Cofactor Characterization and Mechanistic Studies of CDP-6-deoxy-$\Delta^{3,4}$-glucoseen Reductase: Exploration into a Novel Enzymatic C–O Bond Cleavage Event†

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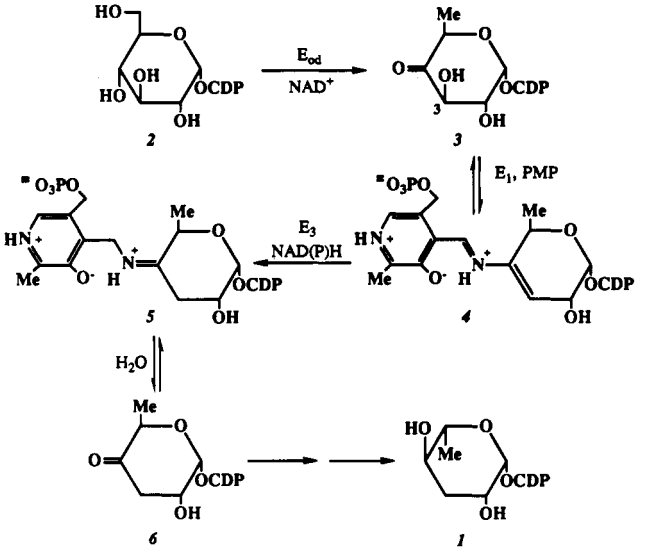
ABSTRACT: The CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3) is a NADH-dependent enzyme which catalyzes the key reduction of the C-3 deoxygenation step during the formation of CDP-ascarylose, a 3,6-dideoxyhexose found in the lipopolysaccharide of *Yersinia pseudotuberculosis*. This highly purified enzyme is also a NADH oxidase capable of mediating the direct electron transfer from NADH to O_2, forming H_2O_2. While previous work showed that E_3 contains no common cofactor, one FAD and one plant ferredoxin type [2Fe-2S] center were found in this study to be associated with each molecule of E_3. The iron–sulfur center is essential for E_3 activity since bleaching of the [2Fe-2S] center leads to inactive enzyme. These results suggest that E_3 employs a short electron-transport chain composed of both FAD and the iron–sulfur center to shuttle electrons from NADH to its acceptor. The order of electron flow, as indicated by EPR measurement with partially reduced E_3, starts with hydride reduction of FAD by NADH. The iron–sulfur cluster, receiving electrons one at a time from the reduced flavin, relays the reducing equivalents via another iron–sulfur center in the active site of E_1 to its final acceptor, the E_1-bound PMP-glucoseen adduct. The participation of a one-electron-carrying iron–sulfur center in this reduction is advantageous since both electrons are dispatched from the same redox state of the prosthetic group, allowing electrons of equal energy to be delivered to the final acceptor. This proposed electron-transport sequence is consistent with the role of E_3 as a $2e^-/1e^-$ switch and provides compelling evidence supporting a radical mechanism for E_3-catalyzed C-3 deoxygenation. In light of the fact that a PMP-glucoseen adduct is the ultimate acceptor receiving electrons from E_3, the catalytic role of E_3 in the biosynthesis of ascarylose clearly constitutes a unique example of biological deoxygenation.

Deoxygenation reactions as catalyzed in biological systems are pivotal transformations and encompass a diverse range of mechanistic themes. Studies of the biosynthesis of ascarylose (**1**; 3,6-dideoxy-L-*arabino*-hexose), a 3,6-dideoxy sugar found specifically in the *O*-antigen of lipopolysaccharides of Gram-negative bacteria (Westphal & Lüderitz, 1960; Lüderitz et al., 1966, 1983; Bishop & Jennings, 1982; Raetz, 1990), have led to the discovery of a novel deoxygenation pathway consisting of two enzymatic steps in which the hydroxyl group at C-3 of **3**, a product of CDP-D-glucose 4,6-dehydratase (E_{od}),[1] is replaced by a hydrogen atom (Gonzalez-Porque, 1986). As depicted in Scheme I, the initial C–O bond cleavage is catalyzed by a pyridoxamine 5′-phosphate (PMP) linked enzyme, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydratase (E_1; Gonzalez-Porque & Strominger, 1972a; Rubenstein & Strominger, 1974a), and the subsequent reduction is mediated by a NADH-dependent catalyst, CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3; Rubenstein & Strominger, 1974b),

Scheme I



giving rise to the actual deoxygenation product, CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**6**), after hydrolysis of the ensuing intermediate, **5**.

Both E_1 and E_3 have been isolated from *Pasteurella pseudotuberculosis*[2] (Gonzalez-Porque & Strominger, 1972b). Although it was recently discovered that E_1 not only is PMP dependent but also contains one plant ferredoxin [2Fe-2S] center (Thorson & Liu, 1993), detailed stereochemical studies have revealed that E_1, despite its having evolved an unusual

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[1] Abbreviations: CDP, cytidine diphosphate; E_{od}, CDP-D-glucose 4,6-dehydratase (CDP-D-glucose oxidoreductase); E_1, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase; E_3, CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase; MMO, methane monooxygenase; PMP, pyridoxamine 5′-phosphate; PLP, pyridoxal 5′-phosphate; TBA, 2-thiobarbituric acid; DCPIP, 2,6-dichlorophenolindophenol; TCA, trichloroacetic acid; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; FAD, flavin adenine dinucleotide; FMN, flavin adenine mononucleotide; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FDR, ferredoxin–$NADP^+$ reductase; PDR, phthalate dioxygenase reductase.

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

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role for the PMP cofactor, retains all the essential elements of catalysis common to other vitamin B_6 phosphate dependent enzymes (Shih et al., 1991; Weigel et al., 1992a,b).

While the putative product of E_1 catalysis, CDP-6-deoxy-$\Delta^{3,4}$-glucoseen (**4**), has never been isolated or characterized, reduction of this highly conjugated species by E_3, in the presence of NAD(P)H, has been postulated to proceed through a hydride-transfer mechanism (Rubenstein & Strominger, 1974b). However, this hypothesis is inconsistent with the lack of tritium incorporation from [4-$^3H_2$]NADPH to either the deoxygenated product, **6**, or the regenerated PMP coenzyme. Furthermore, such a direct hydride reduction is quite problematic since it demands that the active sites of E_1 and E_3 be brought into closer proximity than is spatially and sterically favorable. In an effort to clarify these mechanistic ambiguities, we have isolated an "E_3 equivalent" from *Yersinia pseudotuberculosis* which, like its *P. pseudotuberculosis* counterpart, exhibited a weak and featureless absorption above 300 nm (Han et al., 1990). During a recent attempt to improve the purification procedure for large-scale E_3 preparation, we surprisingly discovered that the previously "purified" enzyme was still a mixture of two proteins having similar chromatographic behaviors and nearly identical molecular weights (Miller & Liu, 1992). Since cloning and expression of the DNA containing the gene coding for the major component in that mixture has led to the isolation of a protein lacking any E_3 activity (Lo et al., 1993), it is now evident that the minor protein component, which can be purified by a newly modified procedure, is the desired enzyme. As expected, cloning and expression of the gene coding for the newly purified enzyme afforded a protein exhibiting catalytic characteristics identical to those of the wild-type E_3. Having the homogeneous enzyme in hand has allowed us to carry out a detailed characterization of the cofactor(s) in the active site of E_3. Reported in this paper are the results of these studies and their mechanistic implications for E_3-catalyzed reduction.

EXPERIMENTAL PROCEDURES

General. The bacterial strain *Yersinia pseudotuberculosis* was kindly provided by Dr. Otto Lüderitz of the Max Planck Institute for Immunobiology, Freiburg, Germany. The wild-type CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3) was purified to homogeneity as described before (Miller & Liu, 1992). CDP-D-glucose-4,6-dehydratase (E_{od}) (Yu et al., 1992) and CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase (E_1) (Weigel et al., 1992a) used in assay procedures were isolated from the same *Y. pseudotuberculosis* strain and were kindly supplied by Ms. Yuan Yu and Dr. Theresa Weigel of our group. The E_3 gene (*ascD*) found in a previously produced clone, pYPT1 (Miller & Liu, 1992), was subcloned into the *Bam*HI–*Sac*I sites of pUC19, and the resulting construct, pSFL28 (Lo et al., 1993), after transformation into *Escherichia coli* DH5α, was the source of the recombinant E_3 used in this study. The methane monooxygenase (MMO) reductase was a gift of Professor John Lipscomb of the University of Minnesota. DEAE-Sephacel, phenyl-Sepharose, and Sephadex G-100 were purchased from Pharmacia (Piscataway, NJ). The protease inhibitors used in enzyme purification were prepared as previously described (Han et al., 1990). All protease inhibitors, commercially available enzymes used in the assays, and most biochemicals were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical reagent grade or the highest quality commercially available.

Enzyme Assays. The enzyme assays were performed via previously described methodology. In particular, the thiobarbituric acid (TBA) assay (Weigel et al., 1992a), the gas chromatography–mass spectroscopy (GC–MS) assay (Weigel et al., 1992a), and the NADH:DCPIP oxidoreductase assay (Han et al., 1990) were the three methods utilized primarily for the presented work.

Growth of Cells. An overnight culture of *E. coli* DH5α-pSFL28 grown in LB medium (30 mL) supplemented with ampicillin (100 μg/mL) was diluted 300-fold into the same medium (9 L, without antibiotic) and grown overnight in an incubator–shaker (Lab-Line) with vigorous agitation (140 rpm) at 37 °C. The cells were harvested by centrifugation at 2500*g*, and the collected cells were washed twice with 50 mM potassium phosphate buffer (pH 7.5). A typical yield was 33 g of wet cells per 9 L of culture.

Enzyme Purification. All operations were carried out at 4 °C.

Step 1. Crude Extracts. The collected cells from 9 L of culture (33 g wet weight) were resuspended in 40 mL of 10 mM potassium phosphate buffer (pH 7.5), followed by addition of the protease inhibitor solution. The cells were disrupted by sonication for 2.5 min with a VirSonic Model 300 sonicator. The temperature of the extract was controlled so as not to exceed 5 °C during this process. Cellular debris was then removed by centrifugation at 4000*g* for 20 min.

Step 2. Streptomycin Sulfate Treatment. Streptomycin sulfate (5% aqueous solution) was added dropwise to the crude extract to a final concentration of 0.8%. After the solution was stirred for 1 h, the precipitate was eliminated by centrifugation at 8500*g* for 1 h. The supernatant solution (50 mL) was diluted with 1 mM potassium phosphate buffer (pH 7.5, 5 mL) and carried out on to the next step.

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. The solution was allowed to stir for 3 h, and the precipitated proteins were collected by centrifugation (10000*g*, 20 min). The collected proteins were redissolved in a minimum amount of 50 mM potassium phosphate buffer, pH 7.5. This solution was dialyzed against 2 L of the same buffer for 24 h with four changes of buffer.

Step 4. DEAE-Sephacel Column Chromatography. The solution from step 3 (60 mL) was applied to a column of DEAE-Sephacel (2.5 × 50 cm) preequilibrated with 50 mM potassium phosphate buffer, pH 7.5. The column was washed with the same buffer (1.2 L) and then eluted with a linear gradient of potassium phosphate (50–200 mM, pH 7.5, 2.6 L total). Fractions of 12 mL were collected during the gradient elution. The contents of fractions 90–118 were pooled and concentrated to 24 mL via an Amicon ultrafiltration unit (PM-30 membrane).

Step 5. Phenyl-Sepharose Column Chromatography. The enzyme solution from step 4 was mixed with 2.5 M KCl in 50 mM potassium phosphate buffer (14 mL) to a final concentration of 0.9 M KCl. This solution was then applied to a phenyl-Sepharose column (3.0 × 38 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 (500 mL) and then eluted with a linear gradient of 0.9–0 M KCl (520 mL) in the same phosphate buffer. Fractions of 12 mL were collected throughout. The contents of fractions 35–68 were pooled and concentrated (YM-10 membrane).

Step 6. Matrex Blue A Column Chromatography. The material from step 5 was loaded onto a column of Blue A (2 × 10 cm) and incubated for 45 min. Elution was then begun

with a linear gradient between 0 and 0.7 M KCl in 50 mM potassium phosphate buffer (125 mL each). Fractions of 2 mL were collected. Active fractions were combined (38–60) and concentrated to 0.95 mL (YM-10 membrane).

Step 7. Sephadex G-100 Column Chromatography. The enzyme solution from step 6 was chromatographed on a column (1.5 × 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 2 mL were collected, and the desired protein was found in fractions 55–65. The active fractions were combined, concentrated (YM-10 membrane) and stored in 10-μL aliquots at −85 °C.

HPLC Identification of FAD. Extraction of the flavin coenzyme followed by HPLC analysis was based on a modified procedure of Light et al. (1980). Approximately 4.6 nmol (150 μL) of purified enzyme was extracted with 5% trichloroacetic acid (16 μL of 50% TCA) for 10 min at 0 °C. The supernatant fraction was withdrawn after centrifugation, and the pellet was washed with 50 μL of 5% TCA. The combined TCA extracts were neutralized with 80 μL of 1 M Na_2HPO_4. An aliquot of neutralized extract was separated on an Alltech Econosphere C_{18} reverse-phase HPLC column (46 × 250 mm) utilizing a 5–30% linear acetonitrile gradient in 50 mM ammonium formate, pH 6.5, at 1 mL/min. The elution was followed by monitoring the flavin absorption at 436 nm, and the UV spectrum of the collected peak was recorded concomitantly with a diode array detector.

UV-Vis Spectral Analysis. The cofactors of E_3 were fully characterized by spectral analysis. As summarized below, in order to facilitate this analysis, several extraction methods were employed to separate the flavin cofactor from the iron–sulfur center and the protein.

(*a*) *Boiling.* Enzyme E_3 (1.71 nmol, 1.7 μM) was prepared in 1 mL of 50 mM potassium phosphate buffer, pH 7.5, containing 10 mM $MgCl_2$. The solution was boiled in a water bath for 15 min. The spectrum of the resulting solution was recorded and ascribed to the absorption of flavin. The difference spectrum between native E_3 and boiled E_3 was assigned to the absorption of the [2Fe-2S] center.

(*b*) *Trichloroacetic Acid (TCA) Extraction.* An E_3 solution (1.7 nmol, 50 μL) was extracted with 50% TCA (final concentration = 5%) for 7 min at 0 °C. Following centrifugation, the supernatant was removed and the remaining pellet was washed with a 5% TCA solution (280 μL). The combined extracts were neutralized with 1 M K_2HPO_4 (300 μL) prior to spectral measurement. The resulting spectrum was due to the absorption of flavin, and the difference spectrum between native E_3 and TCA-treated E_3 was assigned to that of the [2Fe-2S] center.

(*c*) *Proteolysis.* An E_3 solution was extracted with TCA as described above. After the protein pellet was washed twice with 0.1% TCA, it was resuspended in 500 μL of 0.1 M potassium phosphate buffer (pH 8.0) containing 10 mg/mL each of trypsin and α-chymotrypsin. After being incubated at 38 °C for 4 h, the reaction mixture was boiled for 5 min and centrifuged to remove undigested protein. Absorbance of the supernatant was recorded to determine the amount of flavin associated with the protein following TCA extraction.

(*d*) *Mersalyl Acid Treatment.* Enzyme E_3 (1.08 nmol, in 800 μL of 50 mM potassium phosphate buffer, pH 7.5) was treated with a solution of sodium mersalyl (10 μL, 4 mM in 0.5 M Tris·HCl, pH 7.6). The absorption of the resulting solution was assigned to the flavin chromophore, and the difference spectrum between native E_3 and mersalyl-treated E_3 was attributed to the [2Fe-2S] center.

Dithionite Titration. All experiments were performed under anaerobic conditions achieved by 8–10 cycles of argon and vacuum carried out over a period of at least 1 h. Dithionite solutions were standardized with riboflavin. Small volumes of dithionite (2.7 μL, 0.469 mM) were titrated into an E_3 solution (4.5 μM, 50 mM potassium phosphate buffer, pH 7.5, 800 μL), mixed, and equilibrated for 10 min prior to measurement of the spectrum. A plot of the changes in absorbance at 454 nm versus the amount of dithionite added gave the reducing equivalents required to fully reduce the enzyme's chromophores at 454 nm.

EPR Spectroscopy. A solution of E_3 (120 μM in 750 μL of 50 mM potassium phosphate buffer, pH 7.5, at 25 °C) was made anaerobic (8–10 cycles of argon and vacuum carried out over a period of at least 1 h) and titrated with standardized dithionite (5 mM, 75 μL). Samples (300 μL) were removed at various points of reduction, transferred anaerobically to EPR tubes, frozen, and stored in liquid nitrogen. Prior to EPR measurement, one sample was allowed to thaw and equilibrate in air to provide the oxidized (native) protein sample. At the one electron reduced stage, a sample was also transferred to a special EPR flat tube designed for aqueous room temperature EPR.

Cryogenic X-band EPR measurements were made on a Varian E-109 spectrometer which was digitally interfaced to a laboratory microcomputer (Lipscomb & Salo, 1983). The spectrometer was equipped with an Oxford Instruments ESR-910 liquid helium cryostat, a Hewlett-Packard 436A power meter, and a Hewlett-Packard 5350B microwave frequency counter. EPR spectral manipulations and integrations were performed using a program written by Dr. David R. Jollie. Spin quantitations were performed at nonsaturating microwave power levels according to reported procedures (Aasa & Vanngard, 1975; Fee, 1978), and the standard used for quantitation was a 1.0 mM $Cu(ClO_4)_2$ solution. Room temperature EPR measurements of the flavin semiquinone were also made on a Bruker ESP 300 with an ER 4111 VT variable-temperature unit on aqueous samples in a specially designed flat EPR sample tube.

Metal Analysis. Metal analysis of this purified protein was performed on an inductively coupled emission spectrometer (ICP-AES) by the Soil Testing Laboratory in the Department of Soil Science, University of Minnesota. The concentrations of the protein samples were determined on the basis of quantitative amino acid hydrolysis carried out at the Microchemical Facility in the Institute of Human Genetics of the University of Minnesota.

Quantitation of Iron and Inorganic Sulfur in E_3. Iron was quantified using the procedure developed by Fish (1988). Inorganic sulfur was assayed as described by Bienert (1983). Enzyme E_3 was quantified by the method of Bradford, using bovine serum albumin as a standard (Bradford, 1976), and confirmed by quantitative amino acid hydrolysis.

Inactivation of the [2Fe-2S] Center. (*a*) *Mersalyl-Treated E_3.* Enzyme E_3 (1.3 μM, in 800 μL of 50 mM potassium phosphate buffer, pH 7.5) was treated with mersalyl acid (15 μL, 4.0 mM in 0.5 M Tris·HCl, pH 7.6) at room temperature. This sample was then used directly in spectrophotometric analysis.

(*b*) *Apo-[2Fe-2S]-E_3.* Mersalyl-treated E_3 made by the above procedure was desalted by overnight dialysis with four changes of buffer (50 mM potassium phosphate buffer, pH 7.5) at 4 °C. After incubation with 2-mercaptoethanol (10 μL, 0.14 μmol) for 1 h, excess reagents were removed by overnight dialysis with four changes of buffer (50 mM

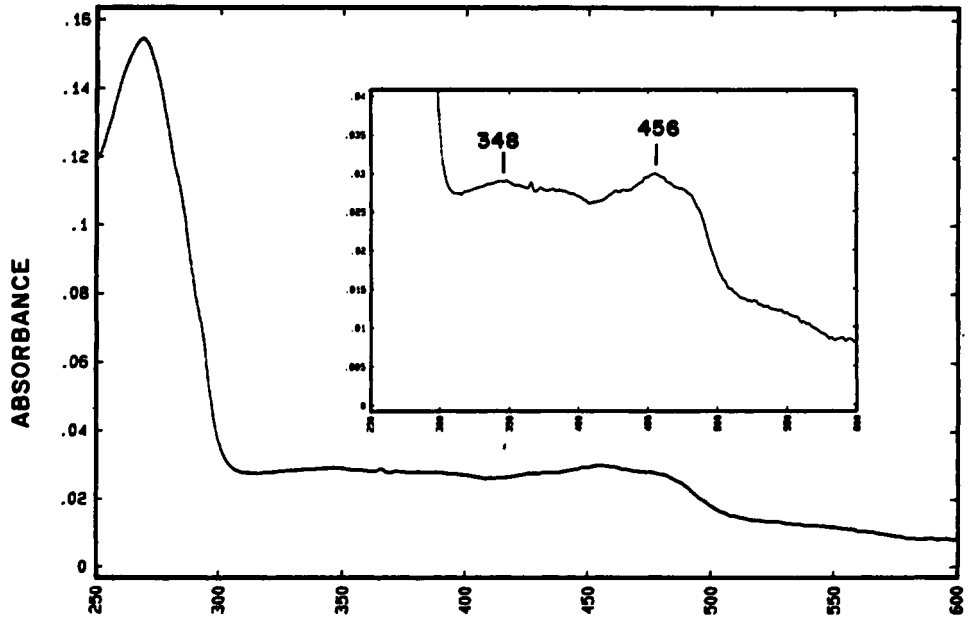


FIGURE 1: Ultraviolet-visible absorption spectrum of E_3 purified from *Y. pseudotuberculosis*. The protein concentration was 9.4 μM in 50 mM potassium phosphate buffer, pH 7.5. The absorption features between 300 and 500 nm are more discernible in the inset.

potassium phosphate buffer, pH 7.5), giving apo-[Fe_2S_2]-E_3.

Anaerobic Reduction of E_1 via E_3 by NADH. Fifty micromolar E_1 and 5 μM E_3 were combined in an anaerobic cuvette in a total volume of 800 μL of 50 mM potassium phosphate buffer containing 1 mM EDTA. The solution was thoroughly degassed by cycling vacuum and argon purge for more than 1 h. In a separate anaerobic flask, a solution of NADH (564 μM, 5 mL total volume) was prepared and degassed by first bubbling argon through the solution followed by similar cycling of vacuum and argon purge. Aliquots of the NADH solution were titrated anaerobically into the enzyme mixture, and the UV-vis spectrum was measured. Only a short equilibration period (<1 min) was required after the addition of NADH as determined by UV-vis. A control in the absence of E_3 was run in parallel.

Alternate Biological Reductants. Two enzymes were used as alternate reductants replacing E_3 in both the TBA and GC–MS product formation assays. The incubation conditions and procedures were identical to the original assays described earlier except for the substitution of E_3 with diaphorase (0.8 nmol in 0.2 M Tris·HCl, pH 7.5, 0.30 M KCl, 0.55 mM FMN, and 4.0 mM BSA) or methane monooxygenase (MMO) reductase (1.4 nmol in 50 mM potassium phosphate buffer, pH 7.5). The yield of product was estimated by comparison of the integration of GC peaks derived from the products (abequitol and paratitol tetraacetate) with those derived from the starting material (glucitol hexaacetate) and the unreacted E_{od} products (fucitol and quinovitol pentaacetate) (Weigel et al., 1992a).

RESULTS

Cofactor Characterization by High-Performance Liquid Chromatography. Extraction of E_3 with trichloroacetic acid was used to unleash noncovalently bound cofactors from the purified protein (Mayhew & Massey, 1969). The resulting extract was examined by HPLC in comparison with authentic standards of riboflavin, FMN, and FAD, which under the separation conditions specified in the Experimental Procedures had retention times of 22.2, 17.0, and 14.7 min, respectively. Since the TCA extract has a HPLC retention time of 14.5 min and also exhibits a characteristic flavin spectrum having absorption maxima at 380 and 450 nm, the isolated cofactor is clearly a FAD. Cofactor identification was confirmed by coinjection with authentic FAD.

Fluorescence Spectroscopy. Consistent with the HPLC results, the fluorescence spectrum of this highly purified enzyme is that of a typical flavoprotein (Ghisla et al., 1974) and thus reaffirms the presence of a FAD cofactor. The excitation spectrum of E_3 has a maximum at 470 nm with smaller peaks at 452, 425, and 380 nm. Interestingly, excitation at 450 nm produces an emission maximum at 518 nm, which is blue shifted by 11 nm from that of the free FAD chromogen (Fox & Walsh, 1982).

UV-Vis Spectral Analysis. The above results have shown that E_3 is clearly a flavoprotein. If the extinction coefficient at 450 nm for the enzyme-bound FAD is the same as that for free FAD (11 300 M^{-1} cm^{-1}), then E_3, on the basis of its UV-visible absorption, should contain approximately 2 mol of FAD per 1 mol of protein (Figure 1). However, unlike the electronic spectra of most other flavoproteins in which a characteristic peak at 350–380 nm is commonly observed, the absorption of E_3 in this region is notably featureless. Such abnormality was found by spectral analysis to be a result of the coexistence of an iron–sulfur cluster whose absorption overlaps with that of the FAD chromophore. Several well-established methods were used to separate and distinguish the two coenzymes. As shown in Figure 2, 1 equiv of FAD was unleashed by boiling the enzyme (Fox & Walsh, 1982), and subtraction of the resulting FAD absorbance from that of the native protein revealed a broad spectrum with absorption maxima at 325, 420, and 454 nm as well as a shoulder at 550 nm that is characteristic of a plant ferredoxin type [2Fe-2S] center (Orme-Johnson & Orme-Johnson, 1978, 1982; Moura & Moura, 1982). Similar results were also obtained by the TCA-precipitation method (Mayhew & Massey, 1969). In light of the fact that no additional flavin cofactor was released from the resulting protein precipitate upon proteolysis (Singer & Edmondson, 1980), the molar absorption coefficient of each chromophore could be readily estimated (ϵ_{454} = 17 560 M^{-1} cm^{-1}; ϵ_{272} = 62 040 M^{-1} cm^{-1}). Comparison of these molar absorption coefficients suggests that each E_3 molecule contains one molecule of FAD together with a single [2Fe-

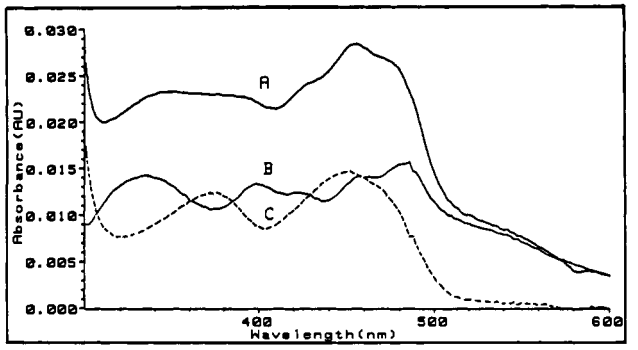


FIGURE 2: Absorption spectra of E_3: (A) native enzyme (1.7 μM) in 50 mM potassium phosphate buffer, pH 7.5; (C) the same sample after 15 min at 100 °C followed by centrifugation; (B) the difference spectrum [(A)–(C)] between the native E_3 and the supernatant of the boiled enzyme.

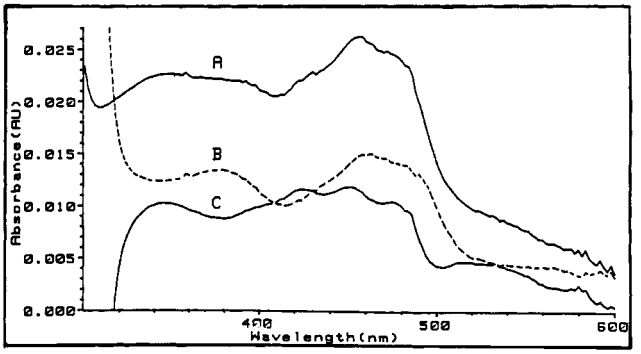


FIGURE 3: Absorption spectra of E_3: (A) native enzyme (1.3 μM) in 50 mM potassium phosphate buffer, pH 7.5; (B) the same sample after treatment with sodium mersalyl (10 μL, 4 mM in 0.5 M Tris-HCl, pH 7.6); (C) the difference spectrum [(A)–(B)] between the native E_3 and the mersalyl-treated enzyme.

2S] cluster (Dawson et al., 1986). A confirming experiment was also performed in which the addition of mersalyl acid that specifically bleaches the iron–sulfur chromophores (Malkin & Rabinowitz, 1966) exposed the FAD absorption as anticipated (Figure 3).

Metal Analysis. Inductive coupled plasma analysis of purified enzyme showed the presence of nearly 2 (1.9) equiv of iron per mole of E_3, suggesting 1 [2Fe-2S] center per enzyme. No other common redox-active metals were found.

Quantitation of Iron and Inorganic Sulfur in E_3. Spectrophotometric quantitation was repeated four times with samples obtained from different enzyme preparations. These analyses revealed 2 equiv of Fe and 2 equiv of S^{2-} per mole of E_3, consistent with 1 [2Fe-2S] center per enzyme.

Redox Titrations. Anaerobic titration with dithionite was used to quantify the equivalents of electrons required for complete reduction of the native enzyme. Since the active site of E_3 contains 1 FAD and 1 [2Fe-2S] center, addition of a total of 3 electron equiv is predicted to give a fully reduced enzyme. As can be seen in Figure 4, titration of E_3 under anaerobic conditions does indeed require 3 electron equiv, or 1.5 equiv of dithionite, to fully reduce the chromophoric absorption at 454 nm. The 1:1 stoichiometry of an iron–sulfur center in association with a FAD cofactor is therefore substantiated.

Electron Paramagnetic Resonance (EPR) Spectroscopy. The presence of an iron–sulfur center in the active site of E_3 was further confirmed by EPR experiments which also made it possible to fully characterize the chemical nature of the metal cluster. The EPR spectra of the oxidized (native), one electron reduced, two electron reduced, and fully reduced states of E_3 are shown in Figure 5. Since the oxidized enzyme is EPR silent, the iron–sulfur center must be a [2Fe-2S] cluster having the two high-spin Fe(III) atoms ($S = 5/2$) antiferromagnetically coupled (Howard & Rees, 1991; Orme-Johnson & Orme-Johnson, 1982; Moura & Moura, 1982). However, one of the iron atoms in the reduced form becomes a high-spin Fe(II) ($S = 4/2$) species giving a net spin of $+1/2$ which makes the cluster EPR active. As expected, a rhombic EPR spectrum of reduced E_3 having g-values at 2.043, 1.960, and 1.877 is consistent with a plant ferredoxin type [2Fe-2S] center (0.88 spin/2 Fe). The resonance observed at 2.002 (0.62 spin/E_3) after the addition of 1 electron equiv is indicative of an organic free radical which clearly arises from residual flavin semiquinone (Campbell & Dwek, 1984). The fact that this free radical signal prevails at 293 K provides strong support for this assignment. Similar g-values (2.002, 2.047, 1.960, and 1.864) have also been observed for MMO reductase, whose active site is known to have one molecule of FAD and one plant-ferredoxin type [2Fe-2S] center (Lund & Dalton, 1985; Fox et al., 1989).

Inactivation of the [2Fe-2S] Center. As shown earlier, the iron–sulfur center of E_3 can be extinguished using mersalyl acid, a mercurial agent which reacts covalently with cysteine and sulfide (Malkin & Rabinowitz, 1966). The resultant mersalyl-treated E_3 was devoid of both CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase and NADH:DCPIP oxidoreductase activities (Table I). The mersalyl-treated E_3 was then desalted by dialysis, incubated with excess 2-mercaptoethanol, and dialyzed again to give apo-[2Fe-2S]-E_3 (Lund & Dalton, 1985). Interestingly, this apo-enzyme showed no glucoseen reductase activity but retained part of its NADH:DCPIP oxidoreductase activity (Table I). These results suggest that the FAD coenzyme alone in E_3 could constitute its oxidoreductase activity, but its competence to oxidize NADH is greatly augmented by the participation of the iron–sulfur center. In contrast to the NADH:DCPIP oxidoreductase activity, the iron–sulfur center is essential for the CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase activity and may play a pivotal role in passing electrons from E_3 to the glucoseen intermediate, **4**.

Anaerobic Reduction of E_1 via E_3 by NADH. To support the "step-down" mechanism of electron transfer between E_3 and E_1, a simple experiment was designed to allow the direct observation of E_1 reduction with NADH mediated by E_3. In this experiment, only a small amount of E_3 was utilized to eliminate any interference of the UV-vis results by the presence of the strong E_3 chromophores. As illustrated in Figure 6, direct E_1 reduction by NADH, mediated by E_3, is observed, while no change in the E_1 chromophore was visible in the absence of E_3 (not shown). The observation that a 2-fold excess of NADH was required for this reduction (Figure 6, inset) suggests that this equilibrium may have to be driven in a forward direction by additional NADH.

Thiol-Directing Reagents. It has previously been shown that E_3 activity is sensitive to thiol-directing agents (Gonzalez-Porque & Strominger, 1972a; Rubenstein & Strominger, 1974b; Han et al., 1990). However, since all these experiments were performed with the impure enzyme, it was necessary to repeat this study to confirm the early observation. As summarized in Table II, in addition to the NEM and DTNB used in the previous studies, dipyridyl disulfides 4,4′-dithiodipyridine and 2,2′-bipyridyl disulfide also inhibit E_3, albeit with different efficiencies which may be attributable to the variation in their pK_a and preferred solvent microenvironment (Brocklehurst, 1982). Inactivation by iodoacetic acid and

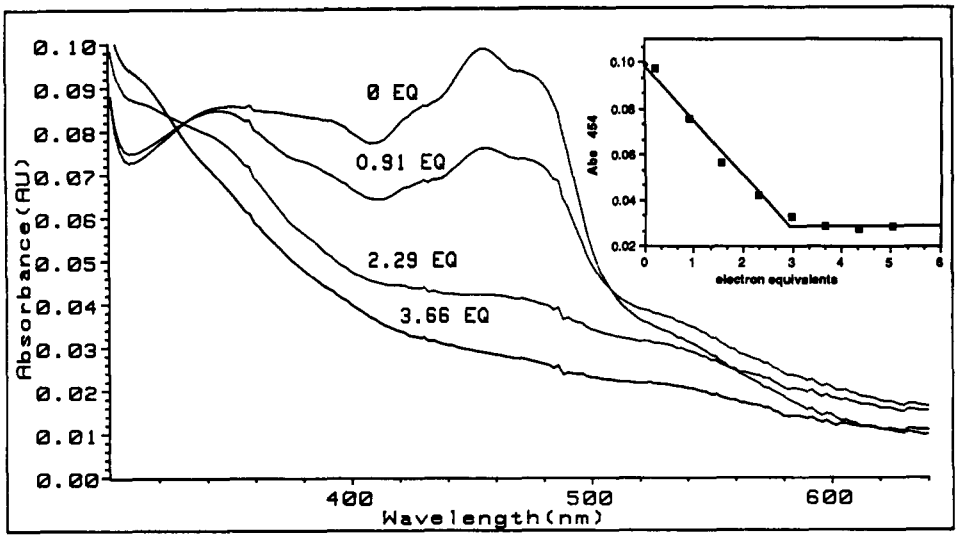


FIGURE 4: Anaerobic titration of E_3 with sodium dithionite: absorption of native E_3 alone (A) and after the addition of 0.91 (B), 2.29 (C), and 3.66 electron equiv of dithionite (D). The protein concentration used in this experiment was 4.5 μM in 50 mM potassium phosphate buffer, pH 7.5. The inset shows the decrease in absorbance at 454 nm plotted against the number of electron equivalents added.

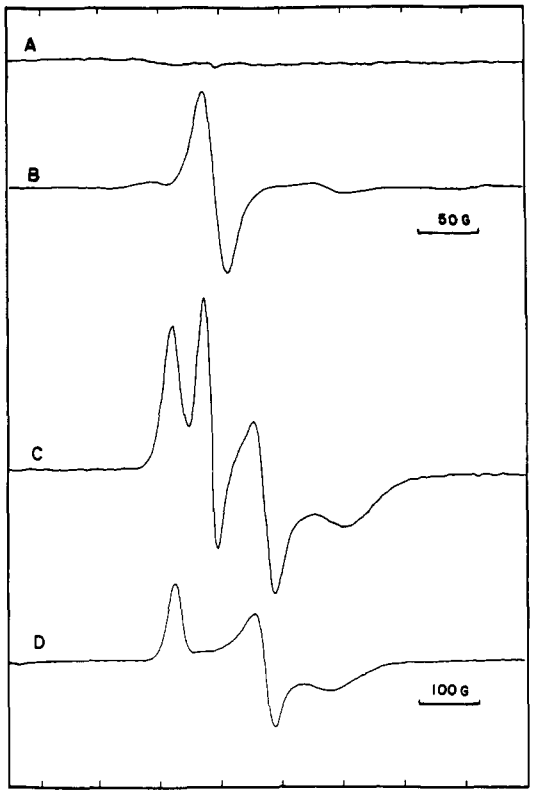


FIGURE 5: Cryogenic X-band EPR of E_3 in 50 mM potassium phosphate buffer (pH 7.5). (A) Fully oxidized E_3 (40 μM; 9.216 GHz; 1 mW; gain, 3.2×10^3; 40 K; modulation amplitude, 10 G). (B) One electron reduced E_3 (40 μM; 9.214 GHz; 125 μW; gain, 1.6×10^3; 10 K; modulation amplitude, 10 G). (C) Two electron reduced E_3 (40 μM; 9.216 GHz; 1 mW; gain, 3.2×10^3; 19 K; modulation amplitude, 10 G). (D) Fully reduced E_3 (40 μM; 9.215 GHz; 1 mW; 6.3×10^3; 40 K; modulation amplitude, 10 G).

iodoacetamide is quite sluggish, which may again be ascribed to unfavorable interactions between the protein and the reagents. Apparently modification of at least one thiol residue, within or close to the active site of E_3, abolishes its activity.

Alternate Reductants. The potential replacement of E_3 by alternate chemical or enzymatic reductants was explored to gain mechanistic insights into its role as a reductase in the biosynthesis of ascarylose. As shown in Table III, attempts to substitute E_3 with the chemical reductants $NaBH_4$ and $NaCNBH_3$ in both the TBA and the GC–MS assay were futile. Neither assay showed any dideoxyhexose product formation. A more fruitful study was the substitution of E_3 with enzymatic reductants. Both diaphorase and MMO reductase exhibited NADH:DCPIP oxidoreductase activity, but they failed to show significant deoxy sugar product formation on the basis of the TBA assay. However, a measurable amount of dideoxyhexose could be detected by the more sensitive GC–MS assay (Table III). It is noteworthy that the GC–MS assay requires incubation with several enzymes and cofactors; data from each experiment must be carefully correlated with that of an incubation performed in parallel with E_3 under identical conditions. Since both diaphorase, a FAD-requiring enzyme, and MMO reductase, a FAD- and [2Fe-2S]-containing enzyme, are competent substitutes for E_3, the successful replacement of E_3 with these electron-shuttle systems strongly suggests an analogous role for E_3 in transferring electrons to the E_1-bound glucoseen intermediate, **4**.

Table I: Treatment of E_3 with Mersalyl Acid[a]

E_3 form	glucoseen reductase activity[b]	NADH:DCPIP oxidoreductase activity[c]
native	100	100
mersalyl-treated E_3	0	0
apo-[2Fe-2S]-E_3	0	12

[a] Detailed procedures used are described in Experimental Procedures. [b] Activity was determined by the TBA assay (Weigel et al., 1992a). [c] Han et al. (1990).

DISCUSSION

There is no doubt that the C-3 deoxygenation catalyzed by E_1 and E_3 is mechanistically the most intriguing step in the biosynthesis of ascarylose. Although E_3 has been purified earlier from *P. pseudotuberculosis* and *Y. pseudotuberculosis*, it was recently found that the previously purified E_3 was still a mixture of two proteins, and the desired enzyme was present only as a minor component (Miller & Liu, 1992). A refurbished purification sequence has now led to the isolation of the genuine E_3 that has been confirmed by cloning and expression of the corresponding gene (*ascD*) (Lo et al., 1993). These findings have rejuvenated our mechanistic studies since many of the early ambiguities can now be resolved.

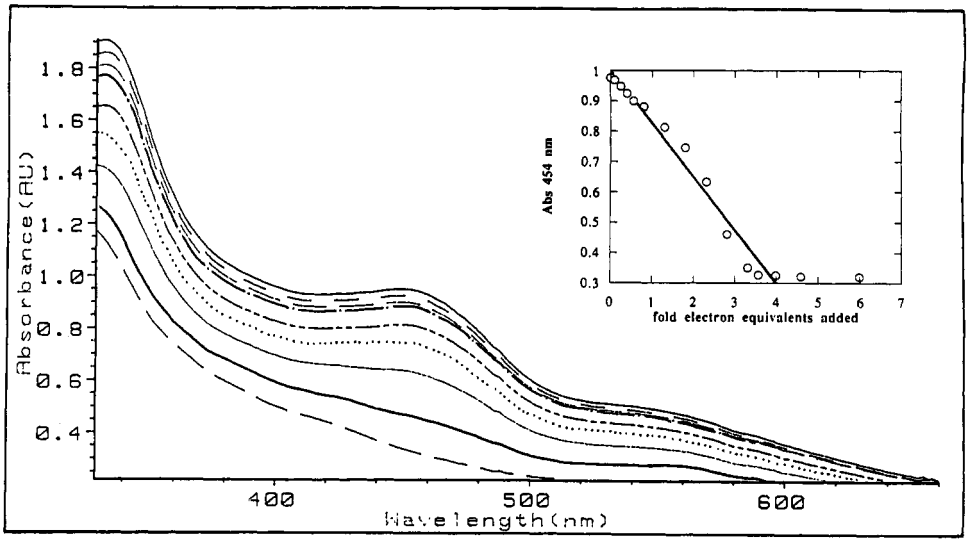


FIGURE 6: Anaerobic reduction of E_1 via E_3 by NADH. The reduction of the [2Fe-2S] chromophore of E_1 (50 μM) is displayed at different points during titration with NADH in the presence of E_3 (5 μM). The number of electron equivalents to fully reduce E_1 was determined by a secondary plot (inset) of the change of absorbance at 454 nm versus the quantity of electron equivalents (2 electron equiv/NADH) added. No E_1 reduction was observed in the absence of E_3.

Table II: Inactivation of E_3 by Thiol-Directing Reagents[a]

reagent	concn (mM)	incubation time (h)	% residual activity
DTNB	0.2	0.5	6
4,4'-dithiodipyridine	0.1	1	2
2,2'-dithiodipyridine	0.1	1	50
iodoacetic acid	1	20	76
iodoacetamide	1	20	66
NEM	20	0.3	9

[a] A typical assay mixture contained 1 nmol of purified enzyme, 100 nmol of NADH, and the indicated concentration of thiol-directing reagent in 1.5 mL of 50 mM potassium phosphate buffer, pH 7.5.

Table III: Substitution of E_3 with Alternate Chemical and Enzymatic Reductants

reductant[a]	% conversion[b]	% efficiency[c]
E_3	45	100
$NaBH_4$	0	0
$NaCNBH_3$	0	0
diaphorase[d]	14	31
MMO reductase[e]	3	7

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

Extraction of this newly purified enzyme with trichloroacetic acid released a chromophoric cofactor which was readily identified as FAD by HPLC. The electronic absorption of FAD could be separated from that of the second prosthetic group by liberating the flavin through boiling or precipitation with trichloroacetic acid to denature the enzyme. Subtraction of the resulting FAD spectra from that of the native protein unveiled a broad absorbance with chromophoric properties indicative of a plant ferredoxin type [2Fe-2S] center. Support for this assignment was provided by Fe quantitation obtained from both ICP analysis and spectrophotometric determination. The S^{2-} quantitation results were also consistent with a [2Fe-2S] cluster. Scrutiny of the molar absorption coefficients indicated that the active site of E_3 consists of a FAD and a [2Fe-2S] center. The necessity of a total of 3 electron equiv of dithionite to fully reduce E_3 under anaerobic conditions further supported a 1:1 stoichiometry of the two coenzymes. While the oxidized form of E_3 is EPR silent, reduction of the enzyme with dithionite induced a rhombic EPR signal which matches the characteristics of a plant ferredoxin type [2Fe-2S] center.

As presented in Table I, the apo-[2Fe-2S]-E_3 showed no sugar reductase activity but retained part of its NADH:DCPIP oxidoreductase capability. These results suggest that the iron–sulfur center must play an indispensable role in E_3 catalysis. It is most likely that FAD is responsible for the initial oxidation of NADH, and the [2Fe-2S] center, which can accommodate only one electron at a time, provides reducing equivalents with a single constant potential to its acceptor (Kamin et al., 1980). On the basis of the observation that the [2Fe-2S] center in E_1 could be effectively reduced by NADH in the presence of E_3 (Figure 6), this iron–sulfur center has been assigned as the proximate acceptor receiving electrons directly from E_3. Since the E_1's iron–sulfur center has been established to be essential for E_1 activity, the electron transfer between the metal clusters of E_3 and E_1 must be an integral part of the overall electron relay from NADH to the PMP-glucoseen complex, **4**. Thus, E_3-catalyzed reduction is formally a step-down process starting from a two-electron donor (NADH) to a one-electron acceptor (E_1's [2Fe-2S] center, O_2, cytochrome *c*, etc.). Such an order of electron transfer is clearly demonstrated by the dithionite titration–EPR experiments (Figure 5) and can also explain the absence of direct hydride transfer seen between NADH and **6** (Rubenstein & Strominger, 1974b).

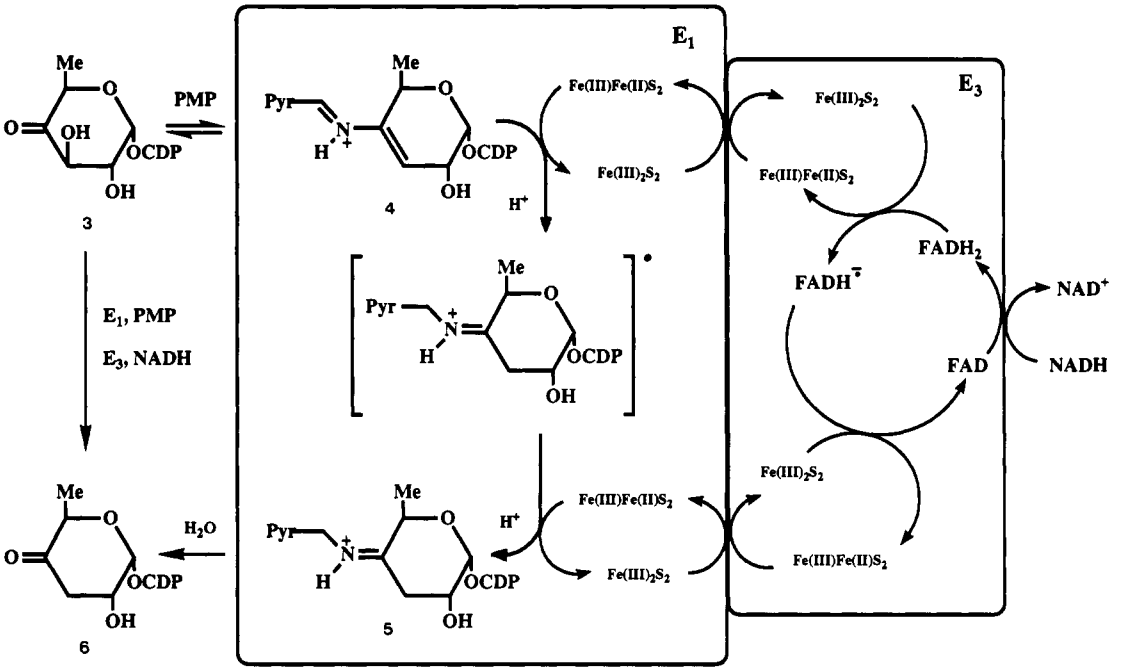
Four other well-characterized enzymes which also incorporate a flavin with a [2Fe-2S] center associated with the same polypeptide are listed in Table IV. It was noted that all of them are members of multicomponent oxygenase systems that employ a short electron-transport chain to mediate electron transfer from an external donor, usually NAD(P)H, to a terminal acceptor, an iron-containing oxygenase. While the oxygenase components of these enzymes show little resemblance to E_1, their reductase components have physical properties remarkably similar to those of E_3, again suggesting

Table IV: Comparison of E_3 with Other [2Fe-2S]-Containing Flavoproteins

physical property	E_3	methane monooxygenase reductase[a]	benzoate oxygenase reductase[b]	NADH: putidamonooxin oxidoreductase[c]	phthalate dioxygenase reductase[d]
molecular weight	39 000	40 000	38 000	42 000	34 000
electron donor	NADH	NADH	NADH	NADH	NADH
flavin cofactor	FAD	FAD	FAD	FMN	FMN
iron–sulfur center	[2Fe-2S]	[2Fe-2S]	[2Fe-2S]	[2Fe-2S]	[2Fe-2S]
absorption spectra	348	340, 398	340, 402	409	330
(λ_{max})	456	458	467	463	462
	550 (sh)[e]	550 (sh)		550 (sh)	550 (sh)
EPR (*g*-values of reduced iron–sulfur center)	2.043, 1.960, 1.877	2.047, 1.960, 1.864	ND[f]	2.032, 1.942, 1.893	2.041, 1.949, 1.900

[a] Fox et al. (1989). [b] Yamaguchi and Fujisawa (1978, 1980). [c] Bernhardt et al. (1975, 1988). [d] Batie et al. (1987). [e] sh = shoulder. [f] ND = not determined.

Scheme II



that E_3 acts as a conduit passing electrons from NADH via the iron–sulfur center of E_1 to the E_1-bound glucoseen intermediate, **4**. On the basis of the physical characteristics of E_3 detailed herein and their similarity to the enzymes listed in Table IV, we can now revise the molecular mechanisms of E_3 catalysis. As depicted in Scheme II, the catalytic cycle is likely to be initiated by a two-electron reduction of FAD by NADH. The reduced FAD may then transfer electrons one at a time to the [2Fe-2S]center. It is the reduced iron–sulfur center that shuttles the reducing equivalents via another iron–sulfur cluster in E_1 to the final acceptor, **4**. The transient flavin semiquinone, after a second cycle of oxidation–reduction with the [2Fe-2S] center, will be oxidized to regenerate FAD. The stepwise electron transfer from FAD via two [2Fe-2S] centers to the glucoseen intermediate, **4**, ensures the formation of a radical intermediate. This intermediate may be a carbohydrate radical species having the unpaired electron at C-3, analogous to its ribosyl counterpart found in the ribonucleotide reductase mechanism (Stubbe, 1989a,b, 1990). A more likely candidate is a PMP radical having its electron density delocalized through the pyridoxamine moiety. The possible intermediacy of a PMP radical may be perceived to be analogous to a pyridoxal phosphate stabilized aziridine radical that has been suggested as a possible transient intermediate in the reaction catalyzed by lysine 2,3-aminomutase (Ballinger et al., 1992a,b). A second electron transfer from the iron–sulfur center yields the Schiff base **5**, which after hydrolysis provides the final E_1–E_3 product **6**. In view of the fact that E_1-catalyzed dehydration represents an unusual example of PMP-dependent catalysis, it is not too surprising that the PMP coenzyme may also play an essential role in facilitating the subsequent reduction mediated by E_3.

Since E_3 utilizes an electron-transport chain consisting of a FAD and a plant ferredoxin type [2Fe-2S] center to shuttle electrons from NADH to E_1, thus reducing the E_1-bound glucoseen intermediate, **4**, E_3 must be able to recognize E_1. Although other enzymes, such as diaphorase and MMO reductase, can replace E_3 in this electron-transport relay, they lack the specificity inherent to E_3 which makes it the preferred reductase in the biosynthesis of ascarylose. The radical nature of this C-3 deoxygenation is reminiscent of the well-known sugar deoxygenation catalyzed by ribonucleotide reductase, albeit the mechanisms of these two deoxygenations are fundamentally distinct (Stubbe, 1989a,b; Bollinger et al., 1991). The insights gained from these studies have provided, for the first time, compelling evidence suggesting that a biological deoxygenation is effected by a new radical process. The unprecedented mechanisms of both E_1 and E_3 distinguish this system as a novel C–O bond cleavage process with the promise of intrigue through future investigation.

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