III, greater than 82% of the tritium label on the dephosphorylated coenzyme was labile during the apotransaminase-catalyzed exchange experiment. These results firmly established that PMP isolated from the enzymatic incubation showed a predominance of tritium incorporation at the *pro-S* position.

Stereochemistry of E_1–E_3-Catalyzed Reprotonation at C-4′ of the PMP–$\Delta^{3,4}$-Glucoseen Complex. It has been well established that the completion of C-3 deoxygenation requires a second enzyme, E_3, whose role is to reduce the nascent PMP–$\Delta^{3,4}$-glucoseen intermediate (**4**) generated in E_1-mediated dehydration. Since E_3 has been characterized as a $2e^-/1e^-$ switch (Han & Liu, 1988; Han et al., 1990), its catalysis is likely to proceed through an electron-transfer process involving formation of a radical intermediate. As the unpaired electron may reside at C-4′ of the putative PMP–glucoseen intermediate, hydrogen incorporation at C-4′ of the PMP cofactor during this reduction may no longer abide by the stereochemical course established for the corresponding reprotonation mediated by E_1 alone. In an attempt to probe this possibility, the procedure used to determine the stereospecificity of E_1-catalyzed PMP reprotonation was repeated in the presence of E_3. The results obtained from these experiments, as shown in Table IV, were similar to those listed in Table III. Namely, the tritium content of the resulting pyridoxamine had decreased to 17% of its initial value after the incubation with apoaspartate aminotransferase. It is apparent that solvent hydrogen incorporation at C-4′ of the regenerated PMP coenzyme consistently occurred at the *pro-S* position whether E_3 was added to the E_1 reaction or not.

Reduction of the E_1 Product by Hydride Reagents. Attempts to chemically reduce the conjugated PMP–$\Delta^{3,4}$-glucoseen product (**4**) were made using sodium borohydride and sodium cyanoborohydride. In each case, the incubation of E_1, PMP, and the CDP-4-keto-6-deoxy-D-glucose substrate was followed by addition of an excess of the reducing agent. The products were then analyzed by both the GC/MS procedure and the TBA method to detect the formation of two-electron reduction products. Unfortunately, both reagents failed to yield detectable 3,6-dideoxysugar products. Since E_1 catalysis undergoes a single turnover in the absence of E_3, it may be difficult with the limited amount of enzyme available to detect product formation whether the reduction had taken place or not.

Table V: Reduction of the E_1 Product by Enzymic Substitutes for E_3

enzyme[a]	% conversion[b]	% efficiency[c]
E_3	45	100
diaphorase[d]	14	31
MMO reductase[e]	3	7

[a] Conditions of incubation and assay are described in Experimental Procedures. [b] Calculated on the basis of the ratio between the integration of abequitol and paratitol tetraacetate peaks versus the integration of all sugar acetate products peaks. [c] Calculated on the basis of the ratio between the conversion using alternate enzyme and the conversion using E_3. [d] The amount of diaphorase used in the incubation was 0.8 molar equiv of that of E_3 used in the parallel experiment. [e] The amount of MMO reductase used in the incubation was 3.75 molar equiv of that of E_3 used in the parallel experiment.

Reduction of the E_1 Product by Enzymatic Substitutes for Enzyme E_3. The potential of using other enzymes to reduce the conjugated PMP–$\Delta^{3,4}$-glucoseen product (**4**) was also explored. Diaphorase and the reductase component of methane monooxygenase were tested for their competence as alternate reductants since both of them are also capable of serving as an NAD(P)H oxidase, as is E_3. Although the results obtained by the TBA assay were ambiguous due to the low yield of the product, analysis by the GC/MS procedure clearly indicated that a small amount of the 3,6-dideoxyhexose product was formed. As shown in Table V, the catalytic competency of diaphorase is only 31% of that of E_3, while that of MMO reductase is less than 10% of E_3 activity. Although the catalytic efficiencies are low, accounting for the low product formation, the successful replacement of E_3 with other electron-shuttle systems does suggest a similar role for E_3 in forming 3,6-dideoxyhexoses. Since E_3 is a more effective catalyst, these results also imply a specific juxtaposition between E_1 and E_3 that facilitates a much more efficient electron transfer from E_3 to E_1.

Discussion

Deoxygenation reactions as catalyzed in biological systems are pivotal transformations and encompass a diverse range of mechanistic themes. Enzyme E_1 represents a unique example which utilizes PMP as the cofactor to catalyze the C–O bond cleavage at C-3, a key step in the biosynthesis of 3,6-dideoxyhexoses. Although enzyme E_1 had already been purified from *Pasturella pseudotuberculosis*[2] by Strominger and coworkers, studies of this enzyme were complicated by the fact that the product of E_1 is a transient species that can be converted to a stable product only after reduction by the subsequent enzyme in the biosynthetic sequence, CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3). Since E_1 and E_3 work closely together, it has been very difficult to separately characterize the catalytic functions of each individual enzyme. The scarcity of the target enzymes in the natural source has also hampered

[2] *Pasturella pseudotuberculosis* is now classified as *Yersinia pseudotuberculosis* (Holt & Krieg, 1984).

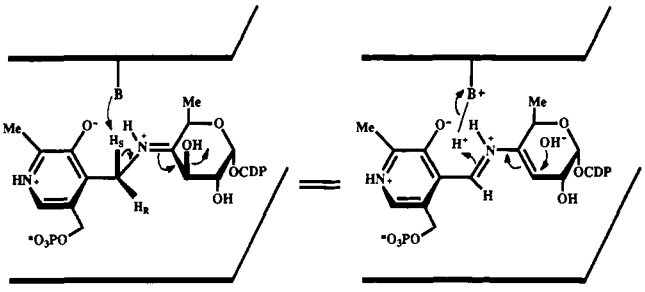
the progress of our early research.

As detailed in the preceding paper (Weigel et al., 1992), enzyme E_1 used in this study was purified from *Y. pseudotuberculosis* and was shown to possess the expected activity. The reversibility of E_1-catalyzed dehydration was revealed by carrying out the enzymatic incubation in buffers prepared from $[^{18}O]H_2O$. The results of GC/MS analysis clearly showed incorporation of ^{18}O into both the C-3 and C-4 positions of the recovered CDP-4-keto-6-deoxy-D-glucose substrate. While the ^{18}O incorporation at C-4 is merely a consequence of the ketone hydration, the ^{18}O incorporation into C-3 unequivocally demonstrates that the E_1-catalyzed reaction is reversible. Similar findings had also been observed for the *P. pseudotuberculosis* enzyme (Rubenstein & Strominger, 1974a).

It is noteworthy that the ^{18}O incorporation experiment not only provided the evidence necessary to reveal the reversibility of E_1 catalysis but also lent credence to the formation of a $\Delta^{3,4}$-glucoseen intermediate (Scheme II). Unfortunately, attempts to trap this E_1 glucoseen intermediate by reduction with either sodium borohydride or cyanoborohydride were futile. It is likely that the nascent E_1 product is labile under these conditions or the proposed intermediate is not accessible to the hydride reagents. The cofactor–glucoseen complex may also undergo a four-electron reduction; however, the fully reduced adduct is no longer a simple sugar molecule and would not be detected by the GC/MS method for analyzing dideoxyhexose formation. Although this fully reduced product may be converted to pyridoxamine by oxidative degradation (Vederas et al., 1979), a single turnover is expected for the E_1 incubation making product detection difficult with a limited supply of enzyme. It must be noted that Rubenstein and Strominger (1974a) used a similar approach to study the nature of PMP binding to E_1 and to probe a covalent attachment between E_3 and intermediate **4** as an essential event of E_3 catalysis. Coincident with our results, reduction with sodium borohydride in both cases failed to yield any covalent adduct.

Since E_1 utilizes the PMP cofactor in a unique manner, it is essential to establish its role in E_1 catalysis. Furthermore, as the specificity imposed by a vitamin B_6 phosphate dependent enzyme ensures that the orientation of the bond to be cleaved is perpendicular to the cofactor–substrate complex (Dunathan, 1966), the fact that the PMP coenzyme is only loosely bound to E_1 (Weigel et al., 1992) has also raised concerns over this enzyme's ability to control its catalysis. Our approach relied on a stereochemical comparison of this unusual deoxygenation with reactions catalyzed by other vitamin B_6 phosphate dependent enzymes. In view of E_1 catalysis being initiated by a C-4′ deprotonation of the PMP–substrate complex, the stereochemical course of this step was examined first, using chemically synthesized (4′*S*)- and (4′*R*)-[4′-^{3}H]PMP as the probes. The data shown in Table II clearly indicated that the stereochemistry of this deprotonation is *pro-S* specific (Shih et al., 1990). This result is in agreement with the stereochemical consistency found with other vitamin B_6 phosphate dependent enzymes, among which only two exceptions are known. The first example is L-alanine aminotransferase (Besmer & Arigoni, 1969) which removes the *pro-R* hydrogen of glycine, an alternative substrate whose *pro-R* hydrogen resides in the same locus as the *pro-S* hydrogen of the natural substrate alanine. The second case is ω-amino acid: pyruvate aminotransferase which abstracts the *pro-R* hydrogen from C-4 of γ-aminobutyrate (GABA) (Burnett et al., 1979). In contrast to the pyruvate-requiring GABA transaminase, all other GABA transaminases are α-oxoglutarate-dependent and show a normal *pro-S* stereospecificity (Palcic & Floss, 1986). Such a distinction in the stereochemical preference observed in the latter case may reflect alternate binding modes of GABA in the active site of these proteins.

Scheme III

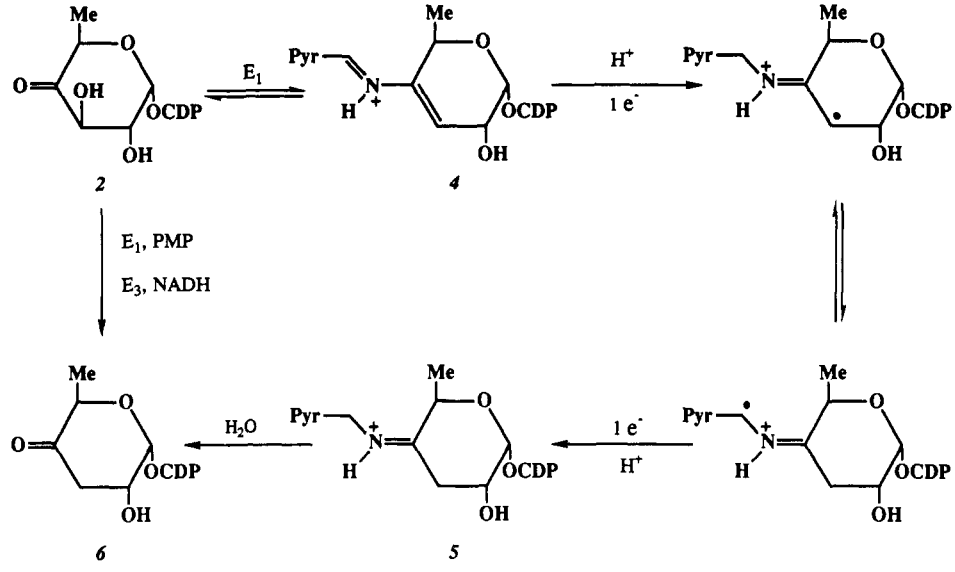


Despite the existence of the aforementioned exceptions, the reactions catalyzed by vitamin B_6 phosphate dependent enzymes have been shown to take place only on a single side, the *si* face, of the cofactor–substrate complex. The fact that reprotonation at C-4′ of the PMP–glucoseen complex in the reverse direction of E_1 catalysis was also found to be *pro-S* stereospecific (Table III) clearly indicated that enzyme E_1, like most of its counterparts, has the *si* face of its cofactor–substrate complex exposed to solvent and accessible to active site catalytic groups. Since a single base located on one face of the π-complex catalyzing reactions in a suprafacial mode can benefit catalysis by limiting the number of potentially rate-limiting diffusion-controlled steps (Palcic & Floss, 1986), the deprotonation and reprotonation of E_1 catalysis may also be mediated by a single active site nucleophilic group. These stereochemical studies have given support to the role postulated for the PMP cofactor in the proposed mechanism, and they also suggest that the active site of E_1 may share features similar to those of other PLP/PMP-linked enzymes which control the orientation of the cofactor–substrate complex. Despite E_1 having evolved an unusual role for the PMP cofactor in the dehydration of CDP-4-keto-6-deoxy-D-glucose, it has managed to retain all the essential elements of catalysis common to other vitamin B_6 phosphate dependent enzymes (Scheme III).

There is no doubt that enzyme E_1 plays a key role in the biosynthesis of 3,6-dideoxyhexoses by catalyzing the reversible C–O bond cleavage at C-3 leading to the formation of a transient glucoseen intermediate; however, E_1 cannot finish C-3 deoxygenation without E_3, which reduces the nascent E_1 product, driving the equilibrium to completion. It was previously suggested that the E_3 reduction may be a direct hydride-transfer process (Rubenstein & Strominger, 1974b; Gonzalez-Porque, 1986), but recent studies carried out in our laboratory have shown that E_3 displays a unique $2e^-/1e^-$ switching capability, and thus this reduction may proceed with a radical mechanism (Han & Liu, 1988; Han et al., 1990). The *pro-S* stereospecificity found for the hydrogen incorporation at C-4′ of PMP in the combined E_1–E_3 reaction does not contravene a stepwise $1e^-/1e^-$ reduction mechanism, since C-4′ reprotonation is expected to occur on a two-electron reduced carbanion species at the end of the E_3 reduction and the constraints imposed by the enzyme(s) may allow the reprotonation to transpire only on the *si* face of the cofactor–glucoseen complex. An investigation of the stereospecificity of hydrogen incorporation at C-3 of the reduced 3,6-dideoxysugar product (**6**) resulting from an enzymatic reaction with both E_1 and E_3 will complement this experiment.

As mentioned earlier, chemical reducing reagents such as $NaBH_4$ and $NaBH_3CN$ were unable to trap the E_1 product. Although a number of possible reasons have been offered, these

Scheme IV



results may simply suggest that these hydride reducing agents cannot substitute for the radical reduction catalyzed by E_3. In an attempt to show that the E_1 product is reduced by a stepwise $1e^-/1e^-$ transfer mechanism, two well-known electron-transfer proteins, diaphorase and the reductase component of methane monooxygenase (MMO), were tested for their ability to serve as E_3 substitutes. While the diaphorase requires FAD for its activity, the MMO reductase is a [2Fe-2S] containing flavoprotein (Fox et al., 1989). Since the assay requires incubation with several enzymes and cofactors, data from each experiment must be carefully correlated with that of an incubation performed in parallel with E_3 under identical conditions. As shown in Table V, based on the GC/MS analysis, both diaphorase and MMO reductase were able to generate a small amount of the dideoxyhexose product, albeit with low catalytic efficiency. These preliminary results clearly indicated that E_3 is a preferred reductase in the biosynthesis of ascarylose that leads to a more efficient flow to complete C-3 deoxygenation. Furthermore, the low yield of product formation in the presence of excess MMO reductase suggested that this enzyme is less capable than diaphorase of passing electron from NADH to reduce the E_1 product. Since momentous thermodynamic changes may occur due to protein–protein interactions as well as binding of substrate (Eich et al., 1985; Lenn et al., 1990), the significance of the distinction of the reducing capability between E_3 and its enzymatic alternates can be comprehended only after a thorough scrunity of the interactions between proteins and substrates in this system and a full characterization of E_3's catalytic center. Although in most biochemical pathways, especially for multicomponent systems, the reductase enzymes are generally incompetent beyond their native systems (Subramanian et al., 1985; Haigler & Gibson, 1990), the fact that other well-known electron-transfer reductases can act as substitutes for E_3 provided compelling evidence supporting our earlier notion that E_3 serves as an electron shuttle mediating electron transfer from NADH to the E_1 product (Scheme IV).

Although E_1–E_3 catalysis appears to be unique with a PMP–glucoseen radical intermediate in the active site of E_1 proposed during the reduction by E_3, there is another vitamin B_6 phosphate dependent enzyme which has also been postulated to proceed via a radical mechanism and the intermediate is likely a pyridoxyl radical (Moss & Frey, 1987). This enzyme is lysine 2,3-aminomutase which catalyzes the conversion of lysine to β-lysine. However, in addition to PLP, this mutase contains iron and sulfide in equimolar amounts, as well as cobalt, zinc, and copper (Song & Frey, 1991; Petrovich et al., 1991). Since enzyme activation in this case requires incubation with excess iron, reducing agents, and *S*-adenosylmethionine under anaerobic conditions, the mechanism of this rearrangement, unlike E_1 catalysis, more closely resembles a vitamin B_{12} dependent reaction. The radical nature of the E_1- and E_3-catalyzed C-3 deoxygenation is also reminiscent of the well-known sugar deoxygenation catalyzed by ribonucleotide reductase (Stubbe, 1989, 1990). In view of the involvement of a tyrosine radical in the reaction of the latter enzyme, the mechanisms of these two sugar deoxygenations are fundamentally distinct.

It is clear that E_1-catalyzed dehydration represents a unique offshoot of PMP-dependent catalysis; however, the stereochemical consistency of E_1 and all other PLP/PMP enzymes suggests that it behaves as a normal vitamin B_6 dependent catalyst. The fact that bond cleavage and bond formation catalyzed by E_1 proceed with uniform stereochemistry, i.e., the *si* face always being utilized, demonstrates that C-3 deoxygenation follows well-established PLP/PMP cofactor chemistry. These results also support Dunathan's hypothesis that this class of enzymes, regardless of its catalytic diversity, evolved from a common progenitor (Dunathan, 1971).

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