Coenzyme B_6 as a Redox Cofactor: A New Role for an Old Coenzyme?

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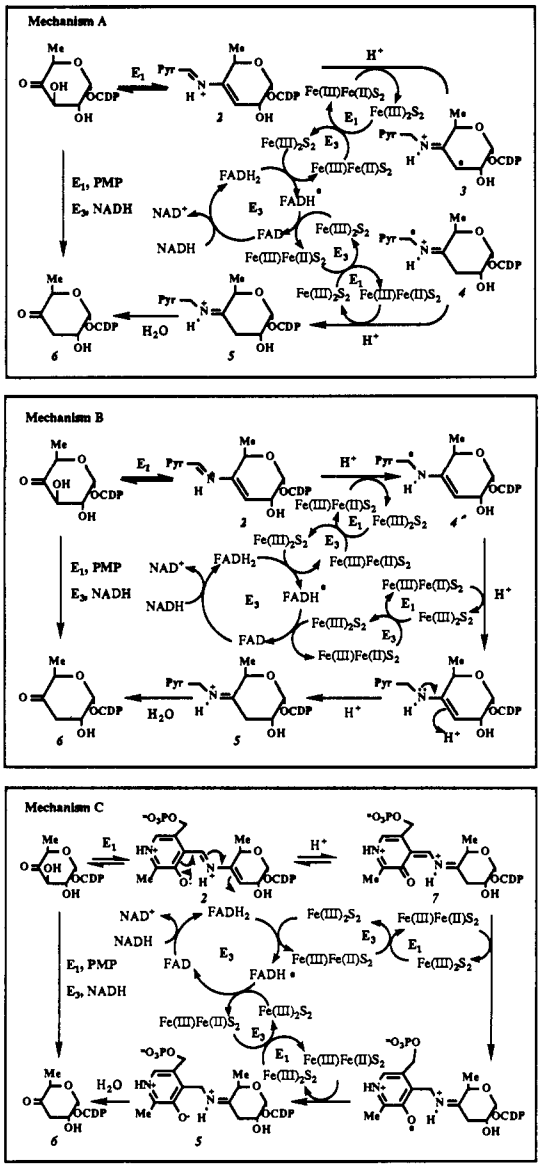
The coenzyme B_6 dependent enzymes are an important class of catalysts which mediate a wide variety of biological transformations involved in the synthesis, degradation, and interconversion of amino acids, such as transamination, decarboxylation, racemization, β- and γ-elimination, and substitution.[1,2] While the aldehyde form of this cofactor, pyridoxal 5′-phosphate (PLP), is the most common form for coenzyme B_6 dependent enzymes, the amine form of this coenzyme, pyridoxamine 5′-phosphate (PMP), has also been shown to play an important role in the reactions mediated by transaminases.[3,4] Early studies of the biosynthesis of 3,6-dideoxyhexoses have discovered a unique PMP-dependent enzyme, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1), which is not a transaminase but is instead a dehydrase catalyzing the carbon–oxygen bond cleavage at C-3,[5] a key step for the formation of CDP-ascarylose (CDP-3,6-dideoxy-L-*arabino*-hexopyranose, **1**).[6] Our recent characterization of the cloned E_1 expressed in *Escherichia coli* has revealed the presence of a redox active [2Fe–2S] center which is essential for E_1 sugar reductase activity.[7] Interestingly, a plant-ferridoxin-type [2Fe–2S] center was also found in CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3),[8] a flavoprotein required for the second phase of C-3 deoxygenation.[9] On the basis of the newly unraveled physical characteristics of these enzymes, the molecular mechanism of E_1/E_3 catalysis can now be formulated. As illustrated in Scheme I, 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. Experimental evidence for the order of this electron relay was previously provided by a simple UV titration of E_1 with NADH, where reduction of E_1 was found to occur only in the presence of E_3.[7] In light of the fact that the iron–sulfur center in E_1 is a well-known one-electron carrier and is imperative for E_1 catalysis, and since a direct hydride reduction has been previously dismissed,[8,9a] reduction of the PMP–glucoseen intermediate **2** most reasonably occurs via a radical mechanism. This reduction may involve a stepwise 1,4 addition of electrons from the E_1 [2Fe–2S] center to **2** (mechanism A), generating either a carbohydrate radical intermediate **3** analogous to its ribonucleotide reductase ribosyl counterpart,[10] or a benzylic radical intermediate **4** which has precedence in the PLP-conjugated

Scheme I



substrate radical found in lysine 2,3-aminomutase catalyzed reaction.[11] A similar mechanism (B) follows reduction at the imino moiety of **2**, and a cognate benzylic radical intermediate **4′** is expected to form. However, a third and clearly the most provocative mechanistic postulation (C) is also conceivable which begins with a tautomerization step generating the PMP-quinoid intermediate **7**, a pseudo-*o*-quinoid species expected to be readily reduced via two one-electron transfers from the E_1 [2Fe–2S] center. The two-electron reduced species **5** will then be hydrolyzed to yield the final E_1–E_3 product **6**. Since the oxygen radical intermediate proposed for mechanism C and the carbinyl radical intermediates postulated for mechanisms A and B are distinct, these mechanisms can be readily differentiated by characterization of the radical intermediate.

In our continuing efforts to explore the details of how E_1 utilizes the reducing equivalents in assisting C–O bond rupture and to secure direct evidence supporting the existence of a radical intermediate, we have carried out a systematic EPR study of E_1 reduced with dithionite in the presence of substrate. Although

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the use of the coupled E_1–E_3 machinery was desired for these experiments, an artificially reduced system was employed to eliminate complications derived from the flavin semiquinone of the reduced E_3. In order to observe the properties of any present organic radical species, the EPR spectrum of this mixture was measured over the temperature range 2–293 K. These studies revealed the presence of a Lorentzian-type absorption with a line width of 1.2 G located at g = 2.007 (Figure 1). When the temperature was lowered, the line width increased gradually, becoming approximately 25 G at 10 K. This signal can be ascribed to a PMP–glucoseen radical since E_1 apoenzyme, that is, without either PMP (obtained via anion-exchange chromatography)[5] or the [2Fe–2S] center (prepared by treatment with mersalyl acid[12] followed by reconstitution with PMP), when reduced with dithionite in the presence of substrate is devoid of this free radical signal. In addition, the observation of the 3,6-dideoxy sugar products (albeit at much lower levels) under identical reduction conditions via a GC–MS assay[5b] further substantiated the catalytic competence of this free radical intermediate. Spin quantitation of the cryogenic signal performed according to reported procedures[13] displayed greater than 0.45 spin/E_1 of organic free radical signal present. As expected, dithionite reduction of a mixture of substrate incubated with PMP in the absence of E_1 is EPR silent.

Previous EPR studies of phenoxy radicals[14] over this temperature range have shown signals with similar characteristics which display no hyperfine structure at 293 K and just slight splitting, attributed to intermolecular coupling, at lower temperatures. Alternatively, a characteristic and dominant EPR spectral feature of π-alkyl radicals and various substituted and nonsubstituted benzyl radicals[15] is the hyperfine splitting tensor from α-proton(s), typically >15 G. In addition, EPR of radicals containing an α-amino group[16] consistently displays ^{14}N hyperfine splitting also of relatively large magnitude (>6 G). Thus, the obvious lack of α-hydrogen hyperfine splitting observed in the E_1–substrate radical spectra (Figure 1B and inset) is most consistent with mechanism C, in which the postulated radical intermediate has a resident unpaired spin at the 3-oxygen of the PMP coenzyme. The formation of a stabilized PMP–glucoseen radical via a one-electron reduction of a redox active pseudo-*o*-quinone intermediate is mechanistically sound, and since the dehydration mediated solely by E_1 is a highly reversible process,[5] such a collaborative stepwise reduction is essential to drive the equilibrium to completion. Thus, in E_1 catalysis, PMP may have diverged from its classical role of purely stabilizing anion chemistry by exhibiting an unprecedented capacity to mediate redox chemistry.[17] In this catalysis, PMP has the dual function of being responsible for the anion-induced dehydration reaction and, by being a participant in the subsequent redox reaction, ultimately receiving electrons from E_3. Since E_1-catalyzed dehydration represents a unique example of PMP-dependent catalysis, the unusual role that PMP provides in facilitating E_3-mediated reduction is not completely unexpected. The elucidation of the new E_1 [2Fe–2S] center[7] and radical intermediate has provided fresh evidence strongly supporting a radical mechanism, thereby establishing the first example of PMP dependent enzymatic redox chemistry. The inherent dependence of E_1 on both PMP and a [2Fe–2S] center makes this catalyst singular in its own class, and very likely brings to light a new and most intriguing role of coenzyme B_6 in redox catalysis.[18]

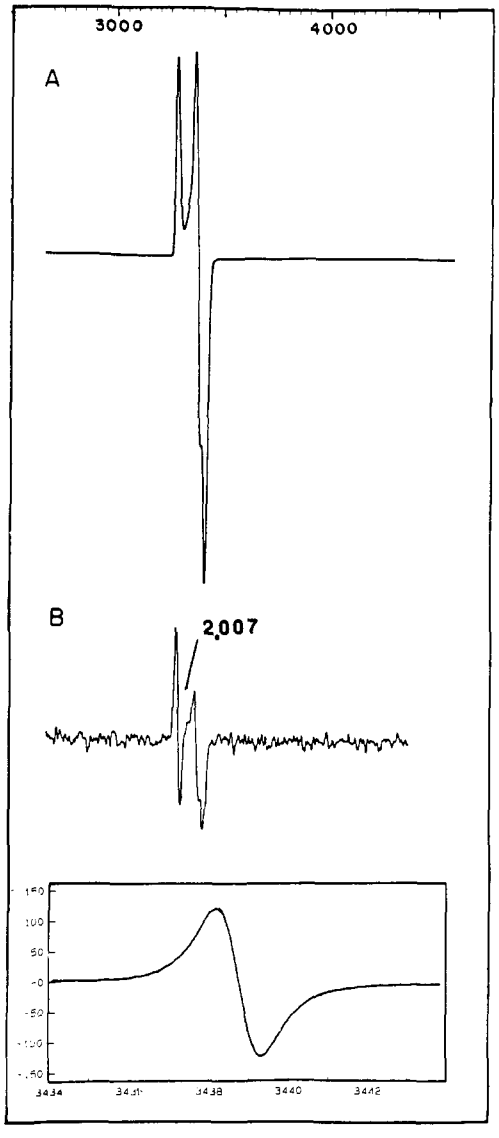

Figure 1. 1. X-band EPR of E_1 substrate intermediate. Cryogenic measurements were made on a Varian E-109 spectrometer digitally interfaced to a laboratory microcomputer and equipped with an Oxford Instruments ESR-910 liquid helium cryostat. Room temperature measurements were made on a Bruker ESP 300 with an ER 4111 VT variable temperature unit on aqueous samples in specially designed flat EPR sample tubes. Dithionite solutions were standardized with riboflavin prior to use, and reduction was carried out under anaerobic conditions. (A) Fully reduced E_1 (120 μM, 9.217 GHz, 5 mW, 40 K). The almost axial signals having g values of 2.007, 1.950, and 1.930 are characteristic for E_1's [2Fe–2S] center.[7] (B) Fully reduced E_1 (120 μM) in the presence of substrate (560 μM, 9.216 GHz, 0.4 μW, 28 K); substrate (CDP-4-keto-6-deoxy-D-glucose) was generated in vitro from CDP-D-glucose with CDP-D-glucose 4,6-dehydratase.[5b] (B, inset) Fully reduced E_1 (120 μM) in the presence of substrate (560 μM, 9.650 GHz, 200 mW, 293 K).

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(17) Another unique but mechanistically different coenzyme B_6 dependent enzyme is lysine 2,3-aminomutase, which contains iron–sulfur centers, cobalt, and PLP and requires *S*-adenosylmethionine for activation.[11]

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