Characterization of a Unique Coenzyme B_6 Radical in the Ascarylose Biosynthetic Pathway

Cheng-Wei T. Chang,[†] David A. Johnson,[†] Vahe Bandarian,[‡] Huiqiang Zhou,[†] Russell LoBrutto,[§] George H. Reed,[‡] and Hung-wen Liu *,[†]

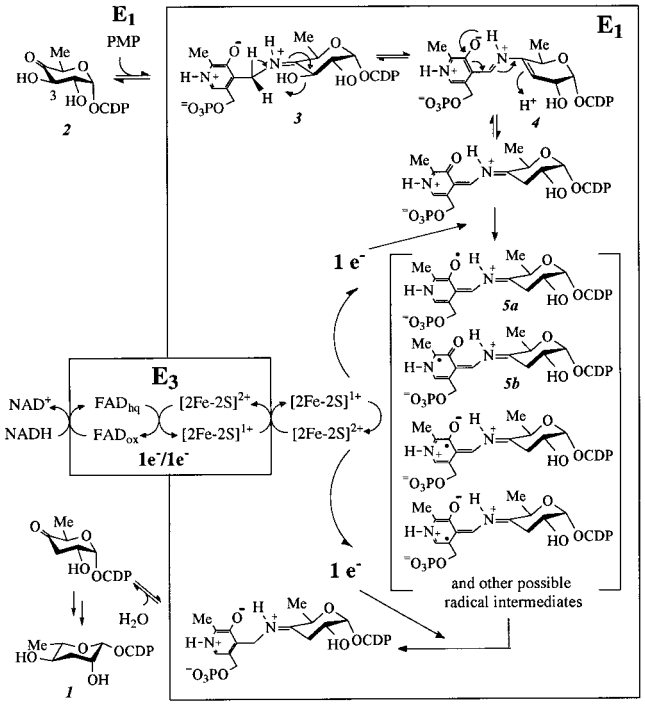
Department of Chemistry, University of Minnesota Minneapolis, Minnesota, 55455
The Institute for Enzyme Research, Graduate School and Department of Biochemistry
College of Agricultural and Life Sciences
University of Wisconsin, Madison, Wisconsin 53705
Department of Plant Biology
Arizona State University, Tempe, Arizona

Received January 6, 2000

Many biological transformations are effected via electron transfer or homolytic bond cleavage, and thus proceed through radical mechanisms.[1] Early studies of the biosynthesis of immunodominant 3,6-dideoxyhexoses found in the lipopolysaccharide of several pathogenic bacterial strains, as exemplified by the biosynthesis of CDP-L-ascarylose (**1**) in *Yersinia pseudotuberculosis* V, have demonstrated that the C-3 deoxygenation step proceeds through a radical mechanism and requires a unique pair of enzymes, E_1[2] and E_3.[2,3] E_1 contains a [2Fe-2S] center and requires pyridoxamine 5′-phosphate (PMP).[4] E_3 contains a flavin and a [2Fe-2S] center and uses NADH as a reductant.[5] In the present work, isotopic labeling of PMP has been combined with EPR techniques to provide unambiguous evidence of a radical being directly associated with the PMP coenzyme in E_1 catalysis.

The catalytic cycle for deoxygenation begins with formation of the Schiff base (**3**) between substrate **2** and PMP in the active site of E_1 (Scheme 1). Subsequent proton abstraction triggers the elimination of 3-OH to give **4**.[4] Transfer of reducing equivalents from NADH via E_3 to reduce the nascent $\Delta^{3,4}$-glucoseen intermediate **4** completes the reaction.[5] Previous studies have revealed the presence of a flavin semiquinone radical in E_3[6] and another organic radical in E_1[7] during transient phases of the reaction. Recent studies led to a model in which the odd electron in the half-reduced intermediate resides primarily on the PMP portion of the PMP−substrate adduct, perhaps as a phenoxyl radical (**5a**).[7]

Scheme 1



To characterize this organic radical, the isotopically labeled forms of PMP, [4′,5′-$^2H_4$]PMP (**6**), and [2′-$^2H_3$]PMP (**7**) have been prepared.[8] Each labeled PMP was used to reconstitute E_1-(apoPMP),[9] and each reconstituted E_1 was used in the coupled E_1−E_3 reaction to prepare samples for CW EPR and pulsed electron nuclear double resonance (pulsed ENDOR) measurements.[10] Figure 1 (inset) shows the CW EPR spectrum of E_1 reconstituted with **6** (heavy line). The signal from the sample with deuterated PMP narrows by approximately 3 G compared with the reference spectrum using E_1 reconstituted with unlabeled PMP (light line). The spin concentration of this organic radical was estimated to be 7.5 μM, which was significantly higher than the maximum possible flavin semiquinone concentration of 0.4 μM calculated on the basis of E_3 concentration in the E_1−E_3 reaction mixture. The observed sharpening effect on the EPR signal is

[†] University of Minnesota.
[‡] University of Wisconsin.
[§] Arizona State University.

(1) (a) Stubbe, J. *Biochemistry* **1988**, *27*, 3893−3900. (b) Frey, P. A. *Chem. Rev.* **1990**, *90*, 1343−1357. (c) Brush, E. J.; Kozarich, J. W. In *The Enzymes*, 3rd ed.; Sigman, D. S., Ed.; Academic Press: New York, 1992; pp 318−403. (d) Frey, P. A. *Curr. Opin. Chem. Biol.* **1997**, *1*, 347−356. (e) Stubbe, J.; van der Donk, W. A. *Chem. Rev.* **1998**, *98*, 705−762.

(2) E_1: CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase. E_3: CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase.

(3) (a) Johnson, D. A.; Liu, H.-w. *Curr. Opin. Chem. Biol.* **1998**, *2*, 642−649. (b) Johnson, D. A.; Liu, H.-w. In *Comprehensive Chemistry of Natural Products Chemistry*; Barton, D., Nakanishi, K., Meth-Cohn, O., Eds.; Pergamon: New York, 1999; Vol. 3, pp 311−365. (c) Johnson, D. A.; Liu, H.-w. In *The Biology−Chemistry Interface: A Tribute to Koji Nakanishi*; Cooper, R., Snyder, J. D., Eds.; Marcel Dekker: New York, 1999; pp 351−396. (d) Hallis, T. M.; Liu, H.-w. *Acc. Chem. Res.* **1999**, *32*, 579−588.

(4) (a) Weigel, T. M.; Liu, L.-d.; Liu, H.-w. *Biochemistry* **1992**, *31*, 2129−2139. (b) Weigel, T. M.; Miller, V. P.; Liu, H.-w. *Biochemistry* **1992**, *31*, 2140−2147. (c) Thorson, J. S.; Liu, H.-w. *J. Am. Chem. Soc.* **1993**, *115*, 7539−7540. (d) Lei, Y.; Ploux, O.; Liu, H.-w. *Biochemistry* **1995**, *34*, 4643−4654.

(5) (a) Miller, V. P.; Thorson, J. S.; Ploux, O.; Lo, S. F.; Liu, H.-w. *Biochemistry* **1993**, *32*, 11934−11942. (b) Lo, S. F.; Miller, V. P.; Lei, Y.; Thorson, J. S.; Liu, H.-w.; Schottel, J. L. *J. Bacteriol.* **1994**, *176*, 460−468. (c) Ploux, O.; Lei, Y.; Vatanen, K.; Liu, H.-w. *Biochemistry* **1995**, *34*, 4159−4168. (d) Burns, K. D.; Pieper, P. A.; Liu, H.-w.; Stankovich, M. T. *Biochemistry* **1996**, *35*, 7879−7889.

(6) Gassner, G. T.; Johnson, D. A.; Liu, H.-w.; Ballou, D. P. *Biochemistry* **1996**, *35*, 7752−7761.

(7) (a) Thorson, J. S.; Liu, H.-w. *J. Am. Chem. Soc.* **1993**, *115*, 12177−12178. (b) Johnson, D. A.; Gassner, G. T.; Bandarian, V.; Ruzicka, F. J.; Ballou, D. P.; Reed, G. H.; Liu, H.-w. *Biochemistry* **1996**, *35*, 15846−15856.

(8) Compound **6** was prepared based primarily on an established scheme (Pieper, P. A.; Yang, D.-y.; Zhou, Z.-q.; Liu, H.-w. *J. Am. Chem. Soc.* **1997**, *119*, 1809−1817). Preparation of **7** also followed a previously developed sequence (Yang, D.-y.; Shih, Y.; Liu, H.-w. *J. Org. Chem.* **1991**, *56*, 2940−2946).

(9) The E_1(apoPMP) protein was prepared by dispersing wild-type E_1 (60 mg) throughout the top 8 cm of a column of DEAE-Sepharose (1 × 10 cm) preequilibrated with 50 mM potassium phosphate buffer (pH 7.5) that had been degassed by cycling between an aspirator vacuum and nitrogen bubbling over a period of an hour. The column was washed continuously at 4 °C with the same degassed buffer for 4 days at a flow rate of 2 L per day. The sample was eluted from the column using 20 mM Tris HCl buffer (pH 7.5) containing 0.5 M NaCl, and subsequently desalted and concentrated using an Amicon ultrafiltration unit (YM-10) with 20 mM Tris HCl buffer, pH 7.5.

(10) A single stock solution of E_1(apoPMP) (15% residual PMP content; 675 μM.; ~0.7−1.0 mol Fe/monomer) was used to generate the results reported in this paper. The E_1 substrate **2** (46.9 mM) was prepared as previously described.[7b] Volumes of various stock solutions were used to give a final concentration of 350 μM E_1, 0.4 μM E_3, 7 mM E_1 substrate, 3.5 mM PMP, and 7 mM NADH in the reaction mixture (200 μL total volume). The samples were prepared by incubating the E_1(apoPMP) with all the reaction components except E_3 for a period of 20 min. After the preincubation to reconstitute the E_1 to full activity, the E_3 was added to initiate the reaction, the tube was vortexed, and the solution was transferred to the EPR tube using a glass pipet and then frozen in liquid nitrogen. The estimated time from the addition of E_3 to the freezing of the sample was about 30 s. Once frozen, the samples were subjected to CW- and ENDOR-EPR analyses.

10.1021/ja0000807 CCC: $19.00
Published on Web 04/14/2000

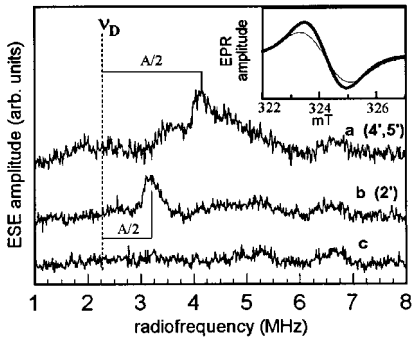
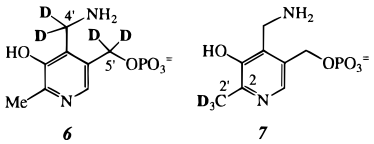


Figure 1. Mims ENDOR spectra obtained from trapped radical in E_1 reconstituted with the following: (a) [4′,5′-$^2H_4$]PMP (**6**); (b) [2′-$^2H_3$]PMP (**7**); and (c) unlabeled PMP. Experimental conditions: instrument, Bruker Elexsys E580; $B_o = 345.7$ mT; $\nu_\epsilon = 9.6989$ GHz; $\tau = 120$ ns; $T = 5$ K; pulse repetition rate = 1 kHz; rf pulse length = 7 μs; total shot repetitions/data point = 2400; shot repetition rate = 1 kHz. The inset shows an overlay of the X-band CW EPR spectra of the E_1-E_3 coupled reaction using E_1 reconstituted with unlabeled PMP (light line) and [4′,5′-$^2H_4$]-PMP (heavy line). The samples were prepared as described in ref 10. EPR conditions: microwave frequency, 9.092 GHz; gain, 2×10^4; temperature, 77 K; and magnetic field modulation amplitude, 0.4 mT.

indicative of the replacement of strongly hyperfine-coupled ^1H by ^2H,[11] specifically the 4′- and/or 5′-^2Hs of PMP.

Pulsed ENDOR was used to characterize **5** further. The Mims[12] ENDOR spectra of the trapped radical in E_1 in complexes with **6**, **7**, and unlabeled PMP are shown in Figure 1, spectra a, b, and



6 *7*

c, respectively. Since spectra 1a and 1b have transitions that are absent in 1c, these signals are assigned to deuterium.[13] Some ^2H transitions occur at frequencies well-separated from that of the ^2H Larmor frequency, a phenomenon characteristic of relatively strong nuclear hyperfine coupling.[14] As expected, variation of the timing parameter τ over a range of small values (100−200 ns) has very little effect on the spectra.

Because **6** is multiply labeled, it was not possible to assign the ^2H resonances in Figure 1a to specific deuterons at either C-4′ or C-5′. If the (typically small) nuclear quadrupole couplings for ^2H are ignored, and if it is assumed that $A/2 < \nu_D$, then the most prominent peak at approximately 4.1 MHz reflects an estimated coupling constant of 3.7 MHz.[15] This translates to a ^1H coupling of about 24 MHz (8.6 G splitting), which would be conspicuous as an inhomogeneous broadening of an EPR signal having a line width of 20 G. Deuteration would diminish this inhomogeneous broadening as observed in the CW EPR spectrum (Figure 1, inset). Figure 1a also shows evidence of a wide range of hyperfine coupling constants. This breadth could arise from the presence of multiple inequivalent ^2H couplings, due to the variation of ^2H orientations with respect to the PMP ring.

At least one ^2H nucleus of **7** gives a sharp ENDOR signal at 3.2 MHz (Figure 1b). A 1.9 MHz ^2H hyperfine coupling constant accounts for this transition, and places the companion signal at 1.3 MHz where it would be difficult to detect. The 1.9 MHz hyperfine coupling translates to a ^1H coupling of just over 12 MHz. The presence of significant electron spin density at C-2 suggests **5b** as one of the possible resonance structures of the radical (Scheme 1), and would give rise to β-^2H couplings with low anisotropy.

It is useful to correlate the appearance of ^2H ENDOR with the loss of the corresponding ^1H transitions. A Mims ENDOR difference spectrum (not shown) was obtained by subtracting the [2′-$^2H_3$]PMP from the natural abundance PMP spectrum. The expected 12 MHz ^1H coupling was not observed despite careful optimization of τ. However, a ^1H coupling of about 7 MHz was found, which must also arise from a methyl proton. Also detected in the difference spectrum were hints of a still weaker ^1H coupling. Clearly, not all of the stronger ^1H couplings are readily detectable by Mims ENDOR. The presence of multiple inequivalent methyl ^1H couplings is to be expected, since methyl group rotation is almost surely frozen out at 5 K. Detailed ENDOR analysis of ^2H and ^1H couplings in the trapped radical is in progress.

The observation of moderately large ^2H hyperfine couplings arising from at least two separate positions within the PMP moiety demonstrates that the organic radical in E_1, which had been characterized kinetically,[7b] is indeed localized to PMP. Though organic radicals have been discovered in a wide variety of enzymatic reactions,[1] the data reported herein represent the first convincing evidence of a radical being immediately associated with PMP. The participation of PMP cofactor in deoxygenation is unique, but the direct involvement of PMP in the subsequent electron transfer reduction via a radical mechanism truly places E_1 in a class by itself. Several E_1 homologues whose sequences show good similarity with that of E_1 are known.[16] Although their catalytic roles remain to be elucidated, E_1 may be the prototype for a new class of coenzyme B_6-dependent enzymes that use an alternate chemistry to catalyze transformations not normally expected for this cofactor.

Acknowledgment. This work is supported by grants from the National Institutes of Health (GM35906 to H.-w.L. and GM35752 to G.H.R.) and the National Science Foundation (BIR-9601774 to R.L). H.-w.L. also thanks the National Institute of General Medical Sciences for a MERIT Award. D.A.J. was a trainee of a NIGMS Biotechnology Training Grant (2 T32 GM08347).

JA0000807

(11) (a) Carrington, A.; McLachlan, A. D. *Introduction to Magnetic Resonance*; Harper and Row: New York, 1967; pp 79−80. (b) Norris, J. R.; Uphaus, R. A.; Crespi, H. L.; Katz, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 625−628.

(12) Mims, W. B. In *Electron Paramagnetic Resonanc*e; Geschwind, S., Ed.; Plenum Press: New York, 1972; pp 263−351.

(13) A weak but quite reproducible pair of transitions is evident in all three traces in Figure 1, at 5.2 and 6.7 MHz. These could be due to strongly coupled ^{14}N or ^1H.

(14) Hoffman, B. M.; DeRose, V. J.; Doan, P. E.; Gurbiel, R. J.; Houseman, A. L. P.; Telser, J. In *Biological Magnetic Resonance*; Berliner, L. J., Reuben, J., Eds.; Plenum Press: New York, 1993; Vol. 13, p 156.

(15) The partner transition for this feature should occur at about 0.4 MHz, but ENDOR is rarely observed at such low frequencies, due to the difficulty of coupling the rf energy into the ENDOR coil.

(16) (a) Thorson, J. S.; Lo, S. F.; Liu, H.-w.; Hutchinson, C. R. *J. Am. Chem. Soc*. **1993**, *115*, 6993−6994. (b) Jiang, X. M.; Neal, B.; Santiago, F.; Lee, S. J.; Romana, L. K.; Reeves, P. R. *Mol. Microbiol*. **1991**, *5*, 695−713. (c) Kessler, A. C.; Haase, A.; Reeves, P. R. *J. Bacteriol*. **1993**, *175*, 1412−1422. (d) Stroeher, U. H.; Jedani, K. E.; Manning, P. A. *Gene* **1998**, *223*, 269−282. (e) Ichinose, K.; Bedford, D. J.; Tornus, D.; Bechthold, A.; Bibb, M. J.; Revill, W. P.; Floss, H. G.; Hopwood, D. A. *Chem. Biol*. **1998**, *5*, 647−659. (f) Westrich, L.; Domann, S.; Faust, B.; Bedford, D.; Hopwood, D. A.; Bechthold, A. *FEMS Microbiol. Lett*. **1999**, *170*, 381−387. (g) Skurnik, M.; Peippo, A.; Ervela, E. Unpublished results; the gene bank accession number is CAB63273.