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# CDP-6-Deoxy-$\Delta^{3,4}$-Glucoseen Reductase from *Yersinia pseudotuberculosis*: Enzyme Purification and Characterization of the Cloned Gene

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**The 3,6-dideoxyhexoses, usually confined to the cell wall lipopolysaccharide of gram-negative bacteria, are essential to serological specificity and are formed via a complex biosynthetic pathway beginning with CDP-D-hexoses. In particular, the biosynthesis of CDP-ascarylose, one of the naturally occurring 3,6-dideoxyhexoses, consists of five enzymatic steps, with CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase ($E_3$) participating as the key enzyme in this catalysis. This enzyme has been previously purified from *Yersinia pseudotuberculosis* by an unusual procedure (protocol I) including a trypsin digestion step (O. Han, V. P. Miller, and H.-W. Liu, J. Biol. Chem. 265:8033–8041, 1990). However, the cloned gene showed disparity with the expected gene characteristics, and upon expression, the resulting gene product exhibited no $E_3$ activity. These findings strongly suggested that the protein isolated by protocol I may have been misidentified as $E_3$. A reinvestigation of the purification protocol produced a new and improved procedure (protocol II) consisting of DEAE-Sephacel, phenyl-Sepharose, Cibacron blue A, and Sephadex G-100 chromatography, which efficiently yielded a new homogeneous enzyme composed of a single polypeptide with a molecular weight of 39,000. This highly purified protein had a specific activity nearly 8,000-fold higher than that of cell lysates, and more importantly, the corresponding gene (*ascD*) was found to be part of the ascarylose biosynthetic cluster. Presented are the identification and confirmation of the $E_3$ gene through cloning and overexpression and the culminating purification and unambiguous assignment of homogeneous $E_3$. The nucleotide and translated amino acid sequences of the genuine $E_3$ are also presented.**

The deoxy sugars, long known as an important class of carbohydrate, are found ubiquitously in nature (7, 16, 47). They are formally derived from common sugars by the displacement of one or more hydroxyl groups with hydrogens. Such a substitution generally induces a dramatic alteration of the biological role of the resulting sugar and is responsible for a fundamental change in the metabolism of the product. Particularly notable are the 3,6-dideoxyhexoses found in the lipopolysaccharides (LPS) of gram-negative bacteria (2). Since LPS is the major surface antigen of the gram-negative cell envelope, this class of dideoxyhexose as the nonreducing end group of LPS has been identified as the key antigenic determinant. In addition, they have also been found to contribute to the serological specificity of many immunologically active polysaccharides (5, 24, 33, 46). Inspired by their specific association with LPS and the intriguing nature of their immunological effects, substantial effort has been devoted to exploring their biosynthetic formation (10, 13). However, although the nature of the precursors of these dideoxy sugars has been well defined and possible routes for their formation have been postulated (17, 26), the only pathway that has been studied at the enzymatic level is the biosynthesis of CDP-ascarylose.

As depicted in Fig. 1, the proposed ascarylose biosynthetic sequence starts with the coupling of glucose-1-phosphate (compound 2) and CTP by D-glucose-1-phosphate cytidylyltransferase ($E_p$) to give CDP-D-glucose (compound 3). This is followed by an intramolecular oxidation-reduction catalyzed by $NAD^+$-dependent CDP-D-glucose 4,6-dehydratase ($E_{od}$). The resulting product, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose (compound 4), is then converted to 3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (compound 7) in two consecutive steps mediated by CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase ($E_1$) and CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase ($E_3$), both of which have been isolated from *Pasturella pseudotuberculosis* (11). The final steps, of which little is known, may be catalyzed by an epimerase and a reductase, a situation similar to that found in the biosynthesis of many 6-deoxyhexoses (40). The culminating step of this biosynthetic sequence is the C-3 deoxygenation catalyzed by $E_1$, a pyridoxamine 5′-phosphate (PMP)-linked catalyst, and the NAD(P)H-dependent $E_3$.

While recent studies of the catalytic role of $E_1$ have revealed that this dehydrase, despite its having evolved a unique role for the PMP cofactor, retains all the essential elements of catalysis common to other vitamin $B_6$ phosphate-dependent enzymes (34, 38, 44, 45), the detailed mechanism of $E_3$ has remained largely unresolved. In light of $E_3$'s dependence on NAD(P)H, this enzyme is expected to deliver a hydride from its cofactor to reduce the glucoseen intermediate 5. However, incubation with [4-$^3H_2$]NADPH resulted in no tritium incorporation in product 7 or the regenerated PMP coenzyme, suggesting an indirect hydride transfer or a stereospecific washout. More-

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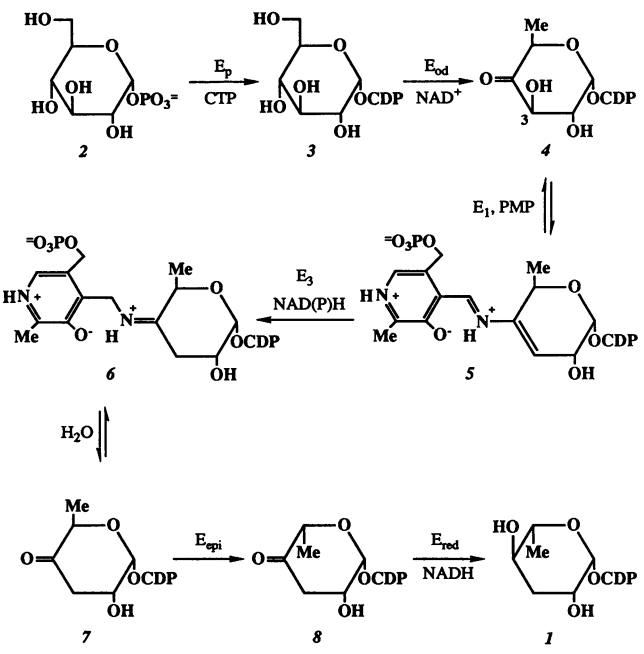


FIG. 1. Biosynthesis of ascarylose.

over, both the 4*R* and 4*S* hydrogens of NADPH were found to be labile in this reduction step, making $E_3$ the only enzyme that lacks prochiral recognition of the two diastereotopic methylene hydrogens on the coenzyme (35). Since the net distance for an effective hydride transfer is approximately 0.55 Å (0.055 nm) (14, 48), for $E_3$ to directly reduce the $E_1$-bound intermediate 5, the active sites of these two enzymes must thus be brought into close proximity. This scenario, however, invokes a potentially substantial and unfavorable steric interference. It is also important to note that $E_3$ is capable of oxidizing NAD(P)H in the presence of oxygen (12, 35). However, unlike other known NAD(P)H oxidases, which are flavin dependent, the reported $E_3$ does not contain any chromophoric group with an absorption above 300 nm. All of these unusual properties of $E_3$ are not readily explicable and thus challenge our understanding of this important biological reduction.

In an effort to elucidate the mechanism by which the sugar deoxygenation and/or the coenzyme NADH oxidation are affected, we have purified an $E_3$ equivalent (14, 15), by monitoring its NADH:dichlorophenolindophenol (DCPIP) oxidoreductase activity, from *Yersinia pseudotuberculosis*. This enzyme showed no obvious absorption above 300 nm and contained no metal constituents, signifying again the absence of any common cofactors as reported earlier by Gonzalez-Porque and Strominger (12). Since the oxygen metabolite was determined to be $H_2O_2$, the formation of which is stoichiometrically proportional to the amount of NADH consumed, this enzyme-catalyzed NADH oxidation is clearly an overall two-electron redox process. Our studies also revealed $O_2^{\cdot -}$ as the hypothetical reducing intermediate, suggesting that $H_2O_2$ formation is not a direct two-electron reduction of molecular oxygen but is instead a one-electron reduction followed by dismutation of the nascent superoxide. This finding of $E_3$'s two-electron/one-electron switching capability provides compelling evidence that it operates through a radical mechanism and also suggests the participation of an enzyme-bound organic cofactor which mediates the obligatory two-electron/one-electron conversion. The mechanistic revision of the reduction step from a hydride transfer to an electron relay process not only explains the lack of direct hydride transfer from NAD(P)H in the reduction of the glucoseen-PMP complex 5 but also alleviates the spatial and steric constraints imposed on the delivery of reducing equivalents from $E_3$ to $E_1$.

There is little doubt that further mechanistic studies of $E_3$ require, foremost, the resolution of the structure and function of the putative organic coenzyme. Unfortunately, past attempts to isolate this cofactor were impeded by the limited availability of $E_3$. In our recent effort to develop a large-scale purification scheme for $E_3$, we were surprised to discover that the previously isolated $E_3$ was a mixture of two proteins, with the desired enzyme being a minor component (27). In this paper, we report molecular evidence supporting this unexpected finding, the isolation of the genuine $E_3$ from *Y. pseudotuberculosis*, and the cloning, sequencing, and expression of the $E_3$ gene.

## MATERIALS AND METHODS

**General.** *Y. pseudotuberculosis* was kindly provided by Otto Lüderitz, Max Planck Institute for Immunobiology, Freiburg, Germany. *Escherichia coli* DH5α and HB101 were obtained from Bethesda Research Laboratories (Gaithersburg, Md.). DEAE-Sephacel, phenyl-Sepharose, and Sephadex G-100 were purchased from Pharmacia (Piscataway, N.J.). Zeta-Probe blotting membranes were products of Bio-Rad (Richmond, Calif.), and Duralose membranes, λ EMBL3 (and accompanying *E. coli* host strains), and Gigapack II packaging extracts were from Stratagene (La Jolla, Calif.). The Sequenase version 2.0 DNA sequencing kit, M13 sequencing primers, 7-deaza-dGTP sequencing mixtures, all restriction and DNA-modifying enzymes, and pUC plasmids were purchased from United States Biochemical Corp. (Cleveland, Ohio). Nitrocellulose and Elutip-D minicolumns were products of Schleicher & Schuell (Keene, N.H.), electrophoretic reagents were from Beckman Instruments (Fullerton, Calif.), and [γ-$^{32}$P]ATP (6,000 Ci/mmol) as well as [α-$^{35}$S]dATP (>1,000 Ci/mmol) were purchased from Amersham (Arlington Heights, Ill.). The $E_{od}$ and $E_1$ used in assay procedures were isolated from the same *Y. pseudotuberculosis* strain (44, 49) and were kindly supplied by Yuan Yu and Theresa Weigel of this research group. The protease inhibitors used in enzyme purification were prepared as previously described (15). All protease inhibitors, molecular weight standards, Cibacron blue 3GA-agarose (blue A), and most biochemicals were purchased from Sigma (St. Louis, Mo.). All other chemicals were of analytical reagent grade or the highest quality commercially available. Methods and protocols for recombinant DNA manipulations are generally referenced by Ausubel et al. (3) and Maniatis et al. (25).

**Enzyme $E_3$ assays.** Four different methods were developed to determine the activity of enzyme $E_3$. Since the substrate of $E_3$ is not readily available, it has to be prepared in situ from CDP-D-glucose prior to each assay. The preparation involved the incubation of purified CDP-D-glucose 4,6-dehydratase (7 μg) with CDP-D-glucose (0.25 μmol) and $NAD^+$ (0.25 μmol) in 110 μl of 10 mM potassium phosphate buffer (pH 7.5) at 37°C for 30 min. The dehydratase product formation was determined by measuring its characteristic absorption at 320 nm (ε, 6,500 $M^{-1}$ $cm^{-1}$) under alkaline conditions (31). An aliquot of this solution was then added to a mixture of $E_1$ and $E_3$ with the necessary cofactors to determine the sugar reductase activity of $E_3$.

**(i) Method 1. TBA assay.** The sugar reductase activity of $E_3$

TABLE 1. Summary of enzyme $E_3$ purification from *Y. pseudotuberculosis* by protocol II

| Purification step | Total protein (mg) | Total activity (μmol of DCPIP consumed/min) | Sp act (U/mg of protein) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extracts[a] | 170,000 | 505 | <0.003 | 1 | 100 |
| Streptomycin sulfate | ND[b] | ND | ND | | |
| Ammonium sulfate | 11,460 | 91.5 | 0.042 | 14 | 95.3 |
| DEAE-Sephacel | 1,056 | 16.9 | 0.13 | 43 | 26.7 |
| Phenyl-Sepharose | 19.3 | 4.8 | 2.0 | 667 | 7.5 |
| Blue A | 1.5 | 16.5 | 11.1 | 3,700 | 3.3 |
| Sephadex G-100 | 0.34 | 8.0 | 23.9 | 7,960 | 1.6 |

[a] Obtained from 520 g (wet weight) of cells.
[b] ND, not determined.

was estimated by a procedure analogous to an $E_1$ assay developed earlier by us (44). A routine assay involved the incubation of appropriate amounts of $E_1$ and $E_3$ with an aliquot of the aforementioned dehydratase reaction solution (50 μl), PMP (10 nmol), and NADH (100 nmol) in a total volume of 200 μl of 50 mM potassium phosphate buffer (pH 7.5) at 27°C for 1 h. The resulting product, after reduction ($NaBH_4$, 0.1 mmol, 30 min), hydrolysis (2 N $H_2SO_4$, 100°C, 5 min), and degradation (0.025 N periodic acid, 55°C, 20 min), was treated with thiobarbituric acid (TBA) reagent (6% in water, pH 2, 100°C, 15 min), resulting in the development of a characteristic pink chromophore (8, 43) with maximum absorption at 532 nm (ε, 159,200 $M^{-1}$ $cm^{-1}$) (29).

**(ii) Method 2. GC-MS assay.** The $E_3$ product was generated by using an in vitro system similar to that described above for the TBA assay. After being quenched by $NaBH_4$ (10 μmol, 30 min), the reduced sugar products were boiled for 10 min in the presence of HCl (pH 2.0), neutralized to pH 7.0 with NaOH, and lyophilized to dryness. The solid residue was redissolved in $NH_4OH$ (0.5 M, 600 μl) and treated with a solution of $NaBH_4$ in dimethyl sulfoxide (20 mg in 1 ml) at 40°C for 1.5 h, and the reaction was quenched with glacial acetic acid. Acetic anhydride (6 ml) and 1-methylimidazole (2.5 μmol) were added, and the resulting mixture was stirred for 1 h at room temperature. Routine workup followed by evaporation in vacuo gave a residue which was directly subjected to gas chromatography-mass spectrometry (GC-MS) analysis (44).

**(iii) Method 3. NADH:DCPIP oxidoreductase activity.** The NADH:DCPIP oxidoreductase activity of $E_3$ was determined, as previously described (15), by measuring the rate of electron transfer from NADH to DCPIP (600 nm; ε, 22,000 $M^{-1}$ $cm^{-1}$).

**(iv) Method 4. NADH oxidase activity.** The NADH oxidase activity of $E_3$ was measured by monitoring the rate of the reduction of NADH absorption at 340 nm (ε, 6,220 $M^{-1}$ $cm^{-1}$).

**Protein determination.** Protein concentration was determined by the method of Lowry et al. (23), using bovine serum albumin as the standard. $A_{280}$ was routinely used to monitor column fractions. This method yielded values ca. 10% higher than those obtained by the Lowry assay on identical samples.

**Growth of cells.** An overnight culture of *Y. pseudotuberculosis* was grown in tryptic soy broth medium (3%, 9 liters) in an incubator-shaker (Lab-Line) with vigorous agitation (140 rpm) at 28°C. This inoculum culture was then diluted 12-fold into a 110-liter fermentor (Stainless Steel Products) and grown at 28°C, 100 rpm, pH 7.5. The culture was harvested in the early to mid-logarithmic phase by centrifugation. A typical yield was 400 to 500 g (wet weight) of cells per 110 liters of culture. All cell culture and harvesting procedures were performed at the Biological Process Technology Institute, University of Minnesota.

**Enzyme purification.** All operations were carried out at 4°C. To preclude enzyme inhibition by exogenous trace metals during purification, all buffers contained 1 mM EDTA unless otherwise specified. The results of the purification are summarized in Table 1. It should be noted that enzyme purified by this new protocol (protocol II) is different from the protein isolated by the old procedure (protocol I) which included a trypsin digestion step (15).

**(i) Step 1. Crude extracts.** Cells from 110 liters of culture (520 g [wet weight]) were resuspended in four times their volume (2.1 liters) of 50 mM potassium phosphate buffer (pH 7.5), and then the protease inhibitor solution was added. The cells were disrupted by sonication for 2 min in batches of 200 ml, at 45-s intervals, with a VirSonic model 300 sonicator at 70% output. The temperature of the extracts was carefully controlled so as not to exceed 5°C during this process. Cellular debris was removed by centrifugation (4,420 × *g*, 20 min). The supernatant solution was diluted with the same buffer to 2.9 liters and was designated the crude extract.

**(ii) Step 2. Streptomycin sulfate treatment.** Streptomycin sulfate (5% aqueous solution, 550 ml) was added drop by drop to the crude extract to a final concentration of 0.8%. After standing for an additional hour with stirring, the precipitate was eliminated by centrifugation at 14,000 × *g* for 2 h. The supernatant solution (3.3 liters) was diluted with 1 M potassium phosphate buffer (pH 7.5, 330 ml) and carried on to the next step.

**(iii) Step 3. Ammonium sulfate precipitation.** Solid ammonium sulfate was slowly added to the protein solution from step 2 to give a final concentration of 65% saturation. After addition was complete, the cloudy solution was stirred for another 10 h. The precipitated proteins were collected by centrifugation (4,400 × *g*, 20 min) and were redissolved in a minimum amount of 50 mM potassium phosphate buffer (pH 7.5). This solution was dialyzed against 40 liters of the same buffer for 24 h with four changes of buffer.

**(iv) Step 4. DEAE-Sephacel column chromatography.** The solution from step 3 (1 liter) was applied to a column of DEAE-Sephacel (4.5 by 40 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.5). The column was washed with the same buffer (1 liter) and then eluted with a linear gradient of potassium phosphate (50 to 200 mM, pH 7.5, 5-liter total). Fractions of 16 ml were collected throughout. The contents of fractions 105 to 125 were pooled and concentrated to 32 ml via an Amicon ultrafiltration unit (PM-30 membrane).

**(v) Step 5. Phenyl-Sepharose column chromatography.** The enzyme solution from step 4 was adjusted to 0.9 M KCl by the addition of 2.5 M KCl in 50 mM potassium phosphate buffer

(18 ml). This solution was then applied to a phenyl-Sepharose column (2.5 by 45 cm) which was preequilibrated with KCl (0.9 M) in potassium phosphate buffer (50 mM, pH 7.5). The column was washed with 0.9 M KCl buffer (90 ml) and then eluted with a linear gradient of 0.9 to 0 M KCl (500 ml) in the same phosphate buffer. Fractions of 6 ml were collected. The contents of fractions 72 to 88 were pooled and concentrated (YM-10 membrane).

**(vi) Step 6. Blue A column chromatography.** The material from step 5 was loaded onto a column of blue A (2 by 10 cm) and incubated for 45 min. Elution was then started with a linear gradient between 0 and 0.7 M KCl in 50 mM potassium phosphate buffer (140 ml each). Fractions of 2 ml were collected throughout. Active fractions (fractions 38 to 44) were combined and concentrated to 1.6 ml (YM-10 membrane).

**(vii) Step 7. Sephadex G-100 column chromatography.** The enzyme solution from step 6 was chromatographed on a column (1.5 by 170 cm) of Sephadex G-100 equilibrated with 50 mM potassium phosphate buffer (pH 7.5). The column was then washed with the same buffer. Fractions of 3 ml were collected, and the desired protein was found in fractions 38 to 46. The active fractions were combined, concentrated (YM-10 membrane), and stored at −85°C.

**SDS-PAGE.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to monitor purification of $E_3$ and estimate the relative molecular mass of the protein. Electrophoresis was carried out at room temperature at a constant 120 V (~30 mA) with a 6% stacking gel and 13% resolving gel in the discontinuous buffer system of Laemmli (21). Prior to electrophoresis, the samples were incubated at 100°C for 10 min in a solution containing 0.5% SDS and 5% 2-mercaptoethanol. Gels were stained with the preparation of Vesterberg (41) and destained with acetic acid-ethanol-water (15:20:165 by volume).

**Molecular weight determination.** The subunit molecular weight was determined by SDS-PAGE as described by Laemmli (21). The molecular weight of the native enzyme was determined by gel filtration performed on a column of Sephadex G-100 (1.5 by 170 cm). The column was calibrated by separate chromatographic runs with protein standards. The molecular weight of the purified protein was estimated by the method of Andrews (1).

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

**Isoelectric focusing.** The isoelectric pH of $E_3$ was determined by a Pharmacia Phast-System instrument. A preformed PhastGel IEF 3-9 plate was used as specified by the manufacturer. The protein bands were visualized by silver staining (30).

**Oligonucleotide probes.** The mixed oligonucleotide probes used in the hybridization, SFL2 and SFL4, were designed on the basis of the respective amino acid sequence near the amino terminus of each protein purified by either the old method (protocol I [15]) or the new procedure described herein (protocol II) while considering the necessary degeneracies in the genetic code. The synthetic probes SFL2 (5′-GC[TC]T G[ATGC]AC[AG]TA[TC]TG[TC]TG[AG]TA-3′), SFL4 (5′-AA[CT]GT[ATGC]AA[AG]CT[ATGC]CA[CT]CC-3′), and JST1 (5′-AA[AG]ACCGT[TC]AC[AGTC]TT[TC][GT]C[AG TC]AA-3′, derived from the N-terminal amino acid sequence of the purified $E_{od}$) were labeled to a specific activity of $7 \times 10^8$ dpm/μg with [γ-$^{32}$P]ATP and T4 polynucleotide kinase prior to hybridization.

**Genomic DNA isolation and hybridization.** The *Y. pseudotuberculosis* genomic DNA, isolated by a modified procedure of Ausubel et al. (3), was digested with restriction enzymes (*Hin*dIII for YPT1 and *Bam*HI for YPT2), electrophoresed through a 1% agarose gel, and transferred to a Zeta-Probe membrane. Membranes containing blotted DNA were incubated with the labeled DNA probe overnight at 42°C in 1 mM EDTA–7% SDS–5× Denhardt's solution–0.5 M $Na_2HPO_4$ (pH 7.2) containing 0.5 mg of denatured salmon sperm DNA per ml. The membranes were then washed twice for 30 min each time in 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–1% SDS at 23°C, washed twice for 30 min each time in 0.1× SSC–0.1% SDS at 42°C, and subjected to autoradiography (−80°C).

**Subgenomic library construction.** The subgenomic library was constructed by completely digesting the genomic DNA of *Y. pseudotuberculosis* with *Hin*dIII and then size selecting the 3- to 4-kb fragments, which were identified by Southern hybridization with the mixed probe SFL4. After purification and treatment with alkaline phosphatase, the inserts (300 ng) were ligated with *Hin*dIII-digested λ ZAPII vector (1 μg) overnight at 4°C. The resulting mixture was packaged by using Gigapack II Plus packaging extracts and plated onto bacterial lawns as instructed by the manufacturer. The resulting plaques were bound to nitrocellulose membranes and screened by using labeled SFL4 probe. After transblotting, the filter was washed twice at room temperature (30 min each time) in 1% SDS–2× SSC and twice at 45°C (45 min each time) in 1% SDS–0.1× SSC. The isolated recombinant was then replated, rescreened, and found to be identical to a subgenomic library, pYPT1, constructed on the basis of the hybridization with JST1 derived from $E_{od}$ (27, 40).

Analogously, the subgenomic library YPT2 was constructed by size selection of 15- to 23-kb fragments of *Bam*HI-digested genomic DNA of *Y. pseudotuberculosis*, which was identified by Southern hybridization with the mixed probe SFL2. The subsequent ligation with commercially digested *Bam*HI λ EMBL3 arms (1 μg) and packaging by using Gigapack II Plus packaging mix were done as instructed by the manufacturer. The resulting plaques were bound to Duralose membranes and screened by using the SFL2 probe. The isolated recombinant was then replated, rescreened, and labeled pYPT2.

**Restriction analysis and plasmid construction.** Restriction mapping of the cloned DNA insert was accomplished through the analysis of DNA size patterns on 0.8% agarose gels subsequent to single, double, or triple digestion with various restriction endonucleases. Comparison of patterns with known reference points within the vector DNA allowed for the construction of a linear map of the restriction sites within the cloned insert. The relative position of the $E_3$ gene (*ascD*) within the cloned insert was determined by Southern blotting with radiolabeled SFL4 and DNA sequencing. The expression construct pSFL28 was built by directionally subcloning the insert of pYPT1 into the *Bam*HI-*Sac*I sites of pUC19. Standard recombinant DNA techniques were used for all plasmid constructions.

**DNA sequencing.** Plasmids were isolated from *E. coli* by the alkali method (4). Nucleotide sequences were determined directly from double-stranded templates by the dideoxynucleotide chain termination method of Sanger et al. (36), using Sequenase version 2.0. The nucleotide analog 7-deaza-dGTP was substituted for dGTP in all sequencing manipulations. Sequencing was completed by using nested deletions (18) of pSFL28 generated by exonuclease III, allowing the use of both

M13 forward and reverse universal sequencing primers as well as designed synthetic primers to complete desired regions. Computer analysis of the resulting sequence information was performed with IntelliGenetics software (release 5.4).

**Nucleotide sequence accession number.** The *ascD* sequence will appear in the EMBL/Genbank/DDBJ nucleotide sequence data libraries under accession number L25594.

## RESULTS

**Cloning and overexpression of the gene coding for the protocol I-purified $E_3$.** Purification of $E_3$ by a previously reported procedure (protocol I) yielded amounts of protein sufficient only for preliminary mechanistic studies (15). To facilitate further characterization of this enzyme, cloning and overexpression of the particular gene were pursued. Oligonucleotide (SFL2) screening of 4,000 plaques of YPT2 resulted in the isolation of 65 positive clones (1.63%), and analysis of 5 randomly selected positive plaques revealed identical restriction fragment patterns of the inserted DNA. Further Southern blotting of this DNA led to the isolation of a 3.2-kb *Kpn*I fragment containing the gene coding for the putative $E_3$, which was subsequently ligated into pUC18 (pSFL31) for exonuclease III digestion. Ligation of these deleted pSFL31 inserts into pUC19 followed by transformation and SDS-PAGE analysis revealed one particular construct, pYL36, which readily overexpressed the desired gene product. However, the expressed protein showed no $E_3$ activity by either the GC-MS or NADH:DCPIP oxidoreductase assay. The negative results in the assay for $E_3$ activity from the overexpressed $E_3$ gene clone suggested that the protein isolated by protocol I may have been misidentified as $E_3$.

**Enzyme purification.** Although $E_3$ had been previously isolated by an unusual purification sequence (protocol I), the cloning results reported above strongly suggested that the resulting enzyme, which deceivingly appeared homogeneous at first, was still a mixture of two proteins in which $E_3$ was only a minor component. To further purify the desired enzyme, chromatography on several affinity reagents that often bind to nicotinamide-utilizing enzymes, 2′,5′-ADP–Sepharose (39) and $NAD^+$-agarose (22), was attempted, albeit with no success. Fortunately, it was found that the dye ligand blue A successfully bound and selectively eluted $E_3$. Thus, a blue A column following phenyl-Sepharose was incorporated in the purification sequence. Such a modification rendered the tryptic digestion step unnecessary and, in effect, greatly simplified the purification procedure. As shown in Table 1 and Fig. 2, purification by blue A affinity chromatography followed by gel filtration effected a 7,960-fold enrichment of $E_3$, which is now truly homogeneous. In retrospect, a close examination by SDS-PAGE of the protein purified by the earlier protocol (15) occasionally revealed the presence of two protein bands differing by ~1 to 2 kDa (Fig. 2, lane F). This phenomenon was, however, inconsistent among preparations and was most often barely discernible. Chromatography on blue A apparently removed the major protein contaminant that was previously misidentified as $E_3$.

**Properties of $E_3$. (i) Molecular weight.** The molecular weight of the newly purified and homogeneous $E_3$ was estimated by gel filtration to be 39,000. SDS-PAGE also showed a single band with a molecular weight of 39,000. Thus, the native enzyme is a monomeric protein consisting of a single polypeptide chain.

**(ii) Isoelectric point.** Purified $E_3$ was subjected to electrophoresis on an analytical isoelectric focusing gel as described in Materials and Methods. The pI of $E_3$ was determined to be 4.7.

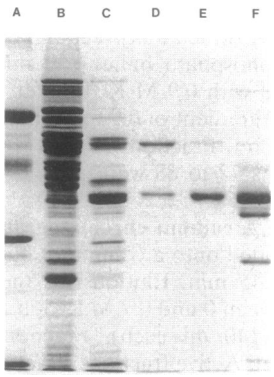


FIG. 2. SDS-PAGE of $E_3$ isolated from *Y. pseudotuberculosis* by protocol II after each step of the purification procedure. Lanes: A, molecular weight standards α-lactalbumin (14,200), carbonic anhydrase (29,000), ovalbumin (45,000), and bovine serum albumin (66,000); B, DEAE-Sephacel chromatography (step 4); C, phenyl-Sepharose chromatography (step 5); D, blue A chromatography (step 6); E, Sephadex G-100 chromatography (step 7); F, $E_3$ partially purified by protocol I. The major protein band consists of two overlapping bands with the desired $E_3$ as a minor component.

**(iii) UV-visible spectrum.** The electronic spectrum of the newly purified $E_3$ has significant adsorption above 300 nm, suggesting the existence of a flavin and possibly an iron-sulfur center.

**(iv) Substrate specificity.** The competence of NADPH as an alternate substrate for $E_3$ was tested by replacing NADH with NADPH in the NADH:DCPIP oxidoreductase activity assay. The rate of the latter was less than 5% of that occurring when NADH was used under identical conditions, indicating that the $E_3$ enzyme preferentially utilizes NADH as a substrate.

**(v) Kinetic parameters for the NADH:DCPIP oxidoreductase activity.** As an NADH:DCPIP oxidoreductase, $E_3$ displayed normal Michaelis-Menten saturation kinetics. The $K_m$ of 37 μM for NADH and $V_{max}$ of 47 μmol $min^{-1}$ $mg^{-1}$ for this catalysis were determined by plotting the data according to the method of Lineweaver and Burk.

**(vi) Alternate electron acceptors.** Enzyme $E_3$ can utilize a variety of alternate electron acceptors for the oxidation of NADH. As shown in Table 2, potassium ferricyanide, ferricytochrome *c*, and DCPIP were the most efficient electron acceptors tested. Interestingly, the quinones menadione and ubiquinone-0 are also good electron acceptors. Flavins, riboflavin, flavin mononucleotide, and flavin adenine dinucleotide are poor electron acceptors, suggesting that if $E_3$ contains a flavin cofactor, it is tightly bound and virtually remains in the active site throughout the purification procedure.

**Gene expression and protein purification of recombinant $E_3$.** A previously produced clone pYPT1 was found to contain the genuine $E_3$ gene (*ascD*), as judged from its consistent hybridization with SFL4. The relative position of the $E_3$ gene within the insert of pYPT1 was located by Southern blotting and DNA sequencing. The initiation codon of the *ascD* gene is only 270 bp away from the vector sequence of pYPT1, and the insert has an opposite orientation to the *lac* promoter of the vector. To match the orientation of the *ascD* gene to the direction of transcription of the *lac* promoter, the *ascD* gene was inverted by directionally subcloning the insert of pYPT1 into the *Bam*HI-*Sac*I sites of pUC19 (Fig. 3). The resulting construct (pSFL28), after transformation into *E. coli* DH5α, readily expressed the desired gene product, as observed by SDS-PAGE. The recombinant $E_3$ expressed in *E. coli*, under

TABLE 2. Utilization of alternate electron acceptors by homogeneous $E_3$ during NADH oxidation[a]

| Added electron acceptor | Concn (μM) | Wavelength observed (nm) | Redox potential ($E'_0$, mV)[b] | Relative velocity (μmol of $2e^-$/min/mg)[c] |
|---|---|---|---|---|
| $O_2$ | 1,300[d] | 340[e] | 820[f] | 1 |
| $K_3Fe(CN)_6$ | 50 | 340 | 420[g] | 41 |
| Cytochrome *c* | 50 | 550 | 250 | 84 |
| DCPIP | 50 | 600 | 220 | 84 |
| Menadione | 50 | 340 | 0.00[h] | 59 |
| Ubiquinone-0 | 50 | 340 | 100 | 52 |
| Ascorbic acid | 530 | 340 | 60 | 0 |
| Riboflavin | 50 | 340 | −200 | 26 |
| Flavin adenine dinucleotide | 50 | 340 | −180 | 9 |
| Flavin mononucleotide | 50 | 340 | −220 | 9 |
| Glutathione | 530 | 340 | −230 | 0 |
| Methylviologen | 40 | 601 | −550 | 0 |

[a] A typical assay contained 44 pmol of the purified enzyme, 100 nmol of NADH, and the indicated amount of added electron acceptor in 1.5 ml of 50 mM potassium phosphate buffer (pH 7.5).
[b] Taken from Walsh (42) unless specified otherwise.
[c] See Materials and Methods for assay conditions.
[d] Buffer saturated with $O_2$ at 30°C was used. The value was recalculated from Sendroy et al. (37).
[e] Reaction was monitored by the consumption of NADH.
[f] Potential corresponds to the reduction of $O_2$ to $H_2O_2$.
[g] Taken from O'Reilly (32).
[h] Taken from Dawson et al. (9).

control of the *lac* promoter, is produced at levels nearly 30 times higher than those in *Y. pseudotuberculosis*, as deduced from amounts of purified proteins. A summary of the five-step purification for the recombinant protein is presented in Table 3. This simplified procedure typically yields >10 mg of purified protein from 6 liters of cell culture of the *E. coli* DH5α (pSFL28) system.

**Properties of the overexpressed $E_3$.** Characterization of the overexpressed $E_3$ from *E. coli* revealed properties identical to those of the wild-type enzyme purified from *Y. pseudotuberculosis.*

**Nucleotide and amino acid sequences of $E_3$.** The nucleotide sequence of the $E_3$ gene (*ascD*) and the deduced amino acid sequence of the corresponding protein are presented in Fig. 4. The DNA sequence predicts a protein of 328 amino acids with a molecular weight of 36,160. This predicted molecular weight is in agreement with the molecular weight of 39,000 determined by SDS-PAGE and gel filtration. While translation is expected to start from ATG, the mature protein must have been posttranslationally modified, since a serine residue instead of a methionine was found as the N-terminal amino acid of the purified $E_3$. Although expression of the protein is under the control of the *lac* promoter in *E. coli* (DH5α), analysis of the *ascD* gene revealed a sequence, 5′-TAATAT-3′, which resembles the *E. coli* consensus Pribnow box (5′-TATAAT-3′) and is positioned 16 to 21 bp upstream from the translational start codon. A sequence resembling the −35 motif of consensus *E. coli* promoters (5′-TTGACA-3′) could also be identified about 20 bp further upstream (5′-TTGAGT-3′). An inverted repeat sequence, located immediately after the stop codon, could act as a transcriptional termination signal or perhaps an mRNA stabilizer. The latter appears to be more likely because of the presence of another open reading frame (ORF) beginning 10 to 20 bp from the stop codon of the *ascD* gene.

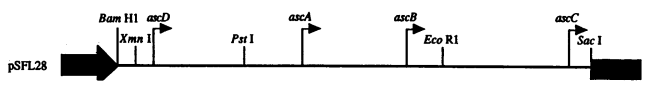


FIG. 3. Partial restriction map of the $E_3$ expression construct (pSFL28). The large arrow indicates the direction of the *lac* promoter, and small arrows indicate the direction of transcription of the genes. The *ascD*, *ascA*, *ascB*, and *ascC* genes encode $E_3$, $E_p$, $E_{od}$, and $E_1$, respectively. Their identities have been established by specific activity assay and N-terminal amino acid analysis of the expressed gene products (40). The restriction sites are represented by thin lines.

## DISCUSSION

Although $E_3$ has been isolated by Gonzalez-Porque and Strominger from *P. pseudotuberculosis* (12), its purification has always been complicated by the lack of a facile and sensitive assay to determine its activity. To circumvent this problem, we have developed a few convenient and sensitive methods to assay $E_3$ activity. Among these, the particular method exploiting $E_3$'s capability to utilize DCPIP as electron acceptor for the oxidation of NADH affords the simplest means of assaying this enzyme's activity. Purification of $E_3$ described herein relied upon the convenient NADH:DCPIP oxidoreductase assay. However, the catalytic role of the isolated enzyme was further analyzed by two additional methods which assay the sugar reductase activity by monitoring the product formation directly. While the TBA assay is very sensitive in detecting the $E_1$-$E_3$ product, it will also respond to 2- and 3-deoxyhexoses. Hence, a definitive verification involves conversion of the $E_1$-$E_3$ product (product 7) to the corresponding alditol acetates, which can be separated and identified by the GC-MS assay. Because product formation by incubation with purified $E_3$ and $E_1$ was confirmed by this assay, enzyme $E_3$ purified on the basis of the NADH:DCPIP oxidoreductase activity was unequivocally demonstrated to be the desired sugar reductase.

As previously reported, initial $E_3$ purification efforts in our group began with the procedure of Gonzalez-Porque and Strominger (12), but this was soon modified to a procedure (protocol I) which led to a 3,600-fold overall purification of $E_3$ (15). While the protein purified by protocol I was valuable for preliminary mechanistic studies, characterization of possible cofactor(s) requires substantial amounts of homogeneous protein which was unattainable by the original protocol. Cloning of the gene coding for this protein was achieved by standard methodology. Although the nucleotide-derived gene product size (39,000 Da) closely matched that found for the putative $E_3$

TABLE 3. Summary of $E_3$ purification from *E. coli* DH5α(pSFL28)

| Purification step | Total protein (mg) | Total activity (μmol of DCPIP consumed/min) | Sp act (U/mg of protein) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Ammonium sulfate[a] | 5,549 | 1,332 | 0.24 | 1 | 100 |
| DEAE-Sephacel | 295.9 | 1,391 | 4.7 | 19.6 | 104 |
| Phenyl-Sepharose | 35.1 | 636 | 18.1 | 75.4 | 47.7 |
| Matrex Blue A | 24.9 | 443 | 17.8 | 74.2 | 33.3 |
| Sephadex G-100 | 13.2 | 428 | 32.4 | 135 | 32.1 |

[a] Obtained from 33 g (wet weight) of cells.

(41,000 Da by SDS-PAGE and gel filtration), inconsistencies began to accumulate upon closer examination. First, with the program FASTDB (6), a comparison of the nucleotide-deduced amino acid sequence of the putative $E_3$ with all sequences available in protein data bases failed to reveal significant homology with any NAD(P)H oxidases or other similar enzymes. Second, a detailed search of the putative $E_3$ gene failed to give any of the common NAD(P)H binding consensus sequences typically found in NAD(P)H-utilizing enzymes. Third, since the $E_3$ gene was expected to be within close proximity of the $E_{od}$ gene, positive hybridization with the isolated YPT2 insert was expected with the $E_{od}$ probe (JST1); however, despite many attempts, no hybridization was observed. Finally, definitive proof of misidentification was found by overexpression of the putative $E_3$ gene product and subsequent assay for activity. No activity was evident by either the GC-MS or NADH:DCPIP oxidoreductase assay. Thus, the protein isolated by protocol I is not $E_3$, and the desired $E_3$ may actually be a minor contaminant of the apparent homogeneous protein obtained from protocol I. This information prompted a thorough reinvestigation of the existing $E_3$ purification protocol.

Modification of the former purification procedure by incorporation of a dye ligand chromatography (protocol II) surpris-

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TTA AGT TTG TAT TGA GTA CGT CTA ATA AAC CTT ACA TAA TAT ATT TGG ACT TAT TTT ATG      3

TCA TTA AAT GTT AAG CTG CAT CCA TCA GGT ATT ATT TTT ACT TCC GAT GGA ACA TCT ACA     63
S   L   N   V   K   L   H   P   S   G   I   I   F   T   S   D   G   T   S   T     20

ATA TTA GAT GCG GCT CTG GAT AGT AAT ATA CAT ATT GAA TAC AGC TGC AAA GAT GGA ACC    123
I   L   D   A   A   L   D   S   N   I   H   I   E   Y   S   C   K   D   G   T     40

TGT GGT TCT TGT AAG GCA ATA TTG ATT TCT GGT GAA GTA GAC AGT GCG GAA AAT ACC TTT    183
C   G   S   C   K   A   I   L   I   S   G   E   V   D   S   A   E   N   T   F     60

TTA ACT GAG GAA GAT GTT GCT AAA GGT GCA ATC CTC ACT TGT TGC TCT AAG GCT AAA TCT    243
L   T   E   E   D   V   A   K   G   A   I   L   T   C   C   S   K   A   K   S     80

GAT ATT GAG TTA GAT GTT AAT TAT TAT CCA GAG TTA AGT CAT ATA CAA AAA AAA ACT TAT    303
D   I   E   L   D   V   N   Y   Y   P   E   L   S   H   I   Q   K   K   T   Y    100

CCA TGT AAA TTA GAT AGC ATT GAA TTT ATT GGT GAA GAT ATT GCC ATT CTC TCC TTA CGT    363
P   C   K   L   D   S   I   E   F   I   G   E   D   I   A   I   L   S   L   R    120

TTG CCA CCA ACG GCC AAA ATA CAG TAT CTG GCG GGC CAA TAC ATT GAT TTA ATT ATT AAT    423
L   P   P   T   A   K   I   Q   Y   L   A   G   Q   Y   I   D   L   I   I   N    140

GGA CAG CGC CGT AGT TAC TCT ATT GCT AAT GCT CCA GGT GGT AAT GGC AAT ATC GAA TTA    483
G   Q   R   R   S   Y   S   I   A   N   A   P   G   G   N   G   N   I   E   L    160

CAC GTA CGT AAA GTT GTT AAT GGT GTA TTC AGC AAC ATC ATT TTT AAT GAG TTA AAA TTA    543
H   V   R   K   V   V   N   G   V   F   S   N   I   I   F   N   E   L   K   L    180

CAG CAG CTT TTG CGA ATT GAA GGC CCG CAA GGG ACC TTT TTC GTT CGT GAA GAT AAT CTC    603
Q   Q   L   L   R   I   E   G   P   Q   G   T   F   F   V   R   E   D   N   L    200

CCT ATT GTT TTT CTT GCT GGT GGA ACA GGT TTT GCA CCA GTG AAA TCA ATG GTT GAG GCG    663
P   I   V   F   L   A   G   G   T   G   F   A   P   V   K   S   M   V   E   A    220

TTG ATC AAT AAG AAT GAC CAA CGG CAG GTT CAT ATC TAT TGG GGA ATG CCT GCA GGG CAT    723
L   I   N   K   N   D   Q   R   Q   V   H   I   Y   W   G   M   P   A   G   H    240

AAT TTC TAT TCT GAC ATT GCC AAT GAG TGG GCT ATA AAA CAC CCT AAC ATT CAT TAT GTG    783
N   F   Y   S   D   I   A   N   E   W   A   I   K   H   P   N   I   H   Y   V    260

CCT GTT GTA TCA GGC GAT GAT AGT ACT TGG ACC GGA GCC ACT GGT TTT GTA CAT CAA GCG    843
P   V   V   S   G   D   D   S   T   W   T   G   A   T   G   F   V   H   Q   A    280

GTG CTT GAA GAT ATA CCC GAT CTC AGC TTA TTT AAT GTT TAT GCC TGT GGT TCA TTA GCT    903
V   L   E   D   I   P   D   L   S   L   F   N   V   Y   A   C   G   S   L   A    300

ATG ATT ACT GCT GCT CGT AAT GAT TTC ATC AAT CAT GGA TTA GCT GAA AAT AAA TTT TTC    963
M   I   T   A   A   R   N   D   F   I   N   H   G   L   A   E   N   K   F   F    320

TCT GAT GCC TTT GTG CCA TCA AAA TAA CTT TGA GAG ATC AAA GTA                      1008
S   D   A   F   V   P   S   K   .                                                328
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FIG. 4. Nucleotide and translated amino acid sequences of $E_3$ (*ascD*). Nucleotide residues are numbered in the 5′-to-3′ direction starting with the translation initiation signal ATG at positions 1 to 3. The deduced amino acid residues are numbered beginning with the amino-terminal serine. The Pribnow box and the promoter sequence which are found 16 and 40 bp, respectively, upstream from the start codon are underlined. Opposing arrows indicate a possible inverted repeat.

ingly resulted in the isolation of a new protein. This 8,000-fold-purified enzyme is a monomeric protein with a molecular weight of 39,000 and, most importantly, has a higher specific activity. It was suspected, therefore, that this newly purified enzyme was the genuine $E_3$. To confirm this conclusion, the N-terminal amino acid sequence of the protein purified by protocol II was used to design an oligonucleotide primer (SFL4) which was used to screen and construct a clone that was found to be identical to a previously constructed clone, pYPT1, known to carry the entire genes for $E_p$ and $E_{od}$. The fact that the gene coding for this newly purified protein is part of the ascarylose gene cluster provides convincing evidence that the enzyme purified by protocol II is the genuine $E_3$. This conclusion was further supported by the fact that the catalytic properties of the *ascD* gene product purified from *E. coli* are identical to those of newly purified wild-type $E_3$. Interestingly, high sequence homology (51%) was found between *ascD* and a previously unidentified gene (ORF 7.6) within the abequose biosynthetic cluster (*rfb*) of *Salmonella typhimurium* (20). Since compound 5 has been suggested as the common intermediate for the biosynthesis of most 3,6-dideoxyhexoses (except for colitose), identification of the gene function of *ascD* has therefore also allowed the assignment of ORF 7.6 in the *Salmonella* O antigen biosynthetic cluster as the corresponding $E_3$ in the abequose biosynthetic pathway (40).

As an NADH:DCPIP oxidoreductase, this newly purified enzyme exhibits a strong preference for NADH over NADPH, and its calculated $K_m$ and $V_{max}$ are substantially higher than those previously reported. In contrast to the protein purified by protocol I, the genuine $E_3$ shows strong absorption above 300 nm, which is characteristic of a flavoprotein. Thus, the above-baseline absorption at higher wavelength observed for the previously purified enzyme is not due to contaminants but rather is an intrinsic property of $E_3$ and suggests the existence of a flavin and/or other cofactors. Apparently, earlier conceived notions on the mystery of $E_3$ catalysis can now be attributed to the complexity of its purification arising mainly from a contaminant whose molecular weight and chromatographic behaviors are similar to those of the genuine $E_3$. With the help of modern biochemical and genetic techniques, we have finally obtained the correct homogeneous enzyme, the purification of which had proved elusive for nearly two decades. Now that the pure enzyme is at hand, we can commence work on the mechanistic details of $E_3$ catalysis.

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