Characterization of the First PMP-Dependent Iron–Sulfur-Containing Enzyme Which Is Essential for the Biosynthesis of 3,6-Dideoxyhexoses[1]

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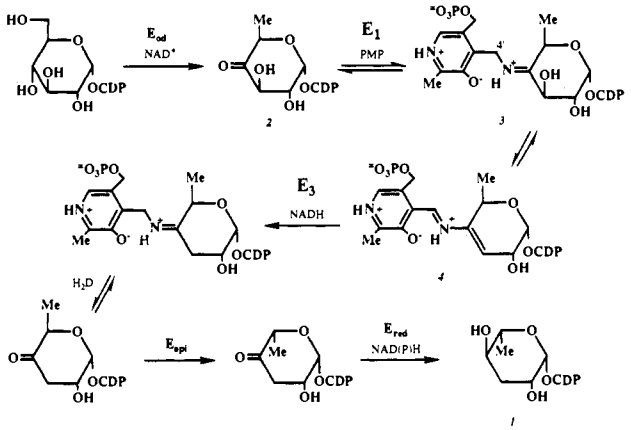
Deoxysugars are an essential class of carbohydrates which exist ubiquitously in nature as mono-, di-, and even trideoxy species, often possessing intriguing biological activities.[2] The 3,6-dideoxyhexoses, in particular, have been found in the lipopolysaccharide of a number of gram-negative cell envelopes where they are the major antigenic determinants and are essential for establishing the serological specificity of many immunologically active polysaccharides.[3] As depicted in Scheme I, studies of the biosynthesis of CDP-ascarylose (CDP-3,6-dideoxy-L-*arabino*-hexopyranose, **1**) have demonstrated that the first phase of the carbon–oxygen bond cleavage at C-3 is catalyzed by a pyridoxamine 5′-phosphate (PMP)-dependent enzyme (CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase, E_1), previously known as CDP-4-keto-6-deoxy-D-glucose-3-dehydrase.[4] A reductive step catalyzed by CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3), which contains both a FAD and a [2Fe–2S] center, has been suggested to constitute the second phase of C-3 deoxygenation (Scheme I).[5] The postulated mechanism for E_1 catalysis follows the well-established vitamin B_6 coenzyme chemistry and is initiated by Schiff base formation between the PMP coenzyme and the C-4 keto group of the substrate, CDP-4-keto-6-deoxy-D-glucose (**2**), followed by a C-4′ proton abstraction from the resulting adduct **3** that triggers the expulsion of the C-3 hydroxyl group.[4] Although initial evidence supporting this mechanistic proposal has been furnished by our recent stereochemical analysis,[4d] more information is required for a complete characterization of this unique deoxygenation which represents the only PMP-dependent catalysis that is not a transamination reaction.

In our continuing efforts to explore the mechanism of this intriguing enzymatic process, we have cloned the gene coding for E_1 (*asc*C) from a *Yersinia pseudotuberculosis* λ gt11 subgenomic library by hybridization with a degenerate oligonucleotide probe designed from the *N*-terminal sequence of the purified wild-type E_1. The enzyme was overexpressed in *Escherichia coli* from a pUC-based plasmid and purified to homogeneity by DE-52 and

Scheme I



G-100 chromatography.[6] A representative yield of E_1 from the recombinant *E. coli* strain was 1.3 g of E_1 per 45 g of wet cells, a quantity previously unobtainable from the wild-type source. Our initial large-scale purification of the recombinant E_1 surprisingly revealed that the enzyme in its concentrated form displays a dark brown color, a trait often associated with metalloproteins. Careful characterization of this recombinant protein has unveiled that E_1 contains, in addition to PMP, a new cofactor with an electronic absorption spectrum (Figure 1) consistent with the ferredoxin-type [2Fe-2S] clusters.[7,8] Preliminary data supporting this assignment were provided by Fe quantitation obtained by both inductively coupled plasma (ICP) emission analysis and a spectrophotometric determination.[9] The quantitation of S^{2-} was also carried out spectrophotometrically[10] with all protein determinations based upon quantitative amino acid hydrolysis. These analyses revealed 1.8 equiv of Fe and 1.6 equiv of S^{2-} per E_1.[11] Subsequent EPR studies showed that E_1 in its oxidized form is EPR silent (Figure 1, inset). However, similar to reduced adrenodoxin[12] or putidaredoxin,[13] fully reduced E_1 displayed an almost axial EPR signal having *g* values of 2.007, 1.950, and 1.930 (0.78 spins/2 Fe) which are prevalent at temperatures > 100 K. These cumulative observations, in conjunction with the fact that 1 electron equivalent of dithionite is needed to fully reduce E_1 under anaerobic conditions, unequivocally established the existence of a [2Fe-2S] cluster.[14] Since the apoenzyme, prepared by treatment with mersalyl acid,[15] was found to retain its capability to abstract a C-4′ proton from **3**, a prerequisite for C-3 deoxygenation, but failed to yield product

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(6) E_1 was eluted from the DE-52 column (2.5 × 15 cm², preequilibrated with 200 mM potassium phosphate, 1 mM EDTA buffer, pH 7.5) with a linear gradient of 200–400 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.5. Gel filtration was accomplished upon Sephadex G-100 (1.5 × 150 cm²) with an elution buffer of 50 mM potassium phosphate, 1 mM EDTA, pH 7.5.

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(8) The UV–vis spectrum of E_1 displays a maximum at 454 nm and a shoulder at 550 nm with the remaining characteristics masked by the PMP absorbance at 334 nm.

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(10) Beinert, H. *Anal. Biochem.* **1983**, *131*, 373.

(11) The molecular weight of E_1 was determined to be 60 000 Da by FPLC equipped with a Superose 12 HR 10/30 column standardized with two separate sets of molecular weight standards. The DNA sequence predicts a protein of 437 amino acids with a mass of 48 371 Da. This predicted molecular weight is in consistent with the subunit molecular weight of 49 000 Da determined by SDS-PAGE. The iron and sulfur contents of E_1 vary among preps, and the numbers reported are an average of the three best purifications.

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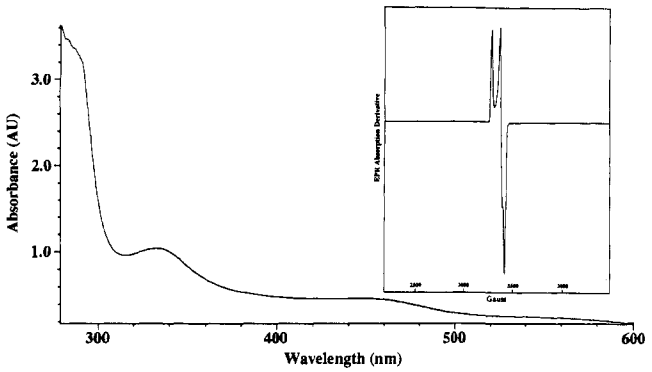

Figure 1. UV–vis absorption spectrum of purified E_1 (protein concentration 73 μM, 50 mM potassium phosphate buffer, pH 7.5). The inset shows cryogenic X-band EPR of dithionite-reduced E_1 in 50 mM potassium phosphate, pH 7.5 (120 μM, 9.215 GHz, 5 mW, 2×10^3, 40 K, modulation amplitude 10 G).

upon incubation with substrate (**2**) and E_3,[16] this newly identified iron–sulfur center must play a catalytic role in E_1 catalysis.

On the basis of the newly unraveled physical characteristics of E_1, the molecular mechanism of E_1/E_3 catalysis has to be revised. The order of electron flow is likely to start with hydride reduction of the FAD in E_3 by NADH. The iron–sulfur cluster of E_3, after receiving electrons one at a time from the reduced flavin, relays the reducing equivalents to its acceptor, the [2Fe-2S] center of E_1. Finally, the reducing equivalents from the reduced iron–sulfur cluster of E_1 are transferred likewise in a one-electron fashion, to the E_1-bound PMP–glucoseen intermediate **4**. This proposed electron transport sequence is mechanistically sound and is consistent with E_3's role as an electron shuttle protein, similar to other reductases such as methane monooxygenase reductase[17] and phthalate dioxygenase reductase.[18] Experimental evidence for the order of this electron relay was provided by a simple UV titration of E_1 with NADH in the presence of a "catalytic" amount of E_3, where reduction of E_1 was found to occur only in the presence of E_3. In light of the fact that the newly identified iron–sulfur center in E_1 is a well-known one-electron carrier and is essential for E_1 catalysis, reduction of the PMP–glucoseen intermediate **4** must occur via a radical mechanism. Since a PMP–glucoseen adduct is the proximate acceptor receiving an electron directly from the iron–sulfur center of E_1, the catalytic role of E_1 in the biosynthesis of ascarylose clearly constitutes a unique example of biological deoxygenation in which the PMP may participate in an unprecedented redox role. Furthermore, the inherent dependence of E_1 on both the PMP and [2Fe-2S] cofactors makes this catalysis singular in its own class. The elucidation and characterization of the new [2Fe-2S] center of E_1 provides fresh evidence strongly supporting a radical mechanism for an E_1-catalyzed reaction. Although the radical nature of this C-3 deoxygenation process is reminiscent of the well-known sugar deoxygenation catalyzed by ribonucleotide reductase,[19] the mechanisms of these two deoxygenations are fundamentally distinct.

(14) This type of iron–sulfur cluster consists of two iron atoms, two atoms of inorganic sulfur, and four cysteine ligands. The two iron atoms in the cluster are in the high-spin Fe(III) state ($S = {}^5/_2$) and coupled antiferromagnetically.[7] However, examination of the deduced amino acid sequence of E_1 revealed none of the typical 2Fe–2S binding motifs, which further accents the uniqueness of E_1.

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(16) E_1 was treated with excess mersalyl acid followed by extensive dialysis in 50 mM potassium phosphate buffer containing 1 mM 2-mercaptoethanol (pH 7.5). This activity of this sample was then analyzed by both the tritium release and GC-MS assay.[4c] In comparison to an identically treated control (in the absence of mersalyl acid), the mersalyl-treated sample was found to retain 96% of its tritium release ability but failed to yield deoxy sugar product (4% determined by an internal standard) upon incubation with substrate and E_3 in the GC-MS assay.

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