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Porcine leukocyte 5- and 12-lipoxygenases are iron enzymes

Peter M.H. Kroneck[1], Christophe Cucurou[1], Volker Ullrich[1], Natsuo Ueda[2], Hiroshi Suzuki[2], Tanihiro Yoshimoto[2], Satoshi Matsuda[2] and Shozo Yamamoto[2]

[1]*Fakultät für Biologie, Universität Konstanz, Postfach 5560, 7750 Konstanz, Germany* and [2]*Department of Biochemistry, Tokushima University, School of Medicine, Tokushima 770, Japan*

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5- and 12-lipoxygenases isolated from porcine leukocytes were investigated by electron paramagnetic resonance at X-band and atomic absorption spectroscopy. For comparison potato 5-lipoxygenase was studied under identical experimental conditions. All three lipoxygenases contained between 0.7 and 0.9 Fe atoms/enzyme molecule. As isolated, both mammalian enzymes exhibited a characteristic EPR signal at low magnetic field with a maximum at $g = 5.20$ indicative of a high-spin ferric iron center. The signal was not affected by the oxidants 12-hydroperoxyeicosatetraenoic acid or arachidonic acid, nor was it affected by the reductant nordihydroguaiaretic acid. In the case of the potato enzyme an intense EPR signal with resonances at $g = 7.50$, 6.39 and 5.84 was only observed after addition of an oxidant, such as 9-hydroperoxyoctadecadienoic acid.

Electron paramagnetic resonance; Iron enzyme; Lipoxygenase; Porcine leukocytes

1. INTRODUCTION

Lipoxygenases are widespread in plants and animals and have recently attracted attention in the medical field since they generate mediators of allergy and inflammation [1]. Therefore, studies on the catalytic mechanism of these enzymes are not only of biochemical interest but also form the basis for developing pharmacologically active inhibitors. Plant lipoxygenases were studied in the past by EPR spectroscopy which for soybean 15-lipoxygenase [2] and potato 5-lipoxygenase [3] revealed a pseudo-axial ferric iron site in the oxidized enzyme. The resting state within the cell seems to be ferrous and hydroperoxides are required to generate the active form of these lipoxygenases [2]. Recently, Ueda et al. [4] and Yokoyama et al. [5] developed an immunoaffinity method to purify the 5- and the 12-lipoxygenases from porcine leukocytes. By this procedure, a sufficient amount of enzyme is obtained to allow, for the first time, an EPR investigation of a mammalian lipoxygenase. The results reported here show that both porcine leukocyte 5- and 12-lipoxygenases contain high-spin ferric centers. For comparison, the corresponding data for potato 5-lipoxygenase were reinvestigated under identical experimental conditions to confirm the differences in the oxidation states as well as in the coordination sites of the iron centers from which variations in mechanistic or regulatory functions may result.

Correspondence address: P.M.H. Kroneck, Fakultät für Biologie, Universität Konstanz, Postfach 5560, W-7750 Konstanz, Germany.

Abbreviations: EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; 12-HPETE, 12-hydroperoxyeicosatetraenoic acid; 9-HPOD, 9-hydroperoxyoctadecadienoic acid; NDGA, nordihydroguaiaretic acid; DETAPAC, diethylenetriaminepentaacetic acid; SP-HPLC, straight phase high performance liquid chromatography

2. MATERIALS AND METHODS

Porcine leukocyte 5- and 12-lipoxygenases were purified to near homogeneity by immunoaffinity chromatography as described previously [4,5]. Potato 5-lipoxygenase was isolated from Bintje variety tubers according to Mulliez et al. [3] except that in the first chromatography step a hydroxyapatite column was used (30 × 2.6 cm, Ultrogel HA IBF Biotechnics). The lipoxygenase was eluted with 50 mM phosphate buffer, pH 6.5, containing 10% glycerol [6].

Enzyme activities were determined on a Kontron Uvikon 810 spectrophotometer at 25°C. The rate of appearance of the arachidonic acid hydroperoxide was measured at 237 nm ($\epsilon = 25\ cm^{-1} \cdot mM^{-1}$). 12-HPETE was prepared by photooxidation of arachidonic acid and purified by SP-HPLC. 9-HPOD was prepared enzymatically by incubation of 100 μM linoleic acid with 10 nM potato 5-lipoxygenase in 0.2 M acetate buffer, pH 5.5, containing 0.1 mM DETAPAC at 0°C under a stream of oxygen. After completion of the reaction, the incubation mixture was acidified to pH 3–4 and passed through a Sep-Pak C-18 cartridge (Waters Associates, Millipore). The hydroperoxide was eluted with 1 ml methanol and stored at −20°C under nitrogen.

Total protein was determined with the bicinchoninic acid (BCA) assay (Pierce). The iron content was obtained by atomic absorption spectroscopy using internal standard calibration with $FeCl_3$ (up to 3 μg/l). Samples (0.1–1 mg/ml protein) were diluted and analyzed on a Perkin Elmer 3030B spectrophotometer equipped with a Perkin-Elmer HGA 600 graphite furnace.

For EPR measurements, approximately 250 μl enzyme was transferred to a quartz tube (O, 4.5 mm) and frozen in liquid nitrogen. After recording the spectrum of the native enzyme, the sample was thawed and 9-HPOD, 12-HPETE or NDGA, dissolved in 1–5 μl methanol, was added. The reaction mixture was rapidly frozen at −145°C in an isopentane cooling bath.

EPR spectra (X-band) were recorded on a Bruker ESP 300 spectrometer equipped with a 100 kHz modulation unit, a frequency counter and a NMR gaussmeter. Depending on the sample 10-20 spectra had to be accumulated for signal averaging by the ESP 300 software. The g-values were calculated by measuring the microwave frequency and the magnetic field. The amount of EPR active lipoxygenase was estimated by double integration of the EPR spectra as described [7]. The low temperature was maintained with the Helitran LTD-110C system (Air Products) operating at 5–10K.

3. RESULTS

0.90 ± 0.17, 0.70 ± 0.09 and 0.71 ± 0.10 Fe atoms/enzyme was determined by atomic absorption spectroscopy for leukocyte 5- and 12-, and potato 5-lipoxygenase, respectively. The specific activity for the three enzymes was 0.2, 1.7, 3.5 μmol·min^{-1}·mg protein^{-1} for 50 μM arachidonic acid as substrate in 10 mM phosphate buffer, pH 7.4, 25°C.

The EPR spectrum of the potato enzyme shows a small Fe(III) signal around $g = 4.3$. In addition, a minor signal in the $g = 6$ region is present which had been assigned to the active Fe(III) center by Mulliez et al. [3].

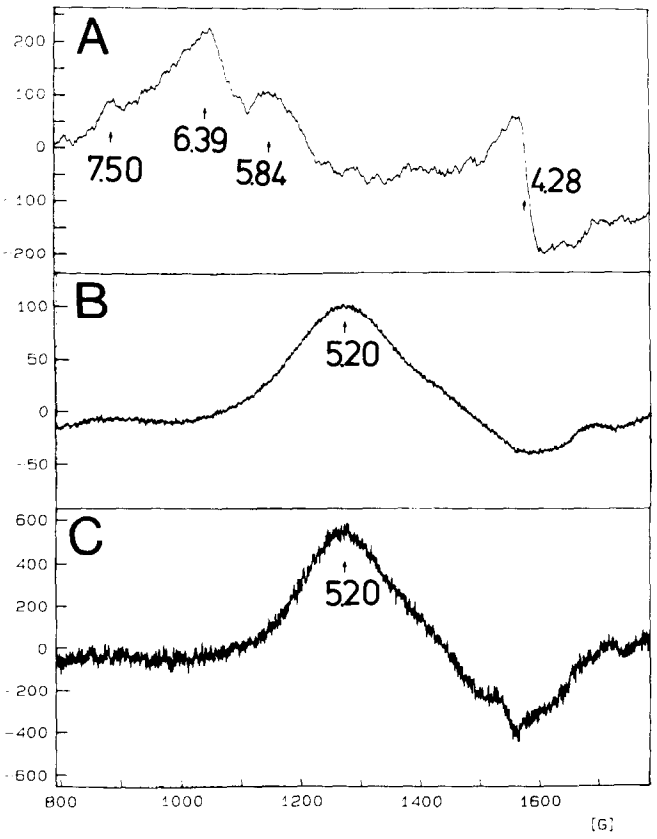

Fig. 1. Electron paramagnetic resonance spectra of potato and porcine leukocyte lipoxygenases. (A) Potato 5-lipoxygenase, 125 μM in 50 mM phosphate buffer, pH 5.15, oxidized by addition of one equivalent of 9-HPOD/enzyme. Microwave frequency 9.31274 GHz; microwave power 2.0 mW; modulation frequency 100 kHz; modulation amplitude 1.0 mT; sweep time 167.7 s; temperature 6K. (B) Porcine leukocyte 5-lipoxygenase, 14.9 μM in 19 mM HEPES buffer, pH 7.5. (C) Porcine 12-lipoxygenase, 1.3 μM in 10 mM HEPES buffer, pH 7.5. Instrument settings as in (A).

Upon addition of one equivalent of 9-hydroperoxyoctadecadienoic acid (9-HPOD) this latter signal increases with a maximum at $g = 6.39$ and shoulders at $g = 7.50$ and 5.84 (Fig. 1A) in good agreement with the signal previously published for the potato enzyme [3] and for soybean 15-lipoxygenase [2,8].

The EPR spectrum of native 5-lipoxygenase from porcine leukocytes recorded under identical experimental conditions (Fig. 1B) exhibits a rather featureless signal at low magnetic field with a maximum around $g = 5.2$ representing close to 50% of the iron determined by atomic absorption spectroscopy. There are no significant signals at $g = 4.3$ and at higher magnetic field in the $g = 3$–1.5 region. The enzyme as isolated appears to be already in the fully active ferric state. No significant changes in the EPR spectrum are found upon activation of the enzyme by addition of 12-HPETE or arachidonic acid, even in the presence of ATP and calcium (data not shown). Furthermore, no reduction of the EPR signal is observed upon incubation of the Fe(III) enzyme with the inhibitor NDGA.

Similar EPR parameters and spin quantity are found for native 12-lipoxygenase isolated from porcine leukocytes (Fig. 1C). Again, no further changes of the resonances at low field are obtained upon addition of 12-HPETE or NDGA, respectively.

4. DISCUSSION

The presence and the role of iron in plant lipoxygenases and particularly the soybean enzyme have been established for a long time [2]. A similar picture now arises for mammalian lipoxygenases. Clearly, the 5- and 12-lipoxygenases from porcine leukocytes both contain close to one atom of iron/enzyme molecule as demonstrated by atomic absorption spectroscopy.

While the native soybean lipoxygenase is silent by EPR [8,9], a high-spin Fe(III) signal is present in the native forms of potato [3] and leukocyte lipoxygenases. Contrarily to the native potato enzyme which is partially in its reduced state since the characteristic Fe(III) EPR signals increase upon addition of 9-HPOD, the stable state of both mammalian lipoxygenases appears to be the fully active Fe(III) form. These variations from one enzyme species to another, and the fact that both the Fe(II) and the Fe(III) states were observed in kinetic investigations of soybean lipoxygenase [2], may suggest that a weak environmental constraint is imposed on the metal center regulating its oxidation state.

Surprisingly, we did not obtain reduction of the mammalian enzymes by addition of NDGA. A probable explanation would be that, under these experimental conditions and especially at this pH, NDGA could only slowly chelate the oxidized iron without reducing it as reported for the interaction of soybean lipoxygenase-1 with several catechols [10]. Models as recently described by Mulliez et al. [11] or EXAFS

studies [12] may help to unravel the ligands of the catalytic iron center.

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