Mechanistic Studies of the Biosynthesis of 3,6-Dideoxyhexoses in *Yersinia pseudotuberculosis*: Purification and Characterization of CDP–4-Keto-6-deoxy-D-glucose-3-dehydrase†

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ABSTRACT: CDP–4-keto-6-deoxy-D-glucose-3-dehydrase (E_1) is a PMP-dependent enzyme which plays an essential role in C–O bond cleavage leading to the formation of 3,6-dideoxyhexoses. Although E_1 catalysis has long been recognized as a unique biological deoxygenation reaction, the catalytic mechanism of this unusual enzyme has never been fully elucidated. The lack of methods that would allow this enzyme's activity to be monitored directly has been an impediment to E_1 purification and has consequently hampered the mechanistic studies. In order to circumvent this problem, we have developed a few convenient and sensitive methods to facilitate the E_1 assay. The first method relies on the fact that E_1-catalyzed dehydration is initiated by a proton abstraction from C-4′ of the PMP–substrate adduct. By using a tritium-labeled cofactor in the incubation that was later quenched with charcoal, the amount of E_1 present could be determined from the amount of released tritium in the supernatant. The second method was designed on the basis of the expectation that E_1 will bind and rupture the C–F bond of a substrate analogue, CDP–4-keto-3,6-dideoxy-3-fluoro-D-glucose, which was derived from CDP–3-deoxy-3-fluoro-D-glucose. Since the bond length and electronegativity of the C–F group are similar to those of a C–OH group, we anticipated that the proposed compound would be processed by E_1, an assumption which was later substantiated. Another assay useful for measuring E_1 activity couples the E_1 transformation with the subsequent reduction step catalyzed by CDP–6-deoxy-$\Delta^{3,4}$-D-glucoseen reductase (E_3) to a thiobarbituric acid (TBA) reaction. Since the condensation product of TBA and malonaldehyde derived from oxidative degradation of the E_1/E_3 product gave a pink chromophore at 532 nm with a known absorption coefficient, the yield of deoxysugar formation could be directly deduced on the basis of the observed absorbance. The most conclusive evidence confirming the role of E_1 was attained by a GC/MS assay which permits an unambiguous identification of the deoxysugar product generated from the E_1 and E_3 reactions. With these convenient and sensitive assays in hand, we have established a sequence of four columns that was effective in consistently producing pure E_1 from *Yersinia pseudotuberculosis*. The overall purification may be as high as 26 000-fold. This purified enzyme consists of a single polypeptide chain in its native form, and the estimated molecular weight is 49 000. Since PMP was easily removed from E_1 by ion-exchange chromatography, the electronic absorption of this highly purified enzyme at neutral pH was primarily the spectrum of a single polypeptide. As expected, purified E_1 was only weakly active unless incubated with PMP in situ or reconstituted with PMP prior to activity assay. These results unequivocally indicated that E_1 is PMP dependent. The stoichiometry of the cofactor to E_1 was determined to be 1.

Deoxysugars are an important class of carbohydrates which have been found ubiquitously in nature (Hanessian, 1966; Butterworth & Hanessian, 1971; Williams & Wander, 1980). These naturally occurring compounds exist as mono-, di-, and even trideoxy species and often possess interesting biological activities. The 3,6-dideoxyhexoses, in particular, have been found in the lipopolysaccharide components of a number of Gram-negative cell envelopes where they have been identified as essential antigenic determinants and contribute to the serological specificity of many immunologically active polysaccharides (Westphal & Lüderitz, 1960; Lüderitz et al., 1966; Bishop & Jennings, 1982). There are five known 3,6-dideoxyhexoses that have been isolated from various Gram-negative species, and the biosynthesis of four of them, paratose, abequose, tyvelose, and ascarylose, has been shown to occur through a complex enzymatic sequence beginning with CDP-D-glucose (Matsuhashi et al., 1966a). The fifth compound, colitose, is derived from GDP-D-mannose via a presumably similar pathway (Health & Elbein, 1962). While the precursors of these unusual sugars may be different, the existence of a common intermediate, a 4-keto-6-deoxy-D-hexose derivative, along the reaction coordinate has been suggested (Matsuhashi et al., 1966b; Glaser & Zarkowsky, 1971; Gabriel, 1973; Grisebach, 1978). The C-3 hydroxyl group of this intermediate is replaced by a hydrogen atom in a deoxygenation step that leads to the formation of these dideoxysugars (Gonzalez-Porque, 1986). Although nature has developed a number of ways to cleave carbon–oxygen bonds encompassing a wide variety of substrates and products, the rupture of the C–O bond at C-3 during the formation of 3,6-dideoxyhexoses appears to proceed via a unique route.

As shown in Scheme I, studies of the biosynthesis of CDP–ascarylose (CDP–3,6-dideoxy-L-*arabino*-hexopyranose) (**1**) have revealed that the unique carbon–oxygen bond cleavage at C-3 is catalyzed by a pyridoxamine 5′-phosphate

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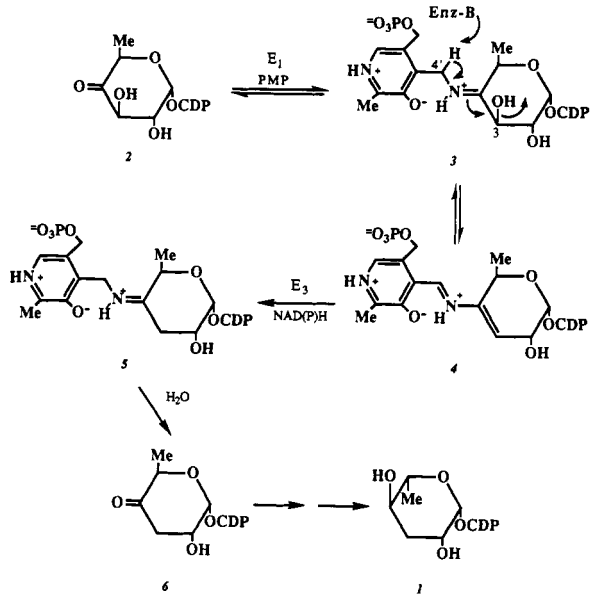
Scheme I



(PMP) dependent enzyme, CDP-4-keto-6-deoxy-D-glucose-3-dehydrase (E_1)[1] (Gonzalez-Porque & Strominger, 1972a,b). This reaction is believed to be initiated by Schiff base formation between the C-4 keto group of the substrate, CDP-4-keto-6-deoxy-D-glucose (**2**), and the PMP cofactor (Rubenstein & Strominger, 1974a). This is followed by the abstraction of a C-4′ hydrogen from the PMP–substrate complex (**3**) leading to a transient carbanion which triggers the expulsion of the C-3 hydroxyl group to produce a $\Delta^{3,4}$-glucoseen product (**4**). While this $\Delta^{3,4}$-glucoseen intermediate (**4**) has never been isolated or characterized, subsequent reduction by an NAD(P)H-dependent enzyme, CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3) (Gonzalez-Porque & Strominger, 1972a), gives rise to the actual deoxygenation product, CDP-4-keto-3,6-dideoxy-D-glucose (**6**) that had been isolated and identified (Rubenstein & Strominger, 1974b). Although the vitamin B_6 phosphate dependent enzymes are extremely versatile, their catalytic functions are predominantly confined to the metabolism of amino acids and amines (Adams, 1976; Evangelopoulos, 1984; Dolphin et al., 1986), with a process involving glycogen phosphorylation as the only exception (Madsen & Withers, 1986; Hajdu et al., 1987; Palm et al., 1990). Even though its aldehyde form, pyridoxal 5′-phosphate (PLP), has been shown to play an essential role in the dehydration mediated by serine dehydrase, which catalyzes the conversion of serine to pyruvate (Cheung & Walsh, 1976), there is no precedent that relies on PMP as the cofactor to carry out a deoxygenation in a biological system. Thus, the C–O bond cleavage found in the biosynthesis of 3,6-dideoxyhexoses is unique. In fact, this system represents the only PMP-dependent reaction that is not a transamination process. While this enzyme had been purified earlier from *Pasturella pseudotuberculosis*[2] by Strominger and his coworkers (Gonzalez-Porque & Strominger, 1972a), its mechanism has never been fully elucidated. In an effort to explore the mechanism by which the sugar deoxygenation reaction is effected, we have purified an enzyme essentially identical to Strominger's E_1 from *Yersinia pseudotuberculosis*, which is also known to have ascarylose as the terminal sugar unit in its lipopolysaccharide (Gorshkova et al., 1983). In this paper, we report the purification and characterization of this dehydrase, while in the following paper we detail the mechanistic studies of its catalyzed deoxygenation.

Experimental Procedures

General. ^1H and ^{13}C NMR spectra were recorded on an IBM NR/200 or NR/300 spectrometer. Chemical shifts are reported in parts per million on the δ scale relative to internal standard (tetramethylsilane, sodium 2,2-dimethyl-2-silapentane-5-sulfonate, or appropriate solvent peaks) with coupling constants given in hertz. NMR assignments labeled with an asterisk may be interchangeable. Flash chromatography was performed in columns of various diameters with J. T. Baker (230–400 mesh) silica gel by elution with the solvents reported. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60 G-254 plates (25 mm) and developed with the solvents mentioned. TLC spots were visualized either with UV light or by dipping into the staining solutions of vanillin/ethanol/H_2SO_4 (1:98:1) or phosphomolybdic acid (7% EtOH solution) and then heating.

Materials. Matrex Blue A dye-ligand was purchased from Amicon Corporation (Lexington, MA). DE-52 was from Whatman (Clifton, NJ) and Sephadex G-100 was from Pharmacia LKB (Piscataway, NJ). DEAE-Sephacel, enzymes used in the assays, molecular weight standards, and most other biochemicals were purchased from Sigma (St. Louis, MO). [^{3}H]$NaBH_4$ 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. 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 pyrophosphorylase (E_p) and CDP-D-glucose oxidoreductase (E_{od}) were isolated from the same *Y. pseudotuberculosis* strain by members of this laboratory (Liu et al., unpublished results).

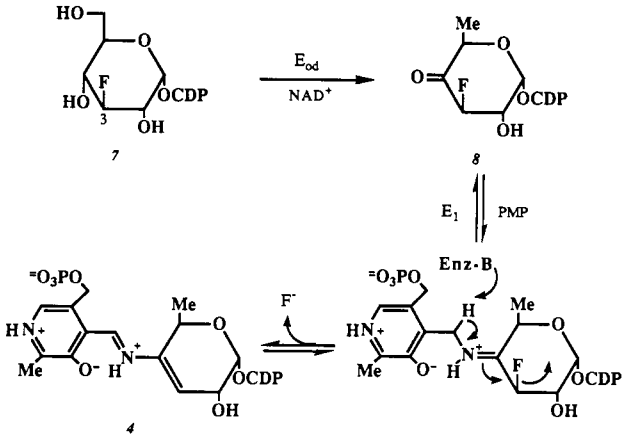
Enzyme E_1 Assays. Four different methods were developed to determine the activity of enzyme E_1. It is worth mentioning that the substrate of E_1 is not readily available; it has to be prepared in situ from CDP-glucose prior to each assay. A general procedure for the preparation of E_1 substrate involves 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 to determine the dehydrase activity.

Method 1: Tritium Release Assay. 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 mixture containing the labeled PMP coenzyme (0.04 μg, 0.15 nmol) and E_1 (10–50 μg) in the same potassium phosphate buffer (final 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 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 (50 μL) was then removed and analyzed by scintillation counting. The readings were calibrated against controls prepared in parallel without CDP-D-glucose.

[1] Abbreviations: E_1, CDP-4-keto-6-deoxy-D-glucose-3-dehydrase; E_3, CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase; E_p, CDP-D-glucose pyrophosphorylase; E_{od}, CDP-D-glucose oxidoreductase; DEAE, (diethylamino)ethyl; DAST, (diethylamino)sulfur trifluoride; TEA, triethylamine; TBA, 2-thiobarbituric acid; DCPIP, dichlorophenolindophenol.

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

Scheme II



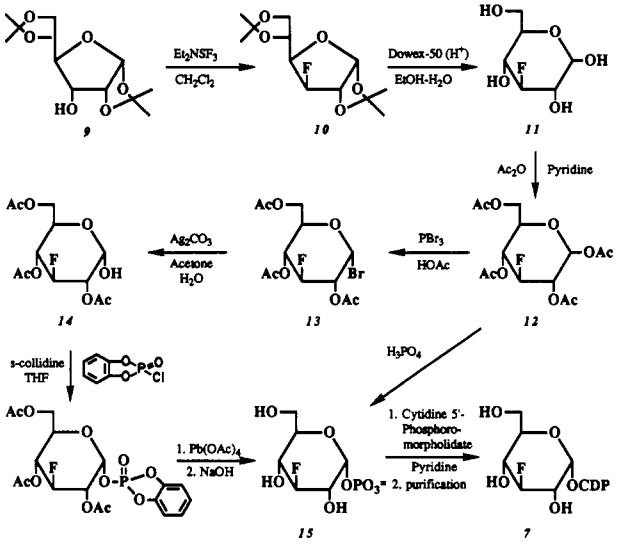
The labeled PMP was synthesized according to a published procedure (Voet et al., 1973; Rubenstein & Strominger, 1974a) with minor modifications. Specifically, PLP (79.6 mg, 0.3 mmol) was dissolved in 3.5 mL of 11 M ammonium hydroxide. To this solution was added [^{3}H]$NaBH_4$ (2.0 mg, 53 μmol, 0.27 Ci/mmol). The reaction was kept in the dark at room temperature for 2 h. Glucose (36 mg, 0.2 mmol) was added to quench any remaining borohydride, and the reaction was allowed to proceed for another 2 h. The solution was then brought to pH 2.0 with concentrated HCl and lyophilized. The remaining powder was redissolved in water, passed through a Dowex 1X4 (OAc^- form, pH 4.5) column, and neutralized with concentrated KOH. The product was purified by HPLC using a Partisil SAX 10 anion-exchange column (4.6 × 250 mm) with a 20-min linear gradient from 30 to 120 mM potassium phosphate buffer at pH 6.6 and a flow rate of 1 mL/min. The retention time for standard PMP was 10.4 min under these conditions.

Method 2: Fluoride Release Assay. As shown in Scheme II, an aliquot (40 μL) of the substrate solution prepared from CDP-3-deoxy-3-fluoro-D-glucose (**7**; 0.24 mg, 0.4 μmol) and NAD^+ (0.26 mg, 0.4 μmol) in potassium phosphate buffer (220 μL) was added to an assay mixture containing PMP (0.04 μg, 0.15 nmol) and E_1 (10–50 μg) in the same buffer (final 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 mixture at the end of the incubation. The resulting solution was mixed vigorously on a vortex mixture for 1 min followed by centrifugation to precipitate the charcoal. The supernatant (50 μL) was then removed and analyzed by a fluoride electrode. A control was also prepared with boiled enzymes. The reading was fitted into a standard curve constructed on the basis of measurements with sodium fluoride of known concentrations.

Preparation of the requisite substrate, CDP-3-deoxy-3-fluoro-D-glucose (**7**), was effected by the sequence depicted in Scheme III. The precursor, 1,2:5,6-di-*O*-isopropylidene-D-allofuranose (**9**), was prepared from glucose by a well-known procedure (Stevens, 1972).

1,2:5,6-Di-O-isopropylidene-3-deoxy-3-fluoro-D-glucofuranose (**10**). 1,2:5,6-Di-*O*-isopropylidene-D-allofuranose (**9**; 5 g, 19.2 mmol) in methylene chloride (60 mL) at −78 °C was treated with (diethylamino)sulfur trifluoride (DAST, 20 mL, 15.2 mmol in 75 mL of methylene chloride). The cooling bath was removed, and the mixture was allowed to stir overnight at room temperature. The addition of DAST followed by stirring was repeated twice to ensure the completion of the substitution. The mixture was then cooled to −78 °C and quenched by the addition of methanol (60 mL) over 3 h via a syringe pump. The resulting mixture was concentrated under reduced pressure. Flash chromatography of the residue on silica gel (10% ethyl acetate in hexane) afforded **10** in 48% yield. ^{1}H NMR ($CDCl_3$): δ 5.93 (1 H; d, J = 3.7 Hz; 1-H), 4.99 (1 H; dd, J = 49.8, 2.3 Hz; 3-H), 4.68 (1 H; dd, J = 10.6, 3.7 Hz; 2-H), 4.27 (1 H; m; 5-H), 4.12 (1 H; dd, J = 8.7, 6.0 Hz; 6-H), 4.03 (1 H; dd, J = 8.7, 4.7 Hz; 6-H), 4.32–3.92 (1 H; m; 4-H), 1.48, 1.43, 1.35, 1.31 (3 H each; s; isopropylidene Me's). High-resolution FAB-MS Anal. Calcd for $C_{12}H_{20}FO_5$ $(M + 1)^+$: 263.1295. Found: 263.1288.

Scheme III



3-Deoxy-3-fluoro-1,2,4,6-tetra-O-acetyl-D-glucose (**12**). Compound **10** (2 g, 7.2 mmol) was dissolved in an ethanolic solution ($EtOH/H_2O$ 2:9, 84 mL) and mixed with a catalytic amount of Dowex 50 (H^+). The reaction was kept at 55 °C overnight. After filtration, the resins were washed with water several times. The combined filtrate and washings were concentrated by a rotatory evaporator to remove ethanol and then lyophilized. The product, 3-deoxy-3-fluoro-D-glucose (**11**) was isolated in quantitative yield as a mixture of α and β isomers. ^{1}H NMR (D_2O, mixture of two isomers): δ 5.29 (1 H; t, J = 3.7 Hz; 1-H of the α isomer), 4.74 (1 H; d, J = 8.2 Hz; 1-H of the β isomer), 4.45 (2 H; dt, J = 53.0, 8.8 Hz; 3-H's of both isomers), 3.90–3.53 (10 H; m; 2-H, 4-H, 5-H, 6-H's of both isomers). ^{13}C NMR (D_2O, mixture of two isomers): δ 99.3 (d, J = 194 Hz; C-3), 98.1 (d, J = 13 Hz; C-1), 97.9 (d, J = 190 Hz; C-3), 95.1 (d, J = 11 Hz; C-1), 77.4 (d, J = 9 Hz), 75.5 (d, J = 17 Hz), 73.6 (d, J = 8 Hz), 72.8 (d, J = 17 Hz), 70.7 (d, J = 17 Hz), 63.1 (d, J = 14 Hz), 60.3 (C-6). The resulting 3-deoxy-3-fluoro-D-glucose (**11**; 94.6 mg, 0.52 mmol) was dissolved in 4.0 mL of pyridine. To this solution was added acetic anhydride (10.6 mmol), and the reaction was stirred at room temperature overnight. Methanol (1.0 mL) was added to quench the reaction, and the excess pyridine was removed by repeated coevaporation with benzene. The product was a mixture of the α and β isomers (1.5:1) which was used directly in the next step without purification. The combined yield was quantitative. ^{1}H NMR ($CDCl_3$): δ 6.31 (0.6 H; t, J = 3.7; 1-H of the α isomer), 5.62 (0.4 H; d, J = 8.4 Hz; 1-H of the β isomer), 5.31–5.14 (1.6 H; m; 2-H of both isomers and 4-H of the α isomer), 4.79 (0.6 H; dt, J = 53.4, 9.4 Hz; 3-H of the α isomer), 4.58 (0.4 H; dt, J = 51.9, 9.1 Hz; 3-H of the β isomer), 4.27–4.19 (2.4 H; m; 6-H's of both isomers and 4-H of the β isomer), 4.10–4.01 (0.6 H; m; 5-H of the α isomer), 3.71 (0.4 H; m; 5-H of the β isomer), 2.09, 2.08, 2.07, 2.06 (1.8 H each; s; acetyl CH_3's), 2.13, 2.09,

2.06, 2.05 (1.2 H each; s; acetyl CH_3's). ^{13}C NMR ($CDCl_3$): δ 170.6, 169.6, 169.1, 169.0, 168.5 (C=O's), 91.6 (d, J = 192 Hz; C-3 of the β isomer), 91.1 (d, J = 11 Hz; C-1 of the β isomer), 89.2 (d, J = 9 Hz; C-1 of the α isomer), 89.0 (d, J = 190 Hz; C-3 of the α isomer), 71.8 (d, J = 8 Hz, C-5)*, 70.2 (d, J = 19 Hz; C-2)*, 69.6 (d, J = 10 Hz; C-5)*, 69.5 (d, J = 15 Hz; C-2)*, 67.7 (d, J = 19; C-4)*, 61.3 (C-6), 20.7, 20.6, 20.5, 20.4 (acetyl CH_3's). High-resolution FAB-MS Anal. Calcd for $C_{14}H_{20}FO_9$ $(M + 1)^+$: 351.1091. Found: 351.1105.

*3-Deoxy-3-fluoro-2,4,6-tri-O-acetyl-α-*D*-glucosyl Bromide* (**13**). The product from the above acetylation reaction (**12**; 57.8 mg, 0.165 mmol) was dissolved in 5.0 mL of acetic acid. Phosphorus tribromide (0.45 g, 1.65 mmol) was added, and the reaction was stirred at room temperature overnight. Methylene chloride was then added, and the mixture was washed twice with ice water, once with sodium bicarbonate, and again with water in sequence. The organic layer was collected, dried over anhydrous sodium sulfate, filtered, and concentrated. Only the α isomer was obtained with a yield of 82%. ^1H NMR ($CDCl_3$): δ 6.56 (1 H; t, J = 3.7 Hz; 1-H), 5.36–5.20 (1 H; m; 2-H), 4.87 (1 H; dt, J = 9.4, 4.0 Hz; 4-H), 4.86 (1 H; dt, J = 54.3, 9.4 Hz; 3-H), 4.31–4.05 (3 H; m; 5-H, 6-H's), 2.12, 2.09, 2.06 (3 H each; s; acetyl CH_3's). ^{13}C NMR ($CDCl_3$): δ 170.6, 169.7, 169.1 (C=O's), 89.4 (d, J = 190 Hz; C-3), 86.2 (d, J = 9 Hz; C-1), 69.7 (d, J = 8 Hz; C-5)*, 69.6 (d, J = 17 Hz; C-2)*, 66.9 (d, J = 19 Hz; C-4)*, 60.8 (C-6), 20.8, 20.6, 20.5 (acetyl CH_3's).

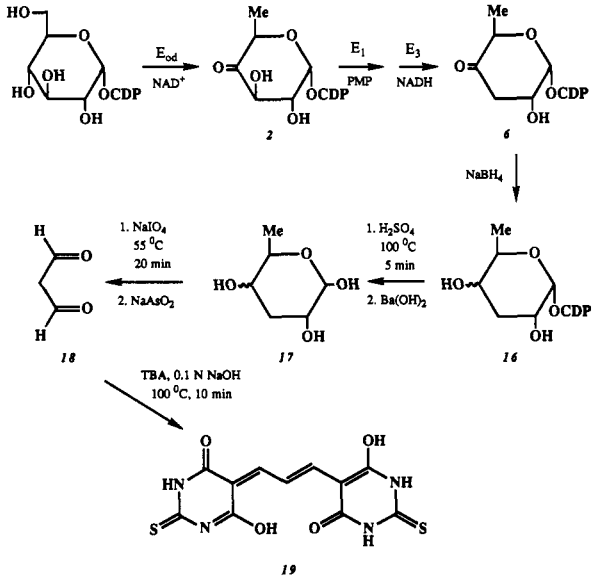
*3-Deoxy-3-fluoro-2,4,6-tri-O-acetyl-*D*-glucose* (**14**). The glucosyl bromide **13** (59.4 mg, 0.16 mmol) was dissolved in 2.0 mL of acetone and a small amount of water (50 μL). After the solution was cooled to 0 °C, freshly prepared silver carbonate (31.9 mg, 0.19 mmol) was added and the reaction was slowly warmed to room temperature. The reaction was run for 2 h, and the precipitate was then filtered and washed with acetone. The filtrate and washings were combined and concentrated. The α anomer was the major product obtained with a yield of 61%. ^1H NMR ($CDCl_3$): δ 5.45 (1 H; br s; 1-H), 5.10 (2 H; m; 2-H, 4-H), 4.64 (1 H; m; 3-H), 4.16 (3 H; m; 5-H, 6-H's), 2.13, 2.10, 2.08 (3 H each; s; acetyl CH_3's). ^{13}C NMR ($CDCl_3$): δ 171.0, 170.3, 169.5 (C=O's), 90.4 (C-1), 89.3 (d, J = 190 Hz; C-3); 71.6–66.9 (C-2, C-4, C-5), 61.8 (C-6), 20.8 (acetyl CH_3's). High-resolution FAB-MS Anal. Calcd for $C_{12}H_{18}FO_8$ $(M + 1)^+$: 309.0986. Found: 309.0976.

*3-Deoxy-3-fluoro-*D*-glucose 1-Phosphate* (**15**). The protected glucose **14** (37.0 mg, 0.12 mmol) in dry tetrahydrofuran (1.0 mL) was added at 0 °C to a THF solution (1.0 mL) of *o*-phenylene phosphorochloridate (68.6 mg, 0.36 mmol) and *s*-collidine (58 μL, 0.44 mmol). The reaction was stirred at 0 °C for 30 min, and the precipitate was then removed. Water (15 μL) and more *s*-collidine (58 μL) were added to the filtrate, and the reaction was allowed to continue. After 30 min at room temperature, the solution was cooled to 0 °C again and refiltered. The THF solvent was removed, and the product was dried thoroughly. The residue was then dissolved in a minimal amount of dioxane and cooled to 12 °C. Lead tetraacetate (97.5 mg, 0.22 mmol) was added, and the reaction was stirred vigorously for 30 min. The dark brown solution was added slowly to 1.0 mL of cold 1 N NaOH and then warmed to room temperature for 2 h. After being cooled back to 0 °C, the mixture was brought to pH 4.0 with Dowex 50 (H^+). Following filtration on a sintered glass funnel, the filtrate was adjusted to pH 7.5. The product was purified on a Dowex 1 (formate form) resin using 0.05 M triethylamine (TEA) buffer, pH 7.5, to wash the column (2 × 12 cm) and a linear gradient from 0.05 to 0.3 M TEA, pH 7.5, to elute the product. The product consisted of a mixture of α and β isomers.

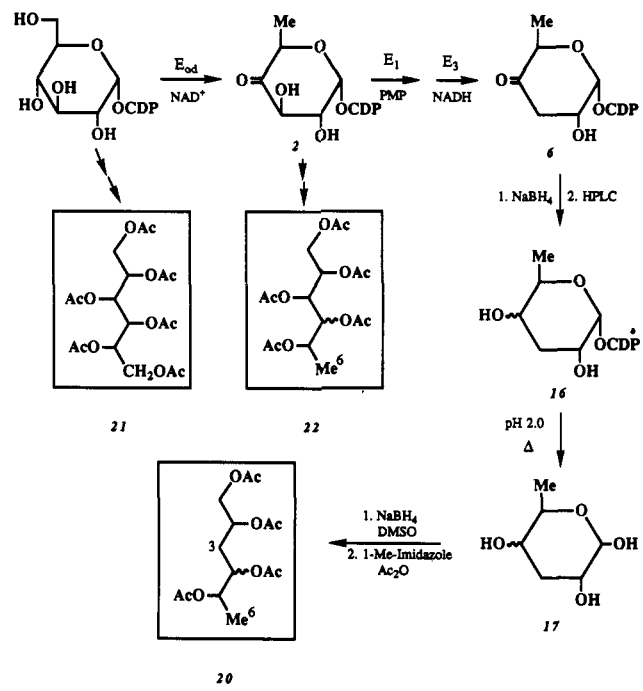
Direct phosphorylation of **12** with 100% phosphoric acid was a facile alternative. Crystalline phosphoric acid (2.8 g) was dried in a round-bottom flask over P_2O_5 at room temperature in vacuo overnight. It was then melted at 50 °C and mixed thoroughly with compound **12** (2 g, 5.71 mmol) by stirring the resulting syrup with a glass rod. The flask containing the mixture was placed in a water bath at 50 °C and evacuated with a vacuum pump. Vigorous gas evolution was noted, and the solid acetate (**12**) was gradually dissolved into solution. After the gas evolution subsided and the solid disappeared (ca. 2 h), the syrup was diluted in 12 mL of dried THF with gentle warming and the resulting solution was poured into 200 mL of ice-cooled 2 N LiOH solution. This mixture was left at room temperature overnight to saponify the acetyl groups. After filtration to remove the lithium phosphate precipitate, the filtrate was passed through a Dowex 50 (H^+) column which was washed with water until the eluate was no longer acidic. The acidic fractions were combined, neutralized with freshly distilled cyclohexylamine, and lyophilized. Precipitation of the desired product was induced by addition of absolute alcohol followed by evaporation in vacuo. When this process was repeated twice, a white solid was collected. This sample was treated with 2-propanol and centrifuged to remove the coproduct, cyclohexylammonium acetate. The supernatant was discarded, and the solid was washed twice in the same manner. The product was filtered, washed with ether, and dried in vacuo over P_2O_5 to yield nearly 200 mg of the product. Since the product might still contain a significant amount of salt, the yield was not calculated. NMR analysis showed that the major isomer has an α configuration at C-1 (α:β 4:1). ^1H NMR (D_2O): δ 5.44 (0.8 H; m; 1-H of the α isomer), 4.47 (0.2 H; m; 1-H of the β isomer), 3.85–3.60 (6 H; m; 2-H, 3-H, 4-H, 5-H, 6-H's), 1.90–1.08 (m; cyclohexylamine signals). Upon passing through a Dowex 50 (H^+) column and washing with water, the counterion of product **15** was exchanged from the original cyclohexylamine salt to the potassium salt form; yield of **15** (from **12**) was approximately 30%. ^1H NMR (D_2O): δ 5.44 (0.8 H; m; 1-H of the α isomer), 4.50 (0.2 H; t, J = 7.0 Hz; 1-H of the β isomer), 3.92–3.58 (6 H; m; 2-H, 3-H, 4-H, 5-H, 6-H's). High-resolution FAB-MS Anal. Calcd for $C_6H_{11}FO_8P$ $(M - 1)^+$: 261.0176. Found: 261.0170.

*CDP-3-Deoxy-3-fluoro-*D*-glucose* (**7**). According to a procedure of Moffatt and Khorana (1961), a solution of dicyclohexylcarbodiimide (824 mg, 4 mmol) in *tert*-butyl alcohol (15 mL) was added dropwise to a refluxing solution of cytidine 5′-phosphate (1 mmol of the free acid) in a mixture of water (10 mL), *tert*-butyl alcohol (10 mL), and purified morpholine (0.34 mL, 4 mmol). The resulting cytidine 5′-phosphoromorpholidate, as a salt of 4-morpholine *N,N′*-dicyclohexylcarboxamidine, (0.5 mmol) was dissolved in anhydrous pyridine (15 mL) and evaporated to dryness in vacuo. The process of dissolution in fresh dry pyridine and evaporation was repeated twice, with argon being admitted into the flask after each evaporation. Meanwhile, an aqueous solution of **15** in its potassium salt form (0.75 mmol) was loaded onto a Dowex 50 (H^+) column which was then thoroughly washed with water. The eluate was made alkaline by the addition of purified pyridine, concentrated in vacuo to about 5 mL, and mixed with a solution of tri-*n*-octylamine (1.13 mmol) in pyridine. This homogeneous solution was evaporated to dryness, and the residue was rendered anhydrous by repeatedly (four times) dissolving it in dry pyridine and evaporating the

Scheme IV



Scheme V



solvent in vacuo. The residue was then redissolved in dry pyridine, and the solution was added to the standby cytidine 5′-phosphoromorpholidate solution. The combined mixture was again evaporated and recharged with pyridine several times and was finally kept in dry pyridine (15 mL) at room temperature for 5 days. All of this manipulation was performed via a vacuum manifold designed by Nordin et al. (1965) with minor modification. It was essential to keep the reaction flask sealed to prevent the possible exposure to moisture during the reaction. The solvent was then evaporated in vacuo, and the residue was suspended in an ether and aqueous acetate solution (ca. 20% more than the amount of the total tri-*n*-octylamine and 4-morpholine *N*,*N*′-dicyclohexylcarboxyamidine present). The aqueous layer was extracted with ether, and the ether layer was back-washed with water. The combined aqueous solution was lyophilized, and the residue thus obtained was purified by paper chromatography (ammonium acetate/ethanol 3:7, 18 h, room temperature). The desired product **7** was located by comparing its R_f value with that of CDP-[U-^{14}C]glucose and was washed out from the paper with water. A similar retention time was also observed by an HPLC assay using a Partisil SAX 10 anion-exchange column (4.6 × 250 mm) with a linear gradient from 0.03 to 0.12 M potassium phosphate buffer (pH 6.6). Fractions containing **7** were combined and lyophilized to dryness. The yield estimated on the basis of the absorbance of the CDP group was approximately 30%. ^{1}H NMR (D_2O): δ 8.06 (1 H; br d, J = 7.0 Hz; cytidine H), 6.06 (0.8 H; br s; 1-H of the α isomer), 5.72 (1 H; br s; 1′-H of ribose), 4.62 (0.2 H; t, J = 9.3 Hz; 1-H of the β isomer), 4.80–4.76, 4.45–4.20, and 4.02–3.80 (14 H; m; glucose and ribose H's). ^{13}C NMR (D_2O): β 166.2 (cytidine C-4), 157.3 (cytidine C-2), 144.5 (cytidine C-6), 96.5 (cytidine C-5), 92.0 (C-1′), 85.4 (C-4′), 75.1 (C-3′), 70.2 (C-2′), 62.8 (C-5′), 98.6 (C-1), 77.2, 72.5, 72.0, 70.3, 67.5 (C-6). High-resolution FAB-MS Anal. Calcd for $C_{15}H_{25}FN_3O_{15}P_2$ $(M + 1)^+$: 568.0745. Found: 568.0745.

Method 3: *TBA Assay*. As delineated in Scheme IV, to an aliquot (20 μL) of the substrate solution [CDP-D-glucose (0.44 mg, 0.75 μmol), NAD^+ (0.05 mg, 75 nmol) in 110 μL of buffer] was added PMP (2.8 μg, 0.01 μmol), NADH (0.53 mg, 0.75 μmol), and enzymes E_1 (50 μg) and E_3 (10 μg) for a total volume of 200 μL. The reaction was kept at 27 °C for 1 h. The resulting product (**6**) as reduced with solid $NaBH_4$ (3.8 mg, 0.1 mmol) for 30 min and the cytidine diphosphate group was removed by hydrolysis with 2 N H_2SO_4 (5 μL) at 100 °C for 5 min. This was followed by neutralization with 0.1 N $Ba(OH)_2$ (30 μL) and centrifugation. The supernatant was collected and treated with 40 μL of a periodate solution (0.025 N sodium periodate in 0.125 N H_2SO_4) at 55 °C for 20 min. The excess oxidant was then quenched with 60 μL of arsenite solution (2% sodium arsenite in 0.5 N HCl). The addition of 200 μL of thiobarbituric acid reagent (6% in water, pH 2.0) followed by heating at 100 °C for 10 min resulted in the appearance of a characteristic pink chromophore (**19**) with maximum absorption at 532 nm (ϵ 159 200) (Nair & Turner, 1984). The absorbance was calibrated against a blank that was prepared in parallel without E_1.

Method 4: *GC/MS Assay*. CDP-D-glucose (0.76 mg, 1.3 μmol) and NAD^+ (0.33 mg, 0.5 μmol) were combined with E_{od} (90 μg) in 10 mM potassium phosphate buffer, pH 7.5 (final volume 100 μL). The reaction was allowed to react at 37 °C for 30 min. Half of this solution (50 μL) was added to 10 mM potassium phosphate buffer (pH 7.5, 200 μL) containing PMP (0.028 mg, 0.1 μmol), NADH (0.53 mg, 0.75 μmol), and enzymes E_1 (100 μg) and E_3 (25 μg) and incubated at 27 °C for 4 h. The product was reduced with $NaBH_4$ (0.38 mg, 0.01 mmol) at room temperature for 30 min. The solution was boiled for 1 min and centrifuged to remove the enzyme precipitate. The supernatant was filtered and the product was purified by HPLC using a Partisil SAX 10 anion-exchange column (4.6 × 250 mm) with a 20-min linear gradient from 30 to 120 mM potassium phosphate buffer (pH 6.6) and a flow rate of 1 mL/min (Liu & Liu, 1989). Under these conditions, standard CDP-D-glucose has a retention time of 5.4 min. Since all the CDP-containing hexose derivatives were eluted with approximately the same retention time, they were collected in a single fraction.

The solution containing the combined enzymatic products (**16** and others) was acidified to pH 2.0 with concentrated HCl, boiled for 10 min, and then neutralized with concentrated KOH to pH 7.0. The mixture was lyophilized to dryness and redissolved in 0.6 mL of 0.5 M NH_4OH. The products were then reduced and acetylated according to a procedure de-

Table I: Summary of Enzyme E_1 Purification from *Yersinia pseudotuberculosis*

purification step	total protein (mg)	sp act. ([^{3}H]PMP)[a]	purification (*x*-fold)	sp act. (TBA)[b]	purification (*x*-fold)
crude extract[c]	486000	ND[d]	ND	ND	ND
streptomycin sulfate	ND	ND	ND	ND	ND
$(NH_4)_2SO_4$	27000	ND	ND	ND	ND
DEAE-Sephacel	137.5	0.53	1	887	1
Matrex Blue A	43.0	1.56	2.9	1183	1.3
DE-52	7.5	3.95	7.5	2042	2.3
G-100	5.0	4.47	8.4	2612	2.9

[a] Activity as measured by the tritium release assay in nanomoles per milligram per hour. [b] Activity as measured by the TBA assay in nanomoles per milligram per hour. [c] From approximately 900 g of wet cells. [d] Not determined.

veloped by Blakeney et al. (1983). As shown in Scheme V, the above sample (**17**) was mixed with 1.0 mL of DMSO to which $NaBH_4$ (21 mg, 0.55 mmol) had been added. The reaction was stirred at 40 °C for 90 min before being slowly quenched with 0.1 mL of glacial acetic acid. 1-Methylimidazole (0.2 mg, 2.5 μmol) and 6.0 mL of acetic anhydride were added, 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. The products were extracted with chloroform three times and the combined organic extracts were washed twice with water, dried over anhydrous sodium sulfate, filtered, and concentrated. The samples were then subjected to GC/MS analysis.

The standard compounds, abequitol tetraacetate (**20**), glucitol hexaacetate (**21**), and fucitol pentaacetate (**22**), were prepared from abequose, glucose, and fucose, respectively, using the same $NaBH_4$/DMSO reduction and 1-methylimidazole/acetic anhydride acetylation procedures described above. *Abequitol tetraacetate* (**20**) ^{1}H NMR ($CDCl_3$): δ 5.14–4.91 (3 H; m; 2-H, 4-H, 5-H), 4.23 (1 H; dd, *J* = 11.9, 3.5 Hz; 1-H), 3.97 (1 H; dd, *J* = 11.9, 5.8 Hz; 1-H), 2.07, 2.07, 2.06, 2.05 (3 H each; s; acetyl CH_3's), 1.82 (2 H; m; 3-H), 1.18 (3 H; d, *J* = 6.4 Hz; 5-Me). ^{13}C NMR ($CDCl_3$): δ 170.4, 170.3, 170.2, 170.1 (C=O's), 70.8 (C-2)*, 70.0 (C-4)*, 67.1 (C-5)*, 65.1 (C-1), 31.5 (C-3), 21.0, 20.9, 20.7, 20.6 (acetyl CH_3's), 16.2 (C-6). *Glucitol hexaacetate* (**21**) ^{1}H NMR ($CDCl_3$): δ 5.34 (2 H; m; 3-H, 4-H), 5.17 (1 H; m; 2-H), 4.97 (1 H; m; 5-H), 2.07, 2.03, 2.02, 2.00, 1.99, 1.98 (3 H each; s; acetyl CH_3's). ^{13}C NMR ($CDCl_3$): δ 170.5, 170.4, 170.3, 170.0, 169.8, 169.7 (C=O's), 69.3 (C-2), 68.6 (C-4), 68.5 (C-5), 68.3 (C-3), 61.7 (C-1), 61.4 (C-6), 20.8, 20.7, 20.7, 20.6, 20.5, 20.4 (acetyl CH_3's). *Fucitol pentaacetate* (**22**) ^{1}H NMR ($CDCl_3$): δ 5.36 (1 H; dd, *J* = 10.0, 2.0 Hz; 3-H), 5.30 (1 H; m; 2-H), 5.15 (1 H; dd, *J* = 9.8, 2.0 Hz; 4-H), 5.05 (1 H; dq, *J* = 6.5, 2.0 Hz; 5-H), 4.25 (1 H; dd, *J* = 11.7, 4.8 Hz; 1-H), 3.81 (1 H; dd, *J* = 11.7, 7.6 Hz; 1-H), 2.10, 2.07, 2.06, 2.03, 2.00 (3 H each; s; acetyl CH_3's), 1.11 (3 H; d, *J* = 6.5 Hz; 5-Me). ^{13}C NMR ($CDCl_3$): δ 170.5, 170.4, 170.3, 170.1, 170.0 (C=O's), 70.2, 67.9, 67.8, 66.8, (C-2, C-3, C-4, C-5), 62.2 (C-6), 21.0, 20.7, 20.6, 20.6, 20.5 (acetyl CH_3's), 16.2 (C-6).

Growth of Cells. An overnight culture of *Y. pseudotuberculosis* was grown in tryptic soy broth medium (3%, 9 L) in an incubator–shaker with vigorous agitation (140 rpm) at 28 °C. This inoculum culture was then diluted 12-fold, placed in a 110-L fermentor, and grown at 28 °C, 100 rpm, pH 7.5. The organisms were harvested in the early logarithmic to mid-logarithmic phase (nearly 4 h after inoculation, OD_{600} = 2.35) by centrifugation. A typical yield was 400–500 g of wet cells/110 L of culture. All cell culture and harvesting procedures were performed by the Biological Process Technology Institute at the St. Paul campus of the University of Minnesota.

Protein Determination. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. The estimation of protein concentration by measuring the $A_{280/260}$ ratio was useful for dilute samples from early purification steps and routine monitoring of column fractions (Kalckar, 1947). This method yielded values approximately 10% higher than the Lowry assay on identical samples.

Enzyme E_1 Purification. All operations were carried out at 4 °C. All buffers contained 1 mM EDTA unless otherwise specified. The results of the purification are summarized in Table I.

Step 1. Crude Extracts. Cells from 110 L of culture (900 g) were resuspended in four times their volume of 50 mM potassium phosphate buffer, pH 7.5 (2.0 L), followed by addition of a protease inhibitor solution (final concentration 1%). This inhibitor solution was prepared by mixing 200 mg of phenanthroline, 200 mg of benzamidine, and 1 mg of phenylmethanesulfonyl fluoride in 1.0 mL of ethanol. The cells were disrupted in batches by sonication at 70% output for 2 min at 45-s intervals with the temperature kept as close to 0 °C as possible. Cellular debris was removed by centrifugation at 4400*g* for 20 min.

Step 2. Streptomycin Sulfate Treatment. Streptomycin sulfate (5% aqueous solution) was added dropwise to the crude extract for a final concentration of 0.8%. After stirring for an additional hour, the precipitate was removed by centrifugation at 14000*g* for 2 h. The supernatant (2.7 L) was diluted with 1.0 M potassium phosphate buffer, pH 7.5 (270 mL).

Step 3. Ammonium Sulfate Precipitation. Solid ammonium sulfate was added slowly to the enzyme solution from step 2 to give a final concentration of 65% saturation. After the addition was complete, the solution was stirred overnight. The precipitated proteins were collected by centrifugation at 14000*g* for 2 h and were then redissolved in a minimal amount of 50 mM potassium phosphate buffer (pH 7.5). This solution was dialyzed against 40 L of the same buffer for 24 h with four solution changes.

Step 4. DEAE-Sephacel Column Chromatography. The enzyme solution from step 3 (600 mL) was applied to a column of DEAE-Sephacel (4.5 × 45 cm) preequilibrated with 50 mM potassium phosphate buffer (pH 7.5). The column was then washed with 2.0 L of 50 mM of the same buffer. A linear gradient of 3.4 L from 50 mM to 0.2 M potassium phosphate buffer (pH 7.5) was used to elute the other enzymes involved in this study (E_p, E_{od}, and E_3). A linear gradient between 800 mL of 0.4 M and 800 mL of 0.6 M potassium phosphate buffer (pH 7.5) was then used to elute E_1 in fractions of 16 mL. The column profile of the last linear gradient elution is illustrated in Figure 1A. The contents of fractions 65–95 were combined.

Step 5. Matrex Blue A Column Chromatography. The material from step 4 was concentrated to 50 mL via an Amicon ultrafiltration unit and PM-30 membrane. The solution was

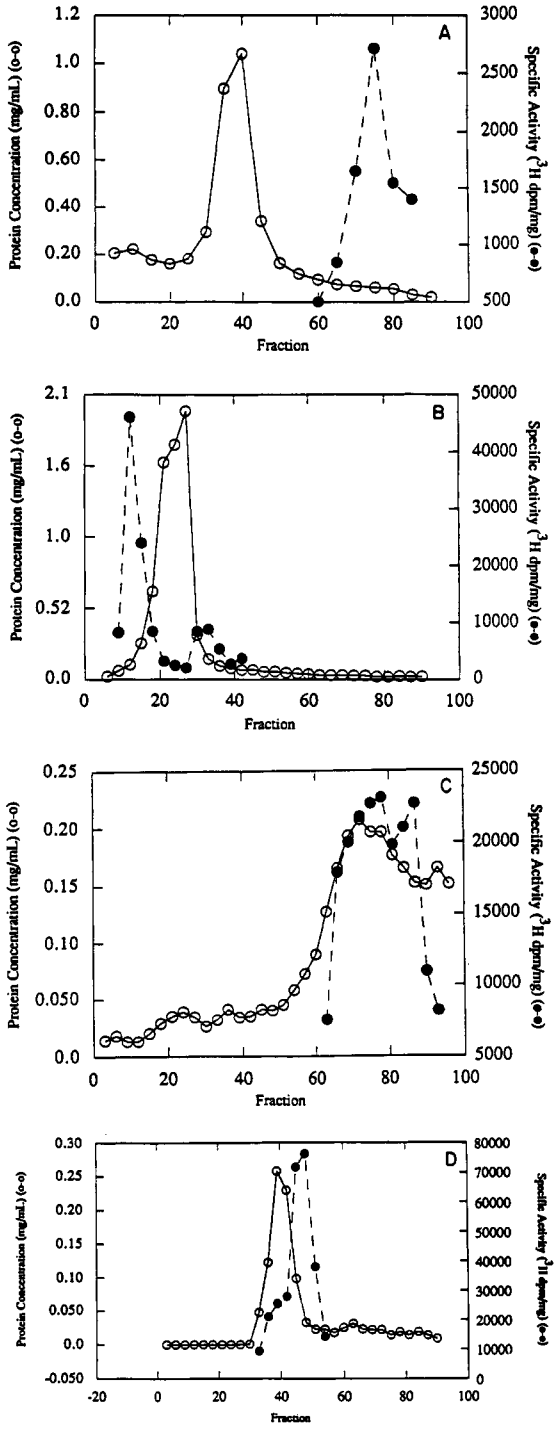


FIGURE 1: Column chromatography of E_1 isolated from *Y. pseudotuberculosis*. Chromatography conditions are discussed in Experimental Procedures. Fractions were monitored for absorbance at 280 nm (O), and aliquots were analyzed for E_1 activity on the basis of the cofactor tritium release assay (●). (A) DEAE-Sephacel chromatography (step 4); (B) Matrex Blue A chromatography (step 5); (C) DE-52 chromatography (step 6); (D) Sephadex G-100 chromatography (step 7).

applied to a Matrex Blue A column (3 × 16 cm) which was preequilibrated with 30 mM potassium phosphate buffer, pH 7.5 (no EDTA). The enzyme was eluted with the same buffer using a flow rate of 25 mL/h, and fractions of 5.0 mL were collected. The column profile is given in Figure 1B. The desired protein was found in fractions 11–21.

Step 6. DE-52 Column Chromatography. The material from step 5 was concentrated to 20 mL using a YM-10 membrane in an Amicon ultrafiltration unit and then applied to the DE-52 column (3 × 12 cm) preequilibrated with 0.2 M potassium phosphate buffer (pH 7.5). The column was washed with 100 mL of 0.2 M potassium phosphate buffer (pH 7.5), and the enzyme was then eluted with a linear gradient between 200 mL of 0.2 M and 200 mL of 0.4 potassium phosphate buffer (pH 7.5). The flow rate was 20 mL/h, and the fraction size was 3.0 mL. The column profile is shown in Figure 1C. Active fractions were combined (fractions 65–91) and concentrated to less than 1 mL using an Amicon ultrafiltration unit and a YM-10 membrane.

Step 7. Sephadex G-100 Column Chromatography. The Sephadex G-100 column (1.5 × 170 cm) was preequilibrated with 50 mM potassium phosphate buffer (pH 7.5) and the protein obtained in step 6 was applied in a volume of less than 1.0 mL. The column was washed with the same buffer. The flow rate was 10 mL/h, and fractions were 3.0 mL. Figure 1D depicts the column profile. The active protein found in fractions 38–53 was collected and stored at −85 °C.

Polyacrylamide Gel Electrophoresis. The relative molecular mass and purity of the enzyme samples were determined using SDS–polyacrylamide gel electrophoresis. The separating and the stacking gel were 13% and 6% polyacrylamide, respectively. Electrophoresis was carried out at a constant 120 V (~30 mA) in the discontinuous system of Laemmli (1970). Prior to electrophoresis, the samples were incubated at 100 °C for 5 min in a solution containing 0.5% sodium dodecyl sulfate and 5% 2-mercaptoethanol. Gels were stained with the preparation of Vesterberg (1971) and destained with acetic acid/ethanol/water (15:20:165 by volume).

Isoelectric Focusing. The isoelectric focusing experiment was run on a Pharmacia PhastSystem instrument using a preformulated PhastGel IEF 3-9 plate. The protein bands were visualized by silver staining (Nauta & Glygax, 1951). The distances from the anode were plotted against the p*I* values of the standards: phycocyanin (4.65), β-lactoglobulin B (5.10), bovine carbonic anhydrase (6.00), human carbonic anhydrase (6.50), equine myoglobin (7.00), human hemoglobin A (7.10), human hemoglobin C (7.50), lentil lectin (7.8, 8.0, 8.2), and cytochrome *c* (9.60).

Molecular Weight Determination. The subunit molecular weight was determined by SDS–polyacrylamide gel electrophoresis as described by Laemmli (1970) using the following standards: α-lactalbumin (14 200), carbonic anhydrase (29 000), ovalbumin (45 000), and bovine serum albumin (66 000). The molecular weight of the native enzyme was determined by gel filtration performed on a column of Sephadex G-100 (1.5 × 170 cm). The column was calibrated by separate chromatographic runs with the following protein standards: cytochrome *c* (12 400), chymotrypsinogen (25 000), ovalbumin (45 000), bovine serum albumin (66 000), and blue dextran (void volume). Samples were eluted in potassium phosphate buffer (10 mM, pH 7.5) while the absorbance was monitored at 280 nm. The molecular weight of the purified protein was estimated by the standard method of Andrews (1964).

Amino-Terminal Analysis. The N-terminal sequence was determined by an Applied Biosystems 470A protein sequencer with an on-line 120A HPLC system. This analysis was carried out at the Microchemical Facility in the Institute of Human Genetics of the University of Minnesota, and the result was confirmed by Dr. Theodore Thannhauser at the Baker Laboratory of Chemistry, Cornell University.

Enzyme E_1:Cofactor PMP Ratio. Enzyme E_1 (0.9 mg) was incubated with [4′-^{3}H]PMP (30.9 nmol, 7.5 mCi/mmol) for 1 h and then applied to a Sephadex G-100 column (1.5 × 170

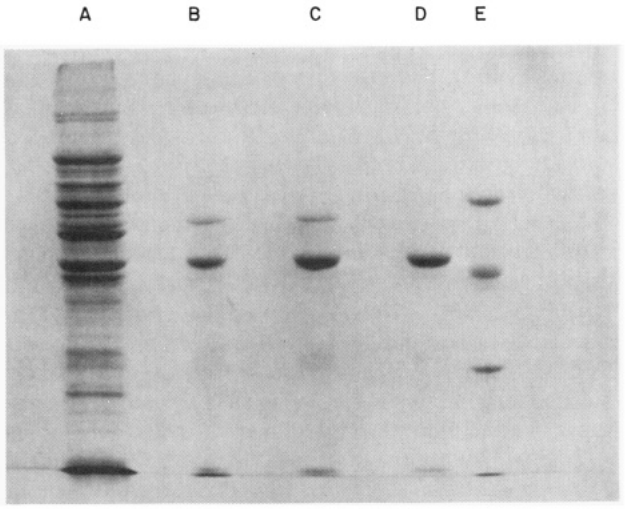


FIGURE 2: SDS gel electrophoresis of E_1 isolated from *Y. pseudotuberculosis* after each step of the purification procedure: (A) DEAE-Sephacel chromatography (step 4); (B) Matrex Blue A chromatography (step 5); (C) DE-52 chromatography (step 6); (D) Sephadex G-100 chromatography (step 7); (E) molecular weight standards. The molecular weight standards are α-lactalbumin (14 200), carbonic anhydrase (29 000), ovalbumin (45 000), and bovine serum albumin (66 000).

cm) preequilibrated with 50 mM potassium phosphate buffer (pH 7.5). The complex was eluted from the column with the same buffer at a flow rate of 10 mL/h and collected in 3.0-mL fractions. Each fraction was checked for protein concentration and radioactivity. Those fractions containing both protein and cofactor were combined and concentrated with an Amicon ultrafiltration unit using a YM-10 membrane.

Kinetic and pH Rate Profile of E_1. The kinetic parameters and pH rate profile were determined using the TBA assay as described above except that the amount of E_3 was increased to 25 μg. For the kinetic experiments, the linearity between product formation and E_1 concentration for this assay was found to be in the range of 1–25 μg of E_1. The amount of E_1 was then kept constant at 8.5 μg, and the concentration of substrate was varied to determined the V_{max} and K_m by Lineweaver–Burk plots.

Results

Enzyme Purification. The crude extract was prepared in much the same way from *Y. pseudotuberculosis* as in the original work reported by Strominger and his co-workers in which E_1 was isolated from *P. pseudotuberculosis* (Gonzalez & Strominger, 1972a). As detailed in Experimental Procedures, following treatment with streptomycin sulfate to remove nucleic acids, the proteins were precipitated with ammonium sulfate and dialyzed. The crude enzyme was then chromatographed on a column of DEAE-Sephacel. This column was useful not only for E_1 purification but also for the separation of all the enzymes involved in the ascarylose biosynthetic pathway (Han et al., 1990). Since E_1 is the only protein which needs a high salt concentration (0.4 M potassium phosphate buffer, pH 7.5) for elution, it can be purified along with enzymes E_p, E_{od}, and E_3 using the same column. The greatest purification was achieved in the next step with the dye-ligand affinity chromatography using a Matrex Blue A column. Interestingly, E_1 itself does not bind to this column, but many of the major protein contaminants from the DEAE-Sephacel step adhered to the Blue A resin. Electrophoretic analysis of the resulting solution (Figure 2) clearly revealed a much cleaner sample. Thus, chromatography of E_1 by a Matrex Blue A column represents a novel example of "negative chromatography" which is crucial for E_1 purification. The protein was then subjected to the second anion-exchange chromatography, DEAE-cellulose (DE-52), which further increased the enzyme's specific activity. The final step, Sephadex G-100 gel filtration, removed the remaining impurities, provided that care was taken not to overload the column. A summary of the results is shown in Table I, and the elution profiles of each column chromatography are depicted in Figure 1. It should be noted that the PMP cofactor is not tightly bound to the enzyme. It is commonly lost in the purification sequence, especially during ion-exchange chromatography. Therefore, the purified E_1 is only an apoenzyme. Nevertheless, E_1 isolated by the above purification sequence is nearly homogeneous.

Properties of CDP–4-Keto-6-deoxy-D-glucose-3-dehydrase (E_1). Molecular Weight. The subunit molecular weight of enzyme E_1 was determined to be 49 000 on the basis of SDS–polyacrylamide gel electrophoresis. However, the apparent molecular weight by gel filtration of the native enzyme was found to be 66 000. Such a discrepancy is likely due to a steric irregularity of E_1 that disturbs the molecular weight measurement. Thus, we believe that enzyme E_1 is a monomeric protein having a molecular weight of 49 000. The concentrations of all E_1 solutions used in the experiments reported in this paper were calculated accordingly.

pI Determination. On the basis of the isoelectric focusing experiment, a p*I* of 6.1 was found for this enzyme.

Amino-Terminal Sequence. The N-terminal amino acid sequence determined from a highly purified sample of E_1 is Met-Ser-Gln-Glu-Glu-Leu-Arg-Gln-Gln-Ile-Ala-Glu-Leu-Val. In fact, this result corresponds perfectly to a sequence of DNA found in a clone which was isolated from a *Hin*dIII size-selected genomic library of *Y. pseudotuberculosis* constructed in our laboratory (Liu et al., unpublished results). This clone contains a gene cluster which encodes for both E_{od} and E_3 as well as part of E_1. A new library containing the whole E_1 gene is being developed in order to clone and overproduce this enzyme.

Ultraviolet–Visible Spectrum. The electronic absorption of this highly purified enzyme at neutral pH is primarily the spectrum of a single polypeptide. A shoulder at 330 nm of the apoenzyme is due to residual PMP bound to E_1. This characteristic absorption becomes more apparent after the enzyme has been reconstituted with the cofactor. Assuming the enzyme-linked PMP has an absorption coefficient of 6500, the ratio of PMP bound to the reconstituted E_1 was estimated to be 1.1:1.

Enzyme:Cofactor Ratio. The stoichiometry of PMP bound to E_1 was determined in a much more accurate manner with a sample prepared by incubating the apoenzyme with labeled [4′-^{3}H]PMP followed by Sephadex G-100 gel filtration. The fractions containing E_1 were collected, and the protein concentration and radioactivity of the combined fractions were determined. The ratio of protein concentration to tritium label was 1.08, clearly indicating that there is 1 cofactor bound per enzyme molecule.

Kinetic Parameters and pH Optimum. The kinetic parameters were determined by coupling E_1 with E_3 and monitoring product formation by the TBA assay. While these measurements were carried out, it was essential to keep E_3 at saturating concentrations so that the results reflected the E_1 contribution. The K_m was determined to be 74.8 μM and V_{max} was measured at 1.0 μmol h^{-1} mg^{-1}. Under the assay conditions, the pH optimum was found to be 7.5. An identical

optimal pH value was obtained for E_3 when it was assayed alone on the basis of its NADH:DCPIP oxidoreductase activity (Han et al., 1990). It should be noted that the [4′-^{3}H]PMP assay is not applicable to study the pH–rate relationship of E_1 activity, since the binding of the cofactor to enzyme and charcoal is pH dependent.

Discussion

The discovery of CDP-4-keto-6-deoxy-D-glucose-3-dehydrase (E_1), a PMP-dependent enzyme that plays an essential role in C–O bond cleavage leading to the formation of CDP-ascarylose, has been important in confirming the common pathway of the biosynthesis of 3,6-dideoxyhexoses and in identifying a new type of biological deoxygenation reaction. Although the target enzyme had been purified by Strominger and his co-workers from *P. pseudotuberculosis*, the catalytic mechanism of this unusual enzyme has never been fully elucidated. The studies described herein and in the following paper encompass our recent efforts in exploring this deoxygenation process in detail. In this paper, we summarize the purification of an "E_1 equivalent" from a similar but different bacterial strain, *Y. pseudotuberculosis*, with an emphasis on the development of enzyme assays and characterization of this enzyme's properties.

As the putative glucoseen product of E_1 (**4**) has never been isolated, there are no obvious methods that would allow this enzyme's activity to be monitored directly. The E_1 assay followed in the original purification was a coupled assay which involved furthering the E_1 conversion by E_3 and the isolation of the resulting product **6**. This method is extremely tedious and time consuming, and it hence has been an impediment to E_1 purification. In order to circumvent this hampering factor, it was necessary to develop a few convenient and sensitive methods to facilitate the E_1 assay. Since sufficient quantities of E_3 essential for the original E_1 assay are not available, the first priority in the early stage of this research was the development of a sensitive and E_3-independent method which could be used for routine checks of E_1 activity in column fractions. On the basis of numerous trials, an expedient method was established that relies on the fact that E_1-catalyzed dehydration is initiated by a proton abstraction from the C-4′ of the PMP–substrate adduct. Since E_1-catalyzed dehydration is highly reversible in the absence of E_3 (Weigel et al., 1992), such a deprotonation simply leads to hydrogen exchange at that locus. By using a tritium-labeled cofactor in the incubation that was later quenched with charcoal, the amount of E_1 present could be determined from the amount of released tritium in the supernatant. It is noteworthy that the C-4′ hydrogen of the enzyme-bound PMP is labile in the absence of substrate; however, the release of tritium is significantly augmented in the presence of substrate. The [4′-^{3}H]PMP used in this assay was obtained from PLP by treatment with ammonium hydroxide and [^{3}H]$NaBH_4$. The requisite cofactor could be synthesized with high specific radioactivity, making this assay quite sensitive. It should be apparent that the reduction step was stereochemically random, the coenzyme generated via this preparation was a racemic and monotritiated mixture. Although it is possible that PMP may also participate in and be utilized by other biological pathways in the crude extract, this assay is facile, sensitive, and most importantly, E_3 independent. As the purified enzyme was later shown to be necessary for C-3 deoxygenation, this confirmation clearly indicated that E_1 was successfully selected by this assay.

The dubiety of the target specificity associated with the tritium release assay was alleviated by a fluoride release assay. This method was designed on the basis of the expectation that E_1 will bind and rupture the C–F bond of a substrate analogue, CDP-4-keto-3,6-dideoxy-3-fluoro-D-glucose (**8**), which is generated in situ from CDP-3-deoxy-3-fluoro-D-glucose (**7**) by E_{od}. Since the bond length and electronegativity of the C–F group are similar to those of a C–OH group, we anticipated that the proposed compound would be processed by E_1. Preparation of the precursor **7** from 3-deoxy-3-fluoro-D-glucose (**11**) was first attempted enzymatically by treatment with a mixture of hexokinase, phosphoglucomutase, and E_p. Inorganic pyrophosphatase was also added to drive the equilibrium to completion (Rubenstein & Strominger, 1974a). However, to our great disappointment, none of the desired product was formed under these conditions despite numerous trials. It was later found that 3-deoxy-3-fluoro-D-glucose 1-phosphate has been reported as a competitive inhibitor for UDP-D-glucose pyrophosphorylase (Wright et al., 1972), and it is possible that an analogous inhibition occurs in the reaction catalyzed by E_p. Synthesis of **7** was eventually accomplished by a chemical approach shown in Scheme III in which the C-3 fluoro moiety was introduced by reacting **9** with the DAST reagent (Card & Reddy, 1983). Phosphorylation of **14** with *o*-phenylene phosphorochloridate and *s*-collidine followed by oxidative deprotection with lead tetraacetate and workup in basic solution afforded the desired 1-phosphate derivative **15** as the major product (Khwaja et al., 1970). Compound **15** could also be satisfactorily prepared by direct phosphorylation of **12** with crystalline phosphoric acid, and the product isolated from this one pot reaction was predominantly the α isomer. Coupling of **15** with cytidine 5′-phosphoromorpholidate based on a procedure of Moffatt and Khorana (1961) and Distler et al. (1961) was used to synthesize the desired sugar nucleotide **7**. The course of the reaction was monitored by HPLC, and the resulting product was isolated in more than 30% yield after purification by paper chromatography. When this purified product **7** was subjected to incubation with excess E_{od} and E_1, fluoride anion was found to be released into the solution whose concentration could be estimated by calibrating the observed readings against a standard curve. Although this assay established the identity of E_1, it is not practically useful due to the excessive effort required to prepare the substrate, the mediocre sensitivity of the fluoride electrode, and perhaps, most importantly, the low catalytic efficiency of E_{od} and E_1 acting on this substrate analogue. Since the fluorine substituent can act only as a hydrogen bond acceptor, and hydrogen bonds involving fluorine are more sensitive to orientation than those involving oxygen or nitrogen (Withers et al., 1988), the insights gained from these studies clearly indicate that the specific interaction between the C-3 hydroxyl group of the substrate and a polar group in the active site is vital for E_p. Although such interactions appear to be less crucial for E_{od} and E_1, further assessment must await a thorough scrutiny of these enzymatic conversions.

Another assay useful for measuring E_1 activity couples the enzymatic transformations to a thiobarbituric acid reaction. The highly conjugated chromophore produced in the reaction between TBA and a ketone/aldehyde is very intense and characteristic and has been commonly used for determining oxidative changes in foods containing unsaturated fatty acids (Ward, 1985). The red pigment produced in the reaction between TBA and oxidized lipids has been shown to be a condensation product of two molecules of TBA and one molecule of malonaldehyde (Nair & Turner, 1984). Because malonaldehyde is also generated in the periodate oxidation of 2- and 3-deoxyhexoses, treatment with TBA to generate the same red chromogen has been used to quantitatively measure

these deoxysugars (Waradekar & Saslaw, 1959; Cynkin & Ashwell, 1960; Lo et al., 1989). The TBA assay that emerged from this principle is illustrated in Scheme IV. Incubation of CDP–D-glucose with E_{od}, enzymes E_1 and E_3, and the necessary cofactors resulted in CDP–4-keto-3,6-dideoxy-D-glucose (**6**) formation. Reduction of the C-4 keto group with sodium borohydride followed by acid hydrolysis to remove the cytidine diphosphate group gave a mixture of 3,6-dideoxy-D-*xylo*-hexose (abequose) and 3,6-dideoxy-D-*ribo*-hexose (paratose), both of which yielded malonaldehyde (**18**) upon treatment with sodium periodate. Since the condensation product of TBA and malonaldehyde (**19**) shows a bright pink color at 532 nm with a known absorption coefficient, the yield of deoxysugar formation can be directly deduced on the basis of the observed absorbance. This assay was found to be linear with respect to the concentration of E_1 when an excess of E_3 was used. Conversely, this assay could also be used to look for the presence of enzyme E_3 when E_1 was used in excess. Since TBA is not a very selective reagent, this assay is best used when both enzymes are in relatively pure form. Incubation with concentrated crude enzyme commonly yielded a complex mixture of products which, upon TBA treatment, gave an ambiguous reddish-brown chromophore. It is worth mentioning that the intensity of the color development varied with the concentration of TBA reagent and the incubation temperature. Nevertheless, when properly executed, this colorimetric method provides a direct, sensitive, and readily interpretable test to assay the formation of the E_1/E_3 product.

The most conclusive evidence confirming the roles of E_1 as well as E_3 was attained by a GC/MS assay which permits an unambiguous identification of the deoxysugar product generated from the E_1 and E_3 reactions. This assay is conceptually akin to a procedure used by Rubenstein and Strominger (1974a) to study the reversibility of E_1 catalysis. As depicted in Scheme V, the deoxysugar product **6** was generated by incubation with enzymes E_{od}, E_1, and E_3. Following ketone reduction and HPLC purification, the resulting compound **16** was subjected to acid hydrolysis, $NaBH_4$ reduction in DMSO, and peracetylation using 1-methylimidazole as a catalyst (Blakeney et al., 1983). This particular reduction and acetylation procedure rendered removal of the borate salts unnecessary and also shortened the reaction times. Owing to the nonstereospecific reduction of the keto moiety in **6** by $NaBH_4$, two 3,6-dideoxyhexitol tetraacetates (**20**), abequitol tetraacetate and paratitol tetraacetate, were obtained from this series of reactions. Formation of these two epimers is indicative of the successful deoxygenation at C-3. Although their GC retention times are barely distinguishable and their mass spectrum fragmentation patterns are the same, these properties are identical to those of the chemically prepared standards. The primary ions in the mass spectra of these alditol acetates are formed by elimination of an acetoxyl group or by cleavage of the alditol chain, while cleavage at deoxy groups is insignificant (Lonngren & Svensson, 1974). The byproducts of this assay include glucitol hexaacetate (**21**) generated from unreacted starting material, and fucitol pentaacetate and quinovitol pentaacetate (both are 6-deoxyhexitol pentaacetates (**22**)), derived from the residual E_{od} product. However, since their GC/MS behaviors are distinct, their existence in the analytical sample imposed no complication to this assay.

With these convenient and sensitive assays in hand, our attention turned to securing the target enzyme. The original purification procedure reported by Strominger and his coworkers (Gonzalez-Porque & Strominger, 1972a) for enzyme E_1 from *P. pseudotuberculosis* began with DEAE-cellulose chromatography followed by Sephadex G-100 gel filtration, a second DEAE-cellulose column, and a preparative gel electrophoresis which resulted in pure protein. Since this purification sequence involved an electrophoresis step, it is not practical for generating useful quantities of enzyme. Furthermore, the bacterial strain *Y. pseudotuberculosis* used in the present study appears to be different from the strain used in Strominger's work (Han et al., 1990). Isolating the target enzyme from a different bacterial source is likely to yield proteins having identical biological roles but distinctly different physical properties. Therefore, the development of new protocols was necessary to purify E_1 from *Y. pseudotuberculosis*. As shown in Figure 2 and Table I, a sequence of four columns was eventually established that was effective in consistently producing pure E_1. On the basis of the tritium release assay using [4′-^{3}H]PMP as the cofactor, enzyme E_1 was purified only 8-fold starting from the first column. When estimated by the TBA assay, an even less impressive value of only 3-fold purification was indicated. The discrepancy in the observed degree of purification may simply reflect the distinction between these two assays, and the accuracy of the specific activities is subject to the detection limitations of these two methods. One should also keep in mind that the PMP–substrate complex is bound tightly to E_1 under the assay conditions and does not freely dissociate; hence, the tritium release assay only measures the first turnover event in which tritium is released from the bound cofactor and exchanged with a solvent hydrogen. Furthermore, the PMP coenzyme used in this assay was only monotritiated in a racemic form. As the initial deprotonation from C-4′ of PMP was found to be a stereospecific process (Shih et al., 1990), only half of the bound cofactor with tritium labeling would have tritium at the right locus. The TBA assay, on the other hand, depends on the contribution from E_3 to generate the deoxygenation product (**6**) and thus is affected by the catalytic efficiency of E_3 as well. Since the detection limit for the TBA assay is 0.1 nmol, the fact that no adduct could be detected in the dilute crude extract and ammonium sulfate samples with this assay suggested that the overall purification may be as high as 26 000-fold.

The purified enzyme from *Y. pseudotuberculosis* consists of a single polypeptide chain in its active form; however, the estimated molecular weight of 49 000 is much smaller than that of E_1 isolated from *P. pseudotuberculosis* by Strominger. This finding sustained our early conjecture that the strains used in Strominger's work and our current study were different (Han et al., 1990). The electronic absorption of this highly purified enzyme at neutral pH is primarily the spectrum of a single polypeptide. While it is well-known that PLP as a cofactor is covalently tied to the protein via an active-site lysine, the PMP coenzyme which lacks the aldehyde group necessary to form the Schiff base is only loosely bound to E_1. Thus, it was easily depleted from E_1 when it was subjected to ion-exchange chromatography. As expected, purified E_1 is only weakly active unless incubated with PMP in situ or reconstituted with PMP prior to activity assay. These results unequivocally indicated that E_1 is PMP dependent. The stoichiometry of the cofactor to E_1 was determined to be 1.

The availability of E_1 in pure form and the successful development of a number of sensitive methods to monitor E_1 catalysis have set the stage for the elucidation of the mechanism of this unique enzyme-catalyzed deoxygenation, which is the subject of the following paper.

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REFERENCES

Adams, E. (1976) *Adv. Enzymol. Relat. Areas Mol. Biol. 44*, 69.

Andrews, P. (1964) *Biochem. J. 91*, 222.

Bishop, C. T., & Jennings, H. J. (1982) in *The Polysaccharides* (Aspinall, G. O., Ed.) Vol. 1, p 291, Academic Press, New York.

Blakeney, A. B., Harris, P. J., Henry, R. J., & Stone, B. A. (1983) *Carbohydr. Res. 113*, 291.

Butterworth, R. F., & Hanessian, S. (1971) *Adv. Carbohydr. Chem. Biochem. 26*, 279.

Card, P. J., & Reddy, G. S. (1983) *J. Org. Chem. 48*, 4734.

Cheung, Y.-F., & Walsh, C. T. (1976) *J. Am. Chem. Soc. 98*, 3397.

Cynkin, M. A., & Ashwell, G. (1960) *Nature (London) 186*, 155.

Disther, J. J., Moffatt, J. G., & Khorana, H. G. (1961) *J. Am. Chem. Soc. 83*, 659.

Dolphin, D., Poulson, R., & Avramovic, O. (1986) *Vitamin B_6 Pyridoxal Phosphate, Chemical, Biochemical, and Medical Aspects*, Wiley-Interscience, New York.

Evangelopoulos, A. E. (1984) *Chemical and Biological Aspects of Vitamin B_6 Catalysis*, A. R. Liss, New York.

Gabriel, O. (1973) in *Carbohydrates in Solution* (Gould, R., Ed.) Advances in Chemistry Series, No. 117, p 387, American Chemical Society, Washington, DC.

Glaser, L., & Zarkowsky, R. (1971) in *The Enzymes, 3rd. Ed.* (Boyer, P., Ed.) Vol. 5, p 465, Academic Press, New York.

Gonzalez-Porque, P. (1986) in *Vitamin B_6 Pyridoxal Phosphate, Chemical, Biochemical, and Medical Aspects* (Dolphin, D., Poulson, R., & Avramovic, O., Eds.) Part B, p 391, Wiley Interscience, New York.

Gonzalez-Porque, P., & Strominger, J. L. (1972a) *J. Biol. Chem. 247*, 6748.

Gonzalez-Porque, P., & Strominger, J. L. (1972b) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1625.

Gorshkova, R. P., Korchahina, N. I., & Ovadov, Yu. S. (1983) *Eur. J. Biochem. 131*, 345.

Grisebach, H. (1978) *Adv. Carbohydr. Chem. Biochem. 35*, 81.

Hajdu, J., Acharya, K. R., Stuart, D. I., McLaughlin, P. J., Barford, D., Oikonomakos, N. G., Klein, H., & Johnson, L. N. (1987) *EMBO J. 6*, 539.

Han, O., Miller, V. P., & Liu, H.-w. (1990) *J. Biol. Chem. 265*, 8033.

Hanessian, S. (1966) *Adv. Carbohydr. Chem. Biochem. 21*, 143.

Health, E. C., & Elbein, A. D. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 1209.

Holt, J. G., & Krieg, N. R. (1984) *Bergey's Manual of Systematic Bacteriology*, Vol. 1, p 498, Williams & Wilkins, Baltimore.

Kalckar, H. (1947) *J. Biol. Chem. 167*, 461.

Khwaja, T. A., Reese, C. B., & Stewart, J. C. M. (1970) *J. Chem. Soc. C*, 2092.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Liu, L.-d., & Liu, H.-w. (1989) *Tetrahedron Lett.* 35.

Lo, S. F., Yu, Y., Yang, D.-y., & Liu, H.-w. (1989) *Carbohydr. Res. 189*, 368.

Lonngren, J., & Svensson, S. (1974) *Adv. Carbohydr. Chem. Biochem. 29*, 41.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Lüderitz, O., Staub, A. M., & Westphal, O. (1966) *Bacteriol. Rev. 30*, 192.

Madsen, N. B., & Withers, S. G. (1986) in *Vitamin B_6 Pyridoxal Phosphate, Chemical, Biochemical, and Medical Aspects* (Dolphin, D., Poulson, R., & Avramovic, O., Eds.) Part B, p 355, Wiley-Interscience, New York.

Matsuhashi, S., Matsuhashi, M., & Strominger, J. L. (1966a) *J. Biol. Chem. 241*, 4267.

Matsuhashi, S., Matsuhashi, M., Brown, J. G., & Strominger, J. L. (1966b) *J. Biol. Chem. 241*, 4283.

Moffatt, J. G., & Khorana, H. G. (1961) *J. Am. Chem. Soc. 83*, 649.

Nair, V., & Turner, G. A. (1984) *Lipids 19*, 804.

Nauta, W. J. H., & Glygax, P. A. (1951) *Stain Technol. 26*, 5.

Nordin, J. H., Salo, W. L., Bevill, R. D., & Kirkwood, S. (1965) *Anal. Biochem. 13*, 405.

Palm, D., Klein, H. W., Schinzel, R., Buehner, M., & Helmreich, E. J. M. (1990) *Biochemistry 29*, 1099.

Rubenstein, P. A., & Strominger, J. L. (1974a) *J. Biol. Chem. 249*, 3776.

Rubenstein, P. A., & Strominger, J. L. (1974b) *J. Biol. Chem. 249*, 3782.

Ruth, J. L., & Cheng, Y.-C. (1981) *Mol. Pharmacol. 20*, 415.

Shih, Y., Yang, D.-y., Weigel, T. M., & Liu, H.-w. (1990) *J. Am. Chem. Soc. 112*, 9652.

Stevens, J. D. (1972) in *Methods in Carbohydrate Chemistry* (Whistler, R. L., & BeMiller, J. N., Ed.) Vol. VI, p 123, Academic Press, New York.

Vesterberg, O. (1971) *Biochem. Biophys. Acta 243*, 345.

Voet, J., Indenlang, P. M., Blank, T., Ulevitch, R., Kallen, R., & Dunathan, H. (1973) *J. Biol. Chem. 248*, 841.

Waravdekar, V. S., & Saslaw, L. D. (1959) *J. Biol. Chem. 234*, 1945.

Ward, D. D. (1985) *Milchwissenschaft 40*, 583.

Weigel, T. M., Miller, V. P., & Liu, H.-w. (1992) *Biochemistry* (following paper in this issue).

Westphal, O., & Lüderitz, O. (1960) *Angew. Chim. 72*, 881.

Williams, N. R., & Wander, J. D. (1980) in *The Carbohydrates: Chemistry and Biochemistry* (Pigman, W., & Horton, D., Eds.) Vol. 1B, p 761, Academic Press, New York.

Withers, S. G., Street, I. P., & Percival, M. D. (1988) in *Fluorinated Carbohydrates, Chemical and Biochemical Aspects* (Taylor, N. F., Ed.) ACS Symposium Series 374, p 59, American Chemical Society, Washington, DC.

Wright, J. A., Taylor, N. F., Brunt, R. V., & Brownsey, R. W. (1972) *J. Chem. Soc., Chem. Commun.*, 691.