Biosynthesis of 3,6-Dideoxyhexoses: New Evidence Supporting a Radical Mechanism for C-3 Deoxygenation

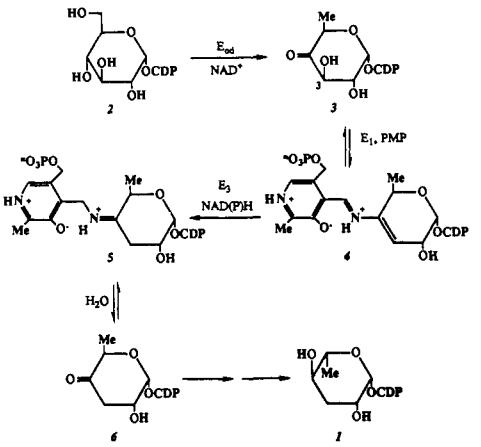
Vaughn P. Miller and Hung-wen Liu*

Department of Chemistry
University of Minnesota
Minneapolis, Minnesota 55455

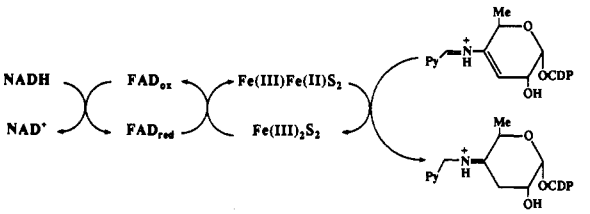
Received November 26, 1991

The 3,6-dideoxyhexoses found in the lipopolysaccharide of Gram-negative bacteria have been shown to be the dominant antigenic determinants and to contribute to the serological specificity of many immunologically active polysaccharides.[1] A biosynthetic pathway for this important class of dideoxy sugars, as exemplified by the formation of ascarylose (**1**, 3,6-dideoxy-L-*arabino*-hexose), has been proposed on the basis of the pioneering work of Strominger and his co-workers (Scheme I).[2] The key step of this postulated sequence is C-3 deoxygenation catalyzed by a pyridoxamine 5′-phosphate linked enzyme, CDP-4-keto-6-deoxy-D-glucose 3-dehydrase (E_1), and a NAD(P)H-dependent catalyst, CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3), both of which have been purified from *Pasturella pseudotuberculosis*.[3] While the catalytic roles of these enzymes have been well defined,[2] the mechanistic details remained to be established. In an effort to elucidate the molecular mechanism of this deoxygenation, we have recently reported the isolation of an "E_3 equivalent" from *Yersinia pseudotuberculosis*[4,5] which, like its *Pasturella* counterpart, can also act as a NADH oxidase converting O_2 to H_2O_2.[2d,3b] Careful analysis revealed that this enzyme-catalyzed H_2O_2 formation was not a direct two-electron transfer process, but was instead a one-electron reduction of molecular oxygen followed by dismutation of the nascent superoxide.[4] These results strongly suggest a radical mechanism for E_3 catalysis. Since E_3 is expected to operate via a single mechanism despite its dual functions, the mechanistic conclusion drawn from its NADH oxidase activity is extrapolated to its sugar reductase activity as well.[4] The two-electron/one-electron switching capability found for this enzyme denotes the participation of active-site-bound cofactors in its catalysis; however, the nature of the putative cofactor(s) remains mysterious because the active enzyme is metal-free and contains no chromophoric groups.[2d,3b,4,6]

Scheme I



Scheme II



In a recent attempt to improve the purification procedure for large-scale E_3 preparation,[7] we surprisingly discovered that the apparently homogeneous enzyme purified by the original protocol[4b] was still a mixture of two proteins having similar chromatographic behaviors and nearly identical molecular weights. Because the amount of the minor component was less than 10% of the total protein, it consistently eluded scrutiny by electrophoretic as well as amino-terminal analysis. Since the DNA fragment containing the gene coding for the minor protein was found to be part of a cluster carrying genes for the entire biosynthetic pathway of ascarylose,[8] it became apparent that this newly identified protein, the minor component of the previously "purified" enzyme, was the genuine E_3.

Interestingly, the electronic absorption of this newly purified E_3 is that of a flavoprotein, and extraction of the purified enzyme with trichloroacetic acid[9] releases 1 equiv of FAD, identified by a modified HPLC procedure.[10] Subtracting the resulting FAD absorbance from that of the native protein revealed a broad spectrum indicative of a plant ferredoxin type [2Fe-2S] cluster[11]

(1) (a) Westphal, O.; Lüderitz, O. *Angew. Chem.* **1960**, *72*, 881. (b) Lüderitz, O.; Staub, A. M.; Westphal, O. *Bacteriol. Rev.* **1966**, *30*, 192. (c) Hanessian, S. *Adv. Carbohydr. Chem. Biochem.* **1966**, *21*, 143. (d) Williams, N. R.; Wander, J. D. In *The Carbohydrates: Chemistry and Biochemistry*; Pigman, W., Horton, D., Eds.; Academic Press: New York, 1980; Vol. 1B, p 761. (e) Bishop, C. T.; Jennings, H. J. In *The Polysaccharides*; Aspinall, G. O., Ed.; Academic Press: New York, 1982; Vol. 1, p 291. (f) Raetz, C. R. H. *Annu. Rev. Biochem.* **1990**, *59*, 129. (g) Cygler, M.; Rose, D. R.; Bundle, D. R. *Science* **1991**, *253*, 442.

(2) (a) Matsuhashi, S.; Matsuhashi, M.; Strominger, J. L. *J. Biol. Chem.* **1966**, *241*, 4267. (b) Matsuhashi, S.; Matsuhashi, M.; Brown, J. G.; Strominger, J. L. *J. Biol. Chem.* **1966**, *241*, 4283. (c) Rubenstein, P. A.; Strominger, J. L. *J. Biol. Chem.* **1974**, *249*, 3776. (d) Rubenstein, P. A.; Strominger, J. L. *J. Biol. Chem.* **1974**, *249*, 3782. (e) Gonzalez-Porque, P. In *Vitamin B_6 Pyridoxal Phosphate, Chemical, Biochemical, and Medical Aspects*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley-Interscience: New York, 1986; Part B, p 392.

(3) (a) Matsuhashi, S.; Strominger, J. L. *J. Biol. Chem.* **1967**, *242*, 3494. (b) Gonzalez-Porque, P.; Strominger, J. L. *J. Biol. Chem.* **1972**, *247*, 6748. (c) Gonzalez-Porque, P.; Strominger, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 1625.

(4) (a) Han, O.; Liu, H.-w. *J. Am. Chem. Soc.* **1988**, *110*, 7893. (b) Han, O.; Miller, V. P.; Liu, H.-w. *J. Biol. Chem.* **1990**, *265*, 8033.

(5) Although *Pasturella pseudotuberculosis* has now been reclassified as *Yersinia pseudotuberculosis*, it is not clear whether the strain used in Strominger's studies and the one used in our current research are identical.

(6) It was speculated[4a] that a likely candidate for the organic cofactor is a quinone type coenzyme known for its capacity to act as a two-electron/one-electron switch and, in general, has a UV absorption at ca. 270–300 nm. On the basis of the results presented herein, it is therefore not surprising that extensive analyses failed to substantiate this conjecture.

(7) A highly selective sequence incorporating DEAE-Sephacel, Phenyl-Sepharose, Matrex Blue A, and Sephadex G-100 chromatography was developed in this study which led to an 8000-fold purification of this protein. Overall yield was 0.3 mg of enzyme per 500 g of wet cells.

(8) A 3–4 kb region of DNA fragments containing the E_3 gene, identified by Southern hybridization of *Hind*III-digested genomic DNA with a mixed oligonucleotide probe derived from the N-terminal amino acid sequence of the purified protein, was cloned into the λZAPII insertion vector. Screening of the resulting recombinant phages with both the E_3-based probe and a similarly derived mixed oligonucleotide from the N-terminal sequence of CDP-D-glucose oxidoreductase (E_{od}) (Yu, Y.; Russell, R. N.; Thorson, J. S.; Liu, L.-d.; Liu, H.-w. *J. Biol. Chem.*, in press) resulted in the isolation of a few positive clones which, after being converted into the plasmid form of the pBluescript vector and then subcloned into pUC plasmids, were used to determine the sequence and orientation of the E_3, E_{od}, E_p (CDP-D-glucose pyrophosphorylase), and E_1 genes (Thorson, J. S.; Lo, S. F.; Liu, H.-w. Unpublished results).

(9) Mayhew, S. G.; Massey, V. *J. Biol. Chem.* **1969**, *244*, 794.

(10) Light, D. R.; Walsh, C. T.; Marletta, M. A. *Anal. Biochem.* **1980**, *109*, 87.

(11) 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 are coupled antiferromagnetically (Bezkorovainy, A. In *Biochemistry of Nonheme Iron*; Plenum Press: New York, 1980; p 343; Palmer G. *Biochem. Soc. Trans.* **1985**, *13*, 548).

0002-7863/92/1514-1880$03.00/0

with absorption maxima at 325, 420, and 455 nm, as well as a shoulder at 550 nm.[12] The chemical nature of the iron–sulfur center was further confirmed as the oxidized enzyme was found to be EPR silent while the fully reduced form exhibited rhombic EPR signals having g values of 2.043, 1.960, and 1.877.[11-13] Since a total of 3 electron equiv of dithionite are required to fully reduce E_3 under anaerobic conditions, the existence of an iron–sulfur center in association with a FAD cofactor in 1:1 stoichiometry is unequivocally established. The iron–sulfur center is essential for E_3 activity as the apoenzyme, prepared by treatment with mersalyl acid,[14] is devoid of any glucoseen reductase activity.

On the basis of the physical characteristics of E_3 and their similarity to other iron–sulfur flavin containing reductases,[15] the molecular mechanisms of its catalysis can now be postulated. As depicted in Scheme II, the order of electron flow is likely to start with hydride reduction of FAD by NADH. The iron–sulfur cluster, receiving electrons one at a time from the reduced flavin, then relays the reducing equivalents to its acceptor, the E_1-bound glucoseen intermediate **4**. This proposed electron-transport sequence is mechanistically sound and is consistent with E_3's role as a two-electron/one-electron switch. 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.[16] In light of the fact that a PMP–glucoseen adduct is the proximate acceptor receiving electrons directly from an iron–sulfur center,[17] the catalytic role of E_3, in association with E_1,[18] in the biosynthesis of ascarylose clearly constitutes a unique example of biological deoxygenation.[19] Although the radical nature of this C-3 deoxygenation process is reminiscent of the well-known sugar deoxygenation catalyzed by ribonucleotide reductase, the mechanisms of these two deoxygenations are fundamentally distinct.[20]

Acknowledgment. We thank Ms. Kim Paulsen and Dr. Charles Pace for their help on anaerobic titration and Mr. Theodore Holman and Professor John Lipscomb for their assistance with EPR measurements. This work was supported by a National Institutes of Health grant (GM 35906). H.-w.L. also thanks the National Institutes of Health for a Research Career Development Award (GM 00559).

(12) (a) Orme-Johnson, W. H.; Orme-Johnson, N. R. In *Iron-Sulfur Proteins*; Spiro, T. G., Ed.; Wiley: New York, 1982, p 67. (b) Moura, I.; Moura, J. J. G. In *The Biological Chemistry of Iron*; Dunford, H. B., Dolphin, D., Raymond, K. N., Sieker, L., Eds., D. Reidel Publishing Co.: New York, 1982; p 179. (c) Orme-Johnson, W. H.; Orme-Johnson, N. R. *Methods Enzymol.* **1978**, *53*, 259.

(13) The co-existence of a free radical signal at g = 2.002 in the EPR spectrum can be ascribed to the residual flavin semiquinone (Campbell, I. D.; Dwek, R. A. In *Biological Spectroscopy*; Benjamin/Cummings: Menlo Park, CA, 1984; p 179).

(14) (a) Malkin, R.; Rabinowitz, J. C. *Biochem. Biophys. Res. Commun.* **1966**, *23*, 822. (b) Lund, J.; Woodland, M. P.; Dalton, H. *J. Biochem.* **1985**, *147*, 297.

(15) (a) Fox, B. G.; Froland, W. A.; Dege, J. E.; Lipscomb, J. D. *J. Biol. Chem.* **1989**, *264*, 10023. (b) Berhardt, F. H.; Bill, E.; Trautwein, A. X.; Twilfer, H. *Methods Enzymol.* **1988**, *161*, 281. (c) Batie, C. J.; LaHaie, E.; Ballou, D. P. *J. Biol. Chem.* **1987**, *262*, 1510.

(16) Kamin, H.; Lambeth, J. D.; Siegel, L. M. In *Flavins and Flavoproteins*; Yagi, K., Yamano, T., Eds.; University Park Press: Baltimore, MD, 1980; Vol. 6, p 341.

(17) Other structurally analogous enzymes are all members of multicomponent oxygenase systems which employ a short electron-transport chain to catalyze electron transfer from an external donor, usually NAD(P)H, to a terminal iron containing oxygenase. The fact that the final acceptor in E_3-catalyzed sugar reduction is a highly conjugated organic molecule makes E_3 a rare, if not a unique, example of this class of enzymes.

(18) (a) Shih, Y.; Yang, D.-y.; Weigel, T. M.; Liu, H.-w. *J. Am. Chem. Soc.* **1990**, *112*, 9652. (b) Weigel, T. M.; Liu, L.-d.; Liu, H.-w. *Biochemistry*, in press. (c) Weigel, T. M.; Miller, V. P.; Liu, H.-w. *Biochemistry*, in press.

(19) The possible intermediacy of a 3,4-glucoseen–PMP in the deoxygenation process has precedent since a pyridoxal phosphate stabilized aziridine radical has been suggested as the central intermediate in the reaction catalyzed by lysine 2,3-aminomutase (Song, K. B.; Frey, P. A. *J. Biol. Chem.* **1991**, *266*, 7651 and references cited therein).

(20) (a) Stubbe, J. *Annu. Rev. Biochem.* **1989**, *58*, 257. (b) Stubbe, J. *J. Biol. Chem.* **1990**, *265*, 5329. (c) Bollinger, J. M., Jr.; Edmondson, D. E.; Huynh, B. H.; Filley, J.; Norton, J. R.; Stubbe, J. *Science* **1991**, *253*, 292.

Electron-Transfer Agents in Metal-Catalyzed Dioxygen Oxidations: Effective Catalysts for the Interception and Oxidation of Carbon Radicals

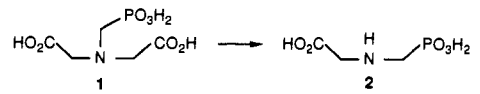
Dennis P. Riley* and Donald L. Fields

Monsanto Company
800 North Lindbergh Boulevard
St. Louis, Missouri 63167

Received October 28, 1991

A key intermediate in metal-catalyzed autoxidations of organic substrates is often an alkyl or benzyl radical.[1] Such intermediates react with triplet molecular oxygen, forming hydroperoxy radicals whose subsequent reactions lead to such products as, e.g., aldehydes from alkyl aromatics, acids from aldehydes,[2] and alcohols and ketones from paraffins.[1] Efficient trapping of such radical intermediates with O_2 before other radical abstraction or recombination reactions occur is important for achieving high selectivity to the desired oxygenated product.

We have reported that the molecular oxygen oxidation of *N*-(phosphonomethyl)iminodiacetic acid (PMIDA), **1**, to yield *N*-(phosphonomethyl)glycine (PMG), **2**, is effectively catalyzed by cobalt(II,III)[3] and vanadium(IV,V)[4] salts in aqueous media.



1 2

This chemistry involves the formation and subsequent trapping by O_2 of an *N*-methylene carbon-centered radical, **3**, generating *N*-formyl-PMG, **4** (Scheme I). Inefficient oxygen trapping of the $NCH_2^{\bullet}$ radical, **3**, leads to the undesired *N*-methyl product, **5**, via H-atom abstraction. With V the oxidation of **1** proceeds at much faster rates, but with lower selectivities than are observed with Co. In both cases selectivity to the desired product **2** increases as O_2 pressure increases. Unfortunately, O_2 pressures over 100 atm ($\sim 1 \times 10^6$ N/m^2) are required to suppress formation of **5** in the V case.[4]

We describe in this report the first well-defined example of the use of a cocatalyst whose role is to efficiently oxidize an intermediate carbon-centered radical to the desired product, and thereby eliminate the need for high oxygen concentrations (pressure) to prevent selectivity-robbing radical processes. The introduction of a cooxidant which can intercept the *N*-methylene radical, **3**, is an attractive alternative if the cooxidant can itself be regenerated with oxygen and if it does not interfere with the primary redox processes involving O_2 oxidation of the metal complex of **1** and the subsequent metal oxidation of bound ligand. Oxidation of **3** to the iminium cation, followed by hydrolysis, would yield the desired product **2** and formaldehyde (Scheme I).

Screening studies were employed with both the cobalt and vanadium catalysts under standard experimental conditions which give 100% conversion in 200 min. For the vanadium system at 75 °C and under 200 psig of O_2, 0.017 mol of **1**/100 mL of H_2O was employed with [$VOSO_4$] = 0.0085 M (pH$_i$ = 1.5). These conditions give a 50% selectivity to desired product, **2**, and ~40% selectivity to **5** in the absence of any cocatalysts.[4] For the cobalt system, screening studies were initiated using 0.088 mol of **1** in 100 mL of H_2O at 90 °C under 200 psig of O_2 with [$CoSO_4$] = 0.015 M (pH$_i$ = 1.5). These conditions give a 50% selectivity to desired product, **2**, and ~40% selectivity to **5** in the absence of any cocatalysts.[4] For the cobalt system, screening studies were initiated using 0.088 mol of **1** in 100 mL of H_2O at 90 °C under 200 psig of O_2 with [$CoSO_4$] = 0.015 M (pH$_i$ = 1.5). Under these conditions the cobalt system gives a 59% selectivity to **2**. Many

(1) Sheldon, R. A.; Kochi, J. K. *Metal-Catalyzed Oxidations of Organic Compounds*; Academic Press: New York, 1982; Chapter 2, pp 17-32.

(2) Riley, D. P.; Getman, D. P.; Beck, G. R.; Heintz, R. M. *J. Org. Chem.* **1987**, *52*, 287.

(3) Riley, D. P.; Fields, D. L.; Rivers, W. *J. Am. Chem. Soc.* **1991**, *113*, 3371.

(4) Riley, D. P.; Fields, D. L.; Rivers, W. *Inorg. Chem.* **1991**, *30*, 4191.

0002-7863/92/1514-1881$03.00/0