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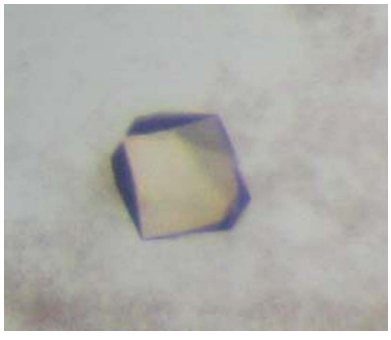
Biosynthesis of a 3,6-dideoxyhexose: crystallization and X-ray diffraction of CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1) for ascarylose biosynthesis

CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1), along with its reductase (E_3), catalyzes the unusual C-3 deoxygenation of CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose to form CDP-3,6-dideoxy-L-*threo*-D-*glycero*-4-hexulose in CDP-ascarylose biosynthesis [Chen *et al.* (1996), *Biochemistry*, **35**, 16412–16420]. This dimeric [2Fe–2S] protein, cloned from the bacteria *Yersinia pseudotuberculosis*, is currently the only known example of an enzyme that uses a vitamin B_6-derived pyridoxamine 5′-phosphate (PMP) cofactor to carry out one-electron chemistry [Agnihotri & Liu (2001), *Bioorg. Chem.* **29**, 234–257]. It also exhibits a [2Fe–2S] cluster-binding motif (C-X_{57}-C-X_1-C-X_7-C) which has not been observed previously [Agnihotri *et al.* (2004), *Biochemistry*, **43**, 14265–14274] The recombinant 97.7 kDa dimer was crystallized in the trigonal space group $P3_2$, with unit-cell parameters $a = b = 97.37$, $c = 142.2$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. A data set has been collected to 1.9 Å resolution. A full MAD data set was also collected at the iron absorption edge that diffracted to 2.0 Å.

1. Introduction

Carbohydrates comprise a large group of physiologically vital biomolecules (Hallis & Liu, 1999). In addition to their paramount importance as an energy source, they are critical biosynthetic precursors and cellular structural elements that are necessary for all life forms (He & Liu, 2002). Deoxysugars represent a unique subclass of carbohydrates that are categorized by the replacement of one or more hydroxyl groups with hydrogen or non-O-linked functionality (Hallis & Liu, 1999). These compounds play a variety of roles. For example, they are ligands for cell–cell interactions, targets for antibodies, antigenic components of bacteria and activating components of secondary-metabolite antibiotics (Allen & Kisailus, 1992; Weymouth-Wilson, 1997). 3,6-Dideoxyhexoses, found in the lipopolysaccharide of many Gram-negative cell envelopes, have also been demonstrated to be the major antigenic determinants of virulence and pathogenesis of *Yersinia pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*, which cause plague, enterocolitis and septicemia, respectively (He & Liu, 2002; Revell & Miller, 2001). E_1 dehydrase, along with its reductase E_3, catalyzes an intriguing C-3 deoxygenation of the CDP-glucose-derived CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose to form CDP-3,6-dideoxy-L-*threo*-D-*glycero*-4-hexulose in CDP-ascarylose biosynthesis (Fig. 1; Chen *et al.*, 1996). Structural elucidation of E_1 has three potential impacts. Firstly, 3,6-dideoxy sugars are important for the virulence and pathogenesis of the *Yersinia* pathogens (Revell & Miller, 2001), so that structure-based E_1 inhibitors could terminate the biosynthesis of 3,6-dideoxy sugars and potentially lead to pathogen death. Secondly, the presence of deoxy sugars is crucial for the pharmaceutical activities of polyketide natural products such as the antibiotic erythromycin and the anticancer compound doxorubicin; structure-based mutants of E_1, should they have relaxed substrate specificity, may be used to produce novel TDP-3,6-dideoxy sugars depending on the substrate compounds offered to the enzyme. Novel TDP-3,6-dideoxy sugars generated in this manner could be offered to glycosyltransferases in an attempt to incorporate them onto natural product aglycone rings and thus change the biological activities of secondary metabolites. Such compounds could then be screened for pharmaceutical activity. Finally, E_1 is currently the only known enzyme that uses a PMP



cofactor for one-electron chemistry and the first known to exhibit a variant [2Fe–2S] cluster-binding motif (C-X_{57}-C-X_1-C-X_7-C; Agnihotri *et al.*, 2004). Structural details will greatly aid in the understanding of how such chemistry proceeds in the active site of this [2Fe–2S] PMP-dependent dehydrase, whose sequence identity (26%) most closely likens it to the pyridoxal 5′-phosphate-dependent ArnB aminotransferase of *Salmonella typhimurium* (PDB code 1mdz). Presented herein is the first crystallographic data set for E_1.

2. Materials and methods

2.1. Materials

Escherichia coli JM105 cells were purchased from Pharmacia (currently Amersham, Piscataway, NJ, USA). Enzymes used in the cloning experiment were obtained from Invitrogen (formerly Gibco BRL, Carlsbad, CA, USA) or Promega (Madison, WI, USA). Antibiotics and biochemicals used in this study were products of Sigma–Aldrich Chemical Co. (St Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA). All chemicals were analytical grade or the highest quality commercially available. Culture-medium ingredients were purchased from Difco (Detroit, MI, USA). DNA minipreps were performed using the Wizard DNA purification kit from Promega. All oligonucleotide primers for PCR amplification of the desired inserts were prepared by Gibco BRL (Grand Island, NY, USA) and used without further purification. Restriction endonucleases were products of Amersham, Gibco BRL or Promega. The DEAE-Sepharose CL6B resin and the MonoQ HR (10/10) column were obtained from Amersham. All electrophoresis materials were purchased from Gibco BRL or Bio-Rad (Hercules, CA, USA).

2.2. Growth of cells

An overnight culture of *E. coli* JM105/pJT18 cells grown at 310 K in LB medium containing ampicillin (100 µg ml^{-1}) was used in a 1000-fold dilution to inoculate 6 l of the same medium. After incu-

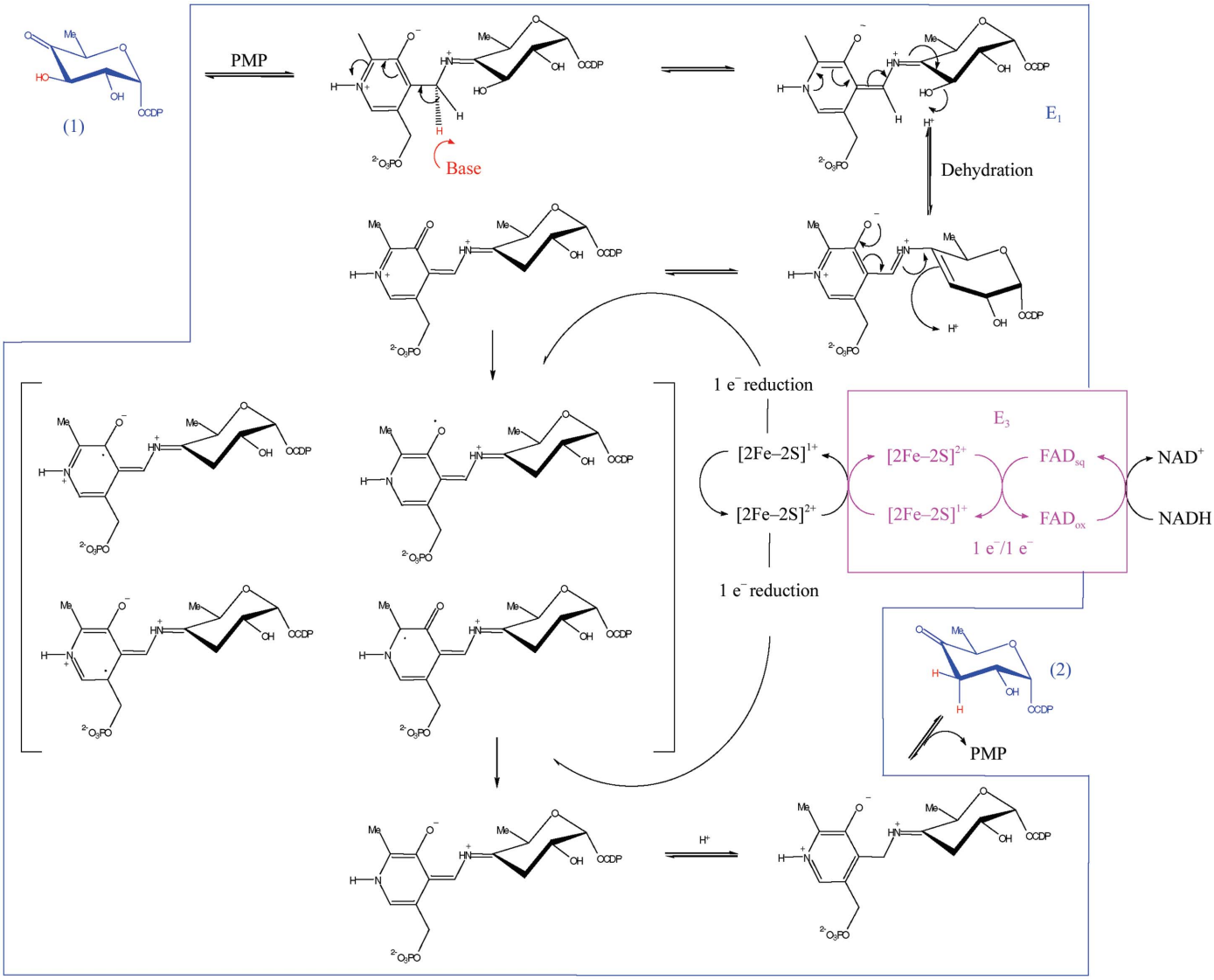


Figure 1
Deoxygenation of CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (1) to form CDP-3,6-dideoxy-L-*threo*-D-*glycero*-4-hexulose (2) catalyzed by E_1 dehydrase and E_3 reductase in CDP-ascarylose biosynthesis. Chemical steps within the E_1 active site are enclosed by a blue border and those undergone in the redox partner E_3 are enclosed by a pink border. Substrate (1) forms a Schiff base in the E_1 active site with the PMP cofactor, interconverting between the hydrated and dehydrated substrate adduct. The product is released upon two single-electron transfers from E_3. NADH is the original electron donor.

Table 1
Data-collection statistics for E_1: MAD and native data sets, each from a unique single crystal.

Values in parentheses are for the highest resolution shell.

	MAD			
	λ1	λ2	λ3	Native
Space group	$P3_2$	$P3_2$	$P3_2$	$P3_2$
Unit-cell parameters (Å)	$a = b = 97.2$, $c = 142.0$			$a = b = 96.814$, $c = 141.644$
Temperature (K)	100	100	100	100
Wavelength (Å)	1.73940	1.74140	1.00866	1.00114
Resolution range (Å)	50–2.2 (2.24–2.20)	50–2.3 (2.34–2.30)	50–2.8 (2.9–2.8)	50–1.92 (1.95–1.92)
Mosaicity (°)	0.53	0.67	0.43	0.65
Unique reflections	75934 (3579)	66931 (2912)	37237 (1852)	114411 (5764)
Redundancy	10.5 (6.0)	9.4 (2.7)	11.6 (11.6)	5.5 (5.0)
Completeness (%)	99.7 (94.8)	99.2 (87.9)	100 (100)	100 (99.9)
R_{merge}	8.5 (65.7)	10.0 (68.3)	8.1 (54.6)	7.6 (76.8)
Mean $I/\sigma(I)$	27.4 (1.85)	22.73 (1.23)	33.6 (5.6)	21.2 (2.34)
Solvent content (%)	70			70

bation for an additional 15 h at 310 K, cells were harvested by centrifugation (4000*g*, 15 min) at 277 K, washed with 50 m*M* potassium phosphate buffer pH 7.5, collected again by centrifugation (4500*g*, 20 min) and stored at 193 K. The typical yield was 6 g of wet cells per litre of culture.

2.3. Purification of recombinant E_1

All purification procedures were carried out at 277 K, except for the FPLC steps, which were carried out at room temperature. Unless otherwise noted, 20 m*M* Tris–HCl buffer pH 7.5 (buffer *A*) was used in all purification procedures. All buffers were degassed and saturated with nitrogen before use.

Thawed cell pellets, resuspended in 100 ml buffer *A* containing 1 m*M* dithiothreitol (DTT), were disrupted by sonication in six 1 min bursts with a 1.5 min cooling period between each burst. Cell debris was removed by centrifugation (27 000*g*, 20 min) and the supernatant was quickly transferred to a flask and flushed with nitrogen. A sufficient quantity of ammonium sulfate was added slowly to the crude extract (115 ml) to bring the extract to 80% saturation and the solution was stirred for 1 h under nitrogen. The precipitated proteins were collected by centrifugation (12 000*g*, 15 min) and resuspended in a minimal amount of buffer *A*. The protein was dialyzed three times against 1 l buffer *A* under nitrogen. Subsequently, protein was loaded onto a DEAE-Sepharose CL6B column (2.5 × 40 cm) equilibrated with buffer *A*. After washing with 200 ml buffer *A*, the column was eluted with a 0–0.4 *M* linear gradient of NaCl in buffer *A* (1 l in total) at a rate of 50 ml h^{-1}. 12 ml fractions were collected during the elution. The desired fractions (fractions 57–62), as detected by SDS–PAGE, were pooled, dialyzed three times against 1 l buffer *A* and concentrated by ultrafiltration through an Amicon YM10 membrane (Millipore Corporation, Bedford, MA, USA). Finally, the protein was further purified by FPLC on a MonoQ HR (10/10) column using buffer *A* and buffer *B* (buffer *A* made 0.5 *M* in NaCl). The elution profile used a linear gradient of 0–75% buffer *B* in 25 min, followed by another gradient of 75–95% buffer *B* in 13 min. 100% buffer *B* was used to wash the column for 10 min. The flow rate was 3 ml min^{-1} and the detector was set at 280 nm. The E_1 protein eluted at 80% buffer *B*. It was concentrated by ultrafiltration through an Amicon YM10 membrane, desalted with buffer *A* and stored at 193 K. The yield of purified E_1 was 80 mg from a 6 l culture.

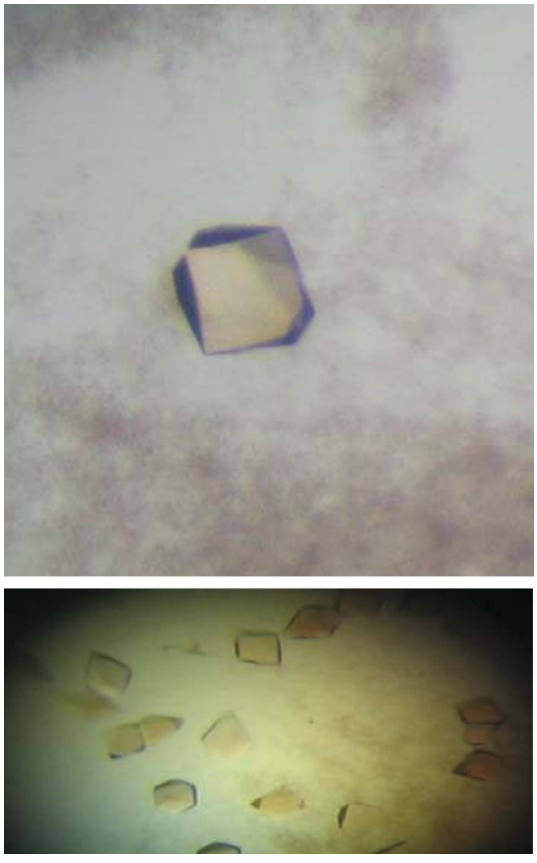


Figure 2
Hexagonal crystals of E_1, grown at 295 K for one week, are approximately 0.1 mm in length.

2.4. Crystallization

Purified E_1 was supplied in 20 m*M* Tris–HCl buffer pH 7.5. The protein sample was diluted in a degassed solution of the same buffer with the addition of 5 m*M* dithiothreitol (DTT). Samples were then filtered with 500 µl Pall Corporation Nanosep MF 0.2 µm centrifuge filters before addition to crystal trays. Appropriate protein concentrations for crystallization screening were determined to be 8 mg ml^{-1} using the precrystallization test (PCT) kit from Hampton Research (Aliso Viejo, CA, USA). Sitting-drop vapor-diffusion method crystallization trays were always prepared in an anaerobic glove box (oxygen $\leq$ 5 p.p.m.) owing to the oxygen-sensitive native [2Fe–2S] cluster. Before going into the glove box, all samples and crystal-screening reagents were degassed (five 30 s cycles at 3.33 kPa, followed by a 15 min stand at 4.0 kPa). All cycles were interspersed with argon flushing. A total of 400 commercial matrix conditions were screened. Thin poorly diffracting needle crystals grew in 0.1 *M* HEPES buffer pH 7.5, 1.0 *M* ammonium sulfate, 2%(*v*/*v*) polyethylene glycol (PEG) 400 using 2 µl sample and 2 µl reservoir solution. These crystals (Fig. 2) were optimized to the high-quality crystals from which the diffraction data were collected using a reservoir solution of 0.1 *M* HEPES pH 7.6, 1.0 *M* ammonium sulfate, 2%(*v*/*v*) PEG 400 and the addition of 2%(*v*/*v*) benzamidine hydrochloride to this mixture as follows: 5 µl protein sample, 1 µl 20%(*w*/*v*) benzamidine–HCl and 4 µl reservoir solution (500 µl per well) at 295 K, lowering the protein concentration to 2 mg ml^{-1}.

2.5. Data collection

Crystals were soaked in a cryoprotectant solution consisting of reservoir cocktail and 30%(*v*/*v*) glycerol and flash-cooled in liquid

nitrogen. Diffraction experiments were carried out at the University of California at Berkeley Advanced Light Source (ALS), beamline 5.0.2. Using the natural fluorescence detector upon X-ray diffraction, a strong iron absorption edge arising from the iron in the native [2Fe–2S] center of E_1 was observed. The native data set was obtained to 1.92 Å resolution. Furthermore, a three-wavelength iron MAD data set (a full data set for each of the peak, inflection and remote wavelengths) was collected. The data were integrated and scaled with *HKL*2000 and *SCALEPACK* (Otwinowski & Minor, 1997). Mosaicity was determined with *HKL*2000 (Otwinowski & Minor, 1997). Data-collection statistics are provided in Table 1.

3. Results and discussion

E_1 is the only known enzyme to require a [2Fe–2S] cluster and a PMP cofactor to modify its substrate. It is also the only known enzyme whose catalysis involves the formation of a PMP-centered radical, making it an unusual dehydrase. In addition, the [2Fe–2S] coordinating cysteines exhibit a previously unseen spacing motif (Agnihotri *et al.*, 2004). In this report, we have purified and crystallized E_1 dehydrase under anaerobic conditions. We found that in the absence of an anaerobic setup, the E_1 crystals disintegrated after one week, presumably owing to oxidation of the [2Fe–2S] cluster. Extensive screening of additives indicates that benzamidine is required in the crystallization in order to obtain high-quality E_1 crystals. From these crystals, high-resolution preliminary X-ray diffraction data have been obtained for E_1, with the added benefit of a naturally occurring iron source in the [2Fe–2S] cluster. This inclusion makes the crystals amenable to structural solution using iron as an anomalous diffractor of X-rays. With a full data set for each of the peak, inflection and remote wavelengths of iron in our crystal, we are well primed to attempt a structural solution using either the SAD or MAD method. The crystal structure of E_1 will undoubtedly offer insight into the environment required for the intriguing chemistry it affects, such as the challenging radical-mediated deoxygenation reaction using PMP and the uniquely bound [2Fe–2S] cluster. The results could impact the development of new antibacterial agents and provide a structural rationale for the chemistry carried out by CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1) and its reductase (E_3).

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