Role of Iron in Lipoxygenase Catalysis

Max O. Funk, Jr.* and Richard T. Carroll

Department of Chemistry, University of Toledo
Toledo, Ohio 43606

John F. Thompson

United States Plant, Soil and Nutrition Laboratory
Agricultural Research Service
United States Department of Agriculture
Ithaca, New York 14853

Richard H. Sands and William R. Dunham

Biophysics Research Division, University of Michigan
Ann Arbor, Michigan 48109

Received February 20, 1990

The enzyme lipoxygenase plays an important role in polyunsaturated fatty acid metabolism in plants and animals. In circulating blood cells, lipoxygenase inaugurates the biosynthesis of the leukotrienes, a family of mediators of medically relevant biological activities.[1] The enzyme has received considerable research attention from chemists in recent years. Lipoxygenase catalyzes a stereospecific autoxidation reaction and has been used in the preparation of synthetically useful chiral moieties.[2] The enzyme also contains an unusual non-heme iron atom in an as yet unidentified structural environment.[3] The role of the iron atom in catalysis has become the focus of recent studies of the mechanism of action of lipoxygenase. In this report, we present a definitive test of whether or not the iron atom cycles between its two most common oxidation states during catalysis by the combined application of EPR and Mössbauer spectroscopy.

The lipoxygenase-1 from soybeans has been available in a highly purified form for some time, and the majority of physical/chemical studies have been carried out on this species.[4] The two current hypotheses for the mechanism of lipoxygenase catalysis are illustrated in Scheme I.[5] The active form of the enzyme contains iron in the ferric state (vide infra). Removal of hydrogen at C-11 as an element of the rate-determining step was clearly indicated by a primary kinetic deuterium isotope effect.[6] Hydrogen atom abstraction with reduction of the iron would result in formation of a pentadienyl radical as an intermediate. This mechanism was proposed to account for the fact that active enzyme when treated with substrate linoleic acid in the absence of oxygen lost its characteristic EPR signal at $g = 6$.[7] The hypothesis is also consistent with other indications that the mechanism involves free-radical intermediates.[8] Recently an alternate mechanism has been proposed in which electron transfer to the iron does not take place.[9] The alternate mechanism involves an organoiron intermediate in place of the pentadienyl radical and iron(II).

Lipoxygenase-1 isolated from soybean seeds and referred to as the native enzyme contains iron(II) in a non-heme environment. This has been demonstrated by magnetic susceptibility,[10] paramagnetic NMR effects,[11] EXAFS,[12] and MCD.[13] When the enzyme is treated with 1 molar equiv of its product, it is converted into an iron(III)-containing species which is responsible for catalysis. The iron(III) is characterized by an EPR signal at $g = 6$ which is sensitive to a number of environmental factors.[14] Upon anaerobic treatment with substrate, the active enzyme is converted into a form that has no $g = 6$ EPR features and has a magnetic susceptibility consistent with high-spin iron(II).[15] The direct observation of the iron atom in lipoxygenase by Mössbauer spectroscopy which is sensitive to both electronic states and ligand environments has not until recently been possible. We have succeeded in obtaining soybean lipoxygenases enriched in the nonnatural isotopes of iron. This was accomplished biosynthetically by the tissue culture of immature soybean seeds in the presence of isotopically enriched iron.[16] The incorporation of iron-57 has made it possible to investigate the iron atom in lipoxygenase in the difficult to characterize iron(II) states by Mössbauer spectroscopy.[17]

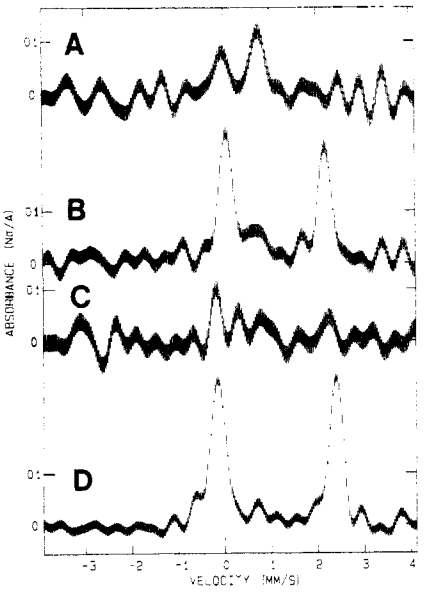
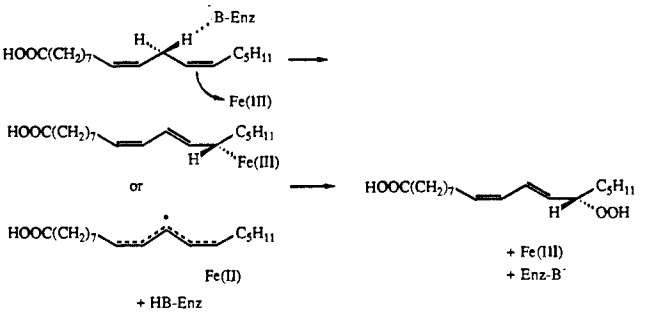


Figure 1. Mössbauer spectra of two product-oxidized lipoxygenases before and after treatment with substrate anaerobically: (A) oxidized P1; (B) oxidized P1 plus substrate anaerobically; (C) oxidized P4; (D) oxidized P4 plus substrate anaerobically. The spectra were taken at 125 K with no applied magnetic field. The spectra all have the same vertical scaling factors. The reference for all measurements was iron metal at room temperature.

Scheme I



(1) (a) Samuelsson, B.; Dahlen, S.-E.; Lindgren, J. A.; Rouzer, C. A.; Serhan, C. N. *Science* **1987**, *237*, 1171-1176. (b) Samuelsson, B.; Funk, C. D. *J. Biol. Chem.* **1989**, *264*, 19469-19472.
(2) Zhang, P.; Kyler, K. S. *J. Am. Chem. Soc.* **1989**, *111*, 9241-9242.
(3) Cox, D. D.; Benkovic, S. J.; Bloom, L. M.; Bradley, F. C.; Nelson, M. J.; Que, L.; Wallick, D. E. *J. Am. Chem. Soc.* **1988**, *110*, 2026-2032.
(4) Theorell, H.; Holman, R. T.; Akeson, A. *Acta Chem. Scand.* **1947**, *1*, 571-576.
(5) Corey, E. J. *Pure Appl. Chem.* **1987**, *59*, 269-278.
(6) Egmond, M. R.; Veldink, G. A.; Vliegenthart, J. F. G.; Boldingh, J. *Biochem. Biophys. Res. Commun.* **1973**, *54*, 1178-1184.
(7) de Groot, J. J. M. C.; Garssen, G. J.; Vliegenthart, J. F. G.; Boldingh, J.; Wever, R.; van Gelder, B. F. *Biochim. Biophys. Acta* **1975**, *377*, 71-79.
(8) de Groot, J. J. M. C.; Garssen, G. J.; Vliegenthart, J. F. G.; Bolding, J. *Biochim. Biophys. Acta* **1973**, *326*, 279-284.
(9) Corey, E. J.; Nagata, R. *J. Am. Chem. Soc.* **1987**, *109*, 8107-8108.
(10) Petersson, L.; Slappendel, S.; Vliegenthart, J. F. G. *Biochim. Biophys. Acta* **1985**, *828*, 81-85.
(11) Cheesbrough, T. M.; Axelrod, B. *Biochemistry* **1983**, *22*, 3837-3840.
(12) Navaratanam, S.; Feiters, M. C.; Al-Hakim, M.; Allen, J. C.; Veldink, G. A.; Vliegenthart, J. F. G. *Biochim. Biophys. Acta* **1988**, *956*, 70-76.
(13) Whittaker, J. W.; Solomon, E. I. *J. Am. Chem. Soc.* **1988**, *110*, 5329-5339.
(14) (a) Nelson, M. J. *J. Am. Chem. Soc.* **1988**, *110*, 2985-2986. (b) Slappendel, S.; Veldink, G. A.; Vliegenthart, J. F. G.; Aasa, R.; Malmstrom, B. *Biochim. Biophys. Acta* **1983**, *747*, 32-36.
(15) Petersson, L.; Slappendel, S.; Feiters, M. C.; Vliegenthart, J. F. G. *Biochim. Biophys. Acta* **1987**, *913*, 228-237.
(16) Funk, M. O.; Carroll, R. T.; Thompson, J. F.; Dunham, W. R. *Plant. Physiol.* **1986**, *82*, 1139-1144.

0002-7863/90/1512-5375$02.50/0

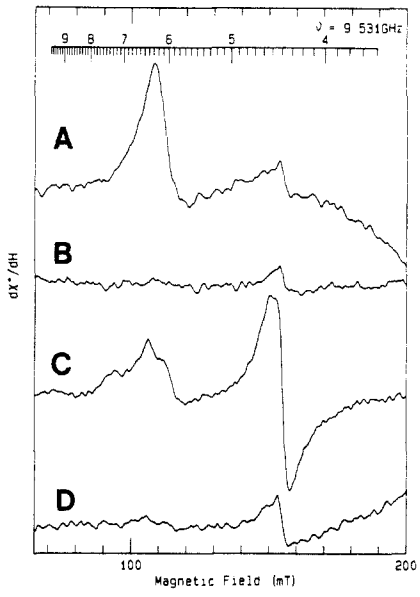


Figure 2. EPR spectra of the Mössbauer samples (Figure 1) of two product-oxidized lipoxygenases before and after treatment with substrate anaerobically: (A) oxidized P1; (B) oxidized P1 plus substrate anaerobically; (C) oxidized P4; (D) oxidized P4 plus substrate anaerobically. The spectra were taken at 40 K, with modulation amplitude = 0.63 mT and microwave power = 10 mW, and represent one 4-min scan each. The spectra all have the same vertical scaling factors.

Samples of two lipoxygenases which had been converted from the native to the iron(III) form and were subsequently treated with linoleic acid in the absence of oxygen were analyzed by Mössbauer and EPR spectroscopy.[18] The results of these experiments are presented in Figures 1 and 2. The isolation procedure we employ provides two lipoxygenases simultaneously: lipoxygenase-1 (P1) which is active at pH 9 and another isoenzyme active only near neutral pH which we operationally refer to as P4.[19] Both native lipoxygenases have Mössbauer parameters which are typical for high-spin iron(II), $\delta/Fe = 1.10$ mm s^{-1} and $\Delta Eq = 3.08$ mm s^{-1}.[20] The active enzyme was generated by treatment with 1 molar equiv of product (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid.[21] This species does not give a well-defined Mössbauer signal under the reported conditions, but can be readily identified by the EPR spectrum of the corresponding Mössbauer sample (Figure 2). When either isoenzyme was treated with linoleic acid anaerobically, iron with Mössbauer parameters characteristic of high-spin iron(II) was obtained: P1, $\delta/Fe = 1.23$ mm s^{-1}, $\Delta Eq = 2.12$ mm s^{-1}; P4, $\delta/Fe = 1.24$ mm s^{-1}, $\Delta Eq = 2.54$ mm s^{-1} (Figure 1). The Mössbauer sample from Figure 1D was subjected to iron determination by atomic absorption spectroscopy and iron isotope ratio determination by mass spectrometry.[22] The integrated intensity of the Mössbauer spectrum in Figure 1D accounts for at least 75% of the iron-57 found to be in the sample. The data are consistent with a mechanism for catalysis in which lipoxygenase iron goes from the iron(III) to the iron(II) state as linoleic acid is oxidized presumably to a pentadienyl radical intermediate. It is interesting to note that the Mössbauer parameters of the substrate-treated enzyme were significantlly different from those of the native enzymes. This is direct evidence that oxidation and substrate binding alter the coordination sphere of the iron atom relative to the native enzyme. Also, the parameters for the two isoenzymes which had been cycled by product and substrate were significantly different from each other. This is consistent with our earlier hypothesis that the isoenzymes may act by similar yet distinguishable catalytic mechanisms.[23]

Acknowledgment. This research was supported financially by the National Science Foundation and the National Institutes of Health. Max Funk was the recipient of a Research Career Development Award from the National Institutes of Health.

(17) Dunham, W. R.; Carroll, R. T.; Thompson, J. F.; Sands, R. H.; Funk, M. O. *Eur. J. Biochem.*, in press.

(18) The purified isoenzymes were dialyzed against phosphate buffer (0.1 M, pH 7.0, 0.1 M NaCl). The oxidation was carried out at room temperature for 10 min using a 1.1 molar ratio of hydroperoxide to enzyme. Samples were loaded into Mössbauer cuvettes and either frozen in liquid nitrogen or transferred to a glove bag purged with argon. The sample was further treated at room temperature with an argon-purged solution of linoleic acid in methanol (<0.5% volume addition) to provide a 2.0 molar ratio of substrate to enzyme. The Mössbauer sample was then frozen immediately in liquid nitrogen.

(19) Draheim, J. E.; Carroll, R. T.; McNemar, T. B.; Dunham, W. R.; Sands, R. H.; Funk, M. O. *Arch. Biochem. Biophys.* **1989**, *269*, 208-218.

(20) Vertes, A.; Korecz, L.; Burger, K. *Mössbauer Spectroscopy*; Elsevier: Amsterdam, 1979; p 47.

(21) Funk, M. O.; Isaac, R.; Porter, N. A. *Lipids* **1976**, *11*, 113-117.

(22) Miller, D. D.; van Campen, D. *Am. J. Clin. Nutr.* **1979**, *32*, 2354-2361.

(23) Hausknecht, E. C.; Funk, M. O. *Phytochem.* **1984**, *23*, 1535-1539.

General Fluorescence Assay for Enzyme-Catalyzed Polyanion Hydrolysis Based on Template-Directed Excimer Formation. Application to Heparin and Polyglutamate

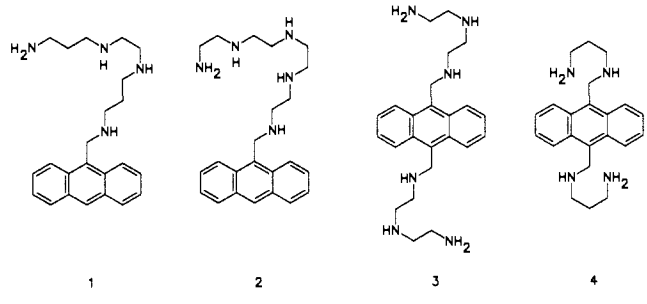
Scott A. Van Arman and Anthony W. Czarnik*

Department of Chemistry, The Ohio State University Columbus, Ohio 43210

Received March 19, 1990

While the hydrolyses of DNA and RNA can be followed by monitoring changes in their UV absorption spectra, the hydrolyses of biological polyanions lacking a chromophore must be accomplished indirectly. Both radiolabeling techniques and coupled enzyme systems are used frequently as indirect methods for enzyme assay. We now report that the activity of hydrolytic enzymes acting on the polyanions heparin and polyglutamate can be monitored by fluorescence using a template directed excimer formation effect obtained with anthrylpolyamine conjugate probes.[1]

Anthrylpolyamines **1–4** were prepared via simple substitution reactions using conditions similar to those reported previously.[2,3] The full emission spectra of anthrylpolyamines **1–4** (all 1 μM) were collected during titration with ds DNA, ss DNA, heparin,



1 2 3 4

and poly-L-glutamate;[4] representative titration data from the monitoring of compound **3** at 422 nm are shown in Figure 1.[5]

(1) Presented at the 23rd Reaction Mechanisms Symposium, Boulder, CO, June 1990.

(2) Wunz, T. P.; Dorr, R. T.; Alberts. D. S.; Tunget, C. L.; Einspahr, J.; Milton, S.; Remers, W. A. *J. Med. Chem.* **1987**, *30*, 1313.

(3) The products were isolated as their HCl salts via a series of aqueous and organic extractions and purified on a column of neutral alumina. Characterizations of **1–4** using ^{1}H NMR, ^{13}C NMR, and high-resolution FAB mass spectrometry were supportive of the structural assignments.

(4) DNAs (calf thymus), heparin (bovine mucosa), and poly-L-glutamate were purchased from Sigma Chemical Company and used as received. All titrations were performed at pH 5.0 in 0.1 M NaOAc buffer with 0.05 mM EDTA.

0002-7863/90/1512-5376$02.50/0