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EPR spectroscopy and electrospray ionization mass spectrometry reveal distinctive features of the iron site in leukocyte 12-lipoxygenase

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Abstract

The procedure for the expression and purification of recombinant porcine leukocyte 12-lipoxygenase using *E. coli* [K.M. Richards, L.J. Marnett, *Biochemistry* 36 (1997) 6692–6699] was updated to make it possible to produce enough protein for physical measurements. Electrospray ionization tandem mass spectrometry confirmed the amino acid sequence. The redox properties of the cofactor iron site were examined by EPR spectroscopy at 25 K following treatment with a variety of fatty acid hydroperoxides. Combination of the enzyme in a stoichiometric ratio with the hydroperoxides led to a g4.3 signal in EPR spectra instead of the g6 signal characteristic of similarly treated soybean lipoxygenase-1. Native 12-lipoxygenase was also subjected to electrospray ionization mass spectrometry. There was evidence for loss of the mass of an iron atom from the protein as the pH was lowered from 5 to 4. Native ions in these samples indicated that iron was lost without the protein completely unfolding.

INTRODUCTION

Polyunsaturated fatty acid metabolism is governed in large measure by just two enzymes, cyclooxygenase and lipoxygenase [1]. They inaugurate the conversion of their substrates, linoleic and linolenic acids in plants and arachidonic acid in animals, into families of metabolites with important biological properties, for example, in the human immune system. The inhibitors of cyclooxygenase are referred to collectively as non-steroidal anti-inflammatory drugs [2]. Lipoxygenase initiates the biosynthesis of leukotrienes, compounds that act as local hormones with a multitude of effects on immune cells [3]. While the two enzymes both catalyze the incorporation of molecular oxygen into polyunsaturated fatty acids with rate limiting homolysis of critical C–H bonds in the substrate, they do so in dramatically different ways. Cyclooxygenase is a heme-protein that employs a tyrosyl radical to abstract hydrogen, while lipoxygenase contains non-heme iron and carries out hydrogen abstraction without the intervention of a protein-based free radical [4].

Much of what is known about lipoxygenase catalysis came from the study of the enzymes found in soybean seeds, where the protein is remarkably abundant [5]. It was also found that

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a lipoxygenase played an important role in the maturation of erythrocytes, and consequently the enzyme was isolated from rabbit reticulocytes [6]. More recently, recombinant DNA technology made it possible to study the enzyme from a wide variety of other, less convenient sources of biological material. For example, the cDNA for a 12-lipoxygenase was obtained from porcine leukocytes, and the protein was subsequently expressed in *E. coli* [7]. The enzyme was investigated primarily by studies of the rate of the catalyzed reaction and the effects of inhibitors and site-specific mutations [8,9].

The role of the redox chemistry of the non-heme iron site in lipoxygenase catalysis was revealed in a series of spectroscopic studies. The enzymes isolated from soybeans contain iron(II) [10]. Treatment with 13-hydroperoxy-9(Z)11(E)-octadecadienoic acid (13-HPOD), the product of the catalyzed reaction, oxidized the cofactor to iron(III) and overcame a characteristic lag phase of the isolated enzyme in kinetic assays, an apparent activating reaction [11]. The activation, however, turned out to be somewhat more involved than just iron oxidation [12]. Redox cycling of the iron was subsequently identified as a central element of the catalyzed reaction [13]. The oxidized form of the enzyme was readily identified because the unshared electrons in the high-spin iron(III) of product oxidized soybean lipoxygenase-1 gave rise to distinctive signals in EPR spectra [14].

The coordination sphere of lipoxygenase iron revealed in the three-dimensional structures determined by X-ray crystallography was distinctive among the non-heme metalloproteins. The ligands were three histidines and uniquely, the carboxyl terminus of the polypeptide typically contributed by an isoleucine [15,16]. In the structures of the soybean lipoxygenases, close to the iron but just beyond a bonding distance, were the side chain from an asparagine as well as a water molecule. In the structure of the rabbit reticulocyte 15-lipoxygenase, a fourth histidine was found at the position corresponding to the asparagines in the soybean enzymes, but in this instance was also a coordinating ligand [17].

Solutions of soybean lipoxygenases treated with a stoichiometric amount of 13-HPOD took on a pale yellow appearance and displayed characteristic signals for high-spin iron(III) in the low temperature EPR spectrum at $g=6$ [18]. Treatment with more than a stoichiometric amount of 13-HPOD caused solutions of soybean lipoxygenases to be blue to purple, and gave rise to EPR signals at $g=4.3$ at the expense of the signals at $g=6$ [18]. The appearance of these signals in the EPR spectra of lipoxygenase samples became the *conditio sine qua non* of the ferric enzyme.

The overall fold of the lipoxygenases consists of two domains, a smaller N-terminal β -barrel domain, and a larger C-terminal mostly α -helical domain. The iron atom in the structures of the soybean and rabbit reticulocyte enzymes was found centrally located in the C-terminal domain. Presumably as a consequence of the central location and the participation of the C-terminal carboxylate as a ligand, the extraction of the metal from lipoxygenase without denaturation has not been straightforward. We previously reported a very narrow set of conditions under which it was possible to remove the iron from soybean lipoxygenase-3 and produce a solution of the apoprotein [19]. More recently we found using electrospray ionization mass spectrometry (ESI-MS) that in solutions of declining pH, soybean lipoxygenase-3 underwent a two state unfolding process at sufficiently low pH (< 3.5) with no evidence for a soluble iron-free protein intermediate [20]. Here we report on the EPR and ESI-MS properties of leukocyte 12-lipoxygenase. The iron in 12-lipoxygenase behaved quite differently from the soybean isoenzymes. For example, it goes through acid-induced unfolding accompanied by the appearance of an iron-free intermediate that retains a native conformation.

MATERIALS AND METHODS

Protein Expression

Rosetta 2 (DE3) cells (Novagen) were transformed with a pET-20b(+) plasmid bearing the cDNA for porcine leukocyte 12-lipoxygenase (a generous gift from Dr. Lawrence J. Marnett, Vanderbilt University). A typical 3 L culture was carried out as follows. LB Medium (1 mL) containing ampicillin ($100 \mu\text{g mL}^{-1}$) and chloramphenicol ($34 \mu\text{g mL}^{-1}$) was inoculated with a single colony of the Rosetta 2 (DE3) cells and the culture was incubated 4 h at 37°C with shaking (220 rpm). An aliquot of the culture ($60 \mu\text{L}$) was used to inoculate fresh LB medium (30 mL) containing ampicillin ($100 \mu\text{g mL}^{-1}$) and chloramphenicol ($34 \mu\text{g mL}^{-1}$). The culture was incubated overnight at 37°C with shaking (220 rpm). The entire culture was then transferred into fresh LB medium (600 mL) containing ampicillin ($100 \mu\text{g mL}^{-1}$) and chloramphenicol ($34 \mu\text{g mL}^{-1}$) and the cells were incubated 4 h at 37°C with shaking (220 rpm). The entire culture was combined with 2400 mL TB medium containing ampicillin ($100 \mu\text{g mL}^{-1}$) and chloramphenicol ($34 \mu\text{g mL}^{-1}$) and the cells were incubated 24 h at 30°C with shaking (200 rpm).

Protein Purification

The cells were collected by centrifugation ($4800 \times g$, 10 min), resuspended in phosphate buffered saline (200 mL), and collected again by centrifugation ($2880 \times g$, 30 min). The cell paste was suspended in lysis buffer (200 mL, 10 mM Bis Tris propane, 1 mM DTT, $60 \mu\text{g mL}^{-1}$ chicken egg white type II-O trypsin inhibitor, $100 \mu\text{g mL}^{-1}$ catalase, $20 \mu\text{g mL}^{-1}$ DNase I, pH 7.0) and stored at -80°C . The cell suspension was thawed on ice and chicken egg white lysozyme was added to achieve a final concentration of 0.4 mg mL^{-1} . The suspension was sonicated 10 times on ice for 30 s each with a 2 min cooling period between bursts. A supernatant for the extract was collected by centrifugation ($184000 \times g$, 1 h). Solid ammonium sulfate was added to the supernatant to achieve 40% saturation, and the protein pellet was obtained by centrifugation ($2880 \times g$, 30 min). The protein was dissolved in Bis Tris propane buffer (0.01 M, pH 7.0) and the solution was further dialyzed three times against this buffer. The protein solution was then loaded on a Q-Sepharose Fast Flow (GE Life Science) anion exchange column (20 mL). The column was washed with Bis Tris propane buffer (0.01 M, pH 7.0) and then eluted at 2.5 mL min^{-1} with a linear gradient (12 column volumes) from 0 to 30% Bis Tris propane buffer (0.01 M, pH 7.0) containing NaCl (1 M). The fractions were surveyed using a catalytic activity assay. The assay was performed using the procedure of Richards, *et al.* [8] except that the temperature was 25°C , and not 30°C . The active fractions were pooled and desalted by washing 5X with Bis Tris propane buffer (0.01 M, pH 7.0) in a 15 mL Amicon Ultra 50,000 MWCO centrifugal filter. The protein solution was applied to a Source 15Q (GE Life Science) anion exchange column and the column was eluted with the same wash and gradient protocol used for the Q-Sepharose column. The chromatographic procedures were conducted on an Akta FPLC (Amersham Biosciences) operated at 4°C .

SDS PAGE was carried out on 10% Bis-Tris mini gels (NuPAGE, Invitrogen) using MES SDS running buffer according to the manufacturer. Proteins were stained with Coomassie Brilliant Blue for visualization. Isoelectric focusing was conducted on a rehydrated Immobiline DryPlate (GE Healthcare) pH 5.6–6.6 using a Pharmacia Multiphor II horizontal apparatus thermostatted to 4°C . The proteins were fixed with trichloroacetic acid/sulfosalicylic acid and stained with Coomassie Brilliant Blue.

Fatty Acid Hydroperoxides

(9Z,11E)-13(S)-Hydroperoxy-9(Z),11(E)-octadecadienoic acid (13-HPOD) and (5Z,8Z,11Z,13E)-15(S)-hydroperoxy-5(Z),8(Z),11(Z),13(E)-eicosatetraenoic acid (15-HPETE) were prepared from linoleic acid and arachidonic acid respectively by the action of soybean

lipoxygenase-1 [21]. (5Z,8Z,10E,14Z)-12(S)-Hydroperoxy-5(Z),8(Z),10(E),14(Z)-eicosatetraenoic acid (12-HPETE) was prepared from arachidonic acid using the purified 12-lipoxygenase [22].

Electrospray ionization mass spectrometry on intact 12-lipoxygenase was performed on a Synapt HDMS (Waters) quadrupole time of flight ion mobility mass spectrometer. Ions of 12-lipoxygenase were generated using a nanoflow electrospray source with home-made nanospray emitters. Working samples of 12-lipoxygenase at 3 μM were prepared by dilution of a 600 μM stock solution (exhaustively desalted by dialysis with ammonium acetate buffer, 0.01 M, pH 7.0) with ammonium acetate buffer (0.01 M) adjusted to the appropriate pH. After dilution samples were equilibrated at room temperature for 30 min prior to mass spectrometry. The instrument parameters were maintained at the following constant values for each experiment: capillary voltage, 2.5 kV; sample cone, 40 V; cone gas, 0 L/hr; trap collision energy, 6 V. Mass were calibrated externally in the range $250 \leq m/z \leq 8000$ using a solution of cesium iodide, and were processed using Masslynx 4.1 software (Waters). All mass spectra were averages of 300 scans and were presented unsmoothed and without background subtraction.

MS/MS following trypsinization was carried out at the Mass Spectrometry and Proteomics facility at Ohio State University using a Micromass Q-ToF II High Resolution electrospray ionization mass spectrometer. The instrument was configured to simultaneously collect MS and MS/MS data on the effluent from a Vydac 2.1×250 mm C18 Mass Spec HPLC column in a Hewlett Packard Series 1100 HPLC. The chromatography column was eluted at 0.1 mL min^{-1} with a 120 min linear gradient from 0.1% formic acid in water to 65% aqueous acetonitrile containing 0.1% formic acid. 12-Lipoxygenase was subjected to reduction, carboxamidomethylation and trypsin digestion by closely following a published procedure [23]. The mass spectra were analyzed using MASCOT by Matrix Science.

Dynamic light scattering measurements were conducted at 20 °C on purified 12-lipoxygenase ($0.5\text{--}1.0 \text{ mg mL}^{-1}$) in Bis Tris propane buffer (10 mM, pH 7.0). Samples were filtered (0.22 μm) and centrifuged prior to analysis on a DynaPro Titan (Wyatt Technology). The data were analyzed using Dynamics Version 7.3. The measurements were carried out on three separate occasions and the values were averaged.

Circular dichroism experiments were conducted on an AVIV Circular Dichroism Model 62DS Spectrometer in a 1 mm pathlength quartz cell at 25 °C constant temperature, or over the range of 25 °C to 85 °C at a scan rate of $90 \text{ }^{\circ}\text{C hr}^{-1}$. Samples of purified 12-lipoxygenase (0.6 mg mL^{-1}) were prepared in Bis Tris propane buffer (10 mM, pH 7.0). For the mean residue molar ellipticity calculations, $n = 662$ residues and $M_r = 74,996 \text{ g mol}^{-1}$.

The iron determinations were carried out using a Perkin-Elmer Model 5100PC atomic absorption spectrophotometer in flame ionization mode. The flame gases were acetylene and air. Measurements were conducted at 248.3 nm with a slit width of 0.2 nm and a lamp current of 5 mA. A standard curve was produced using certified atomic absorption standard iron reference solution ($1,000 \text{ ppm} \pm 1\%$) from Fisher Scientific.

EPR spectroscopy was carried out at 9 GHz on a Bruker Model ESP 300E spectrometer equipped with an Oxford Instruments Model ITC4 cryostat operating at 25 K. The microwave power was 5 mW with a modulation amplitude of 1 mT. The fatty acid hydroperoxides were dispensed into plastic tubes on ice from stock methanol solutions. The methanol was removed with a stream of nitrogen. An aliquot (0.25 mL, 0.2 mM) of 12-lipoxygenase in Bis Tris propane buffer (10 mM, pH 7.0, 1 mM DTT) was added to each tube. The contents of the tubes were mixed and allowed to incubate for 10 min on ice. The samples were transferred into quartz EPR tubes, and the contents were frozen in liquid nitrogen.

RESULTS

The previously published procedure for the expression and purification of 12-lipoxygenase was modified to allow for the isolation of the quantities of protein necessary for physical characterization. Small adjustments to the expression and purification steps described in the experimental section culminated in a 4 to 6 fold increase in the overall yield of protein. The results of the final step are presented in Figure 1. Using the modified procedure resulted in the isolation of approximately 60 mg of highly purified protein with an average specific activity of $8.0 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ from 3 L of cultured bacteria. The measured isoelectric point for the purified protein was 5.65 compared to a calculated value of 5.82. Electrospray ionization mass spectrometry of solutions of 12-lipoxygenase under non-denaturing conditions produced a family of ions (Figure 2A) with charges from 15+ to 19+. The spectrum represents a single species yielding multiple charge states upon electrospray ionization characteristic of a native protein in its folded conformation. Deconvolution without any smoothing produced a mass of 75,007 Da for the single species giving rise to the ions (Figure 2B). The purified protein was also subjected to trypsinization followed by reverse-phase HPLC and tandem electrospray ionization mass spectrometry. In a single experiment, 543 out of 663 (82%) amino acids in 12-lipoxygenase were identified (Swissprot Accession Number **P16469**) on the basis of MS/MS peptide fragmentation patterns. Two trypsin fragments (Val-8 to Arg-40 and Glu-524 to Arg-563) too large to be resolved and sequenced prevented this fraction from being even larger. The iron content of the protein was determined by atomic absorption spectroscopy to be 0.94 ± 0.03 atoms of iron per molecule of 12-lipoxygenase. Dynamic light scattering measurements conducted on solutions of 12-lipoxygenase at 20 °C indicated a monomeric protein. The hydrodynamic radius was 3.76 ± 0.08 nm, which was consistent with a globular protein with a mass of 74.7 kDa. The polydispersity values for these measurements were in the range of 9–12%. Circular dichroism measurements on solutions of the protein (Figure 3) showed, as expected, that 12-lipoxygenase was an α/β protein. It had only modest thermal stability. Variable temperature circular dichroism data from 222 nm were fit to a simple two state unfolding process providing a T_m value of 53.6 °C for the portion of the protein composed of α -helix.

When electrospray ionization mass spectrometry was conducted on solutions of 12-lipoxygenase adjusted to different pH values, the mass spectra shown in Figure 4A were obtained. At pH 5.0 and below, the native ions were joined in the spectra by ions of lower mass, which is common for proteins undergoing acid-induced unfolding [24]. Examining the individual ions more closely revealed that the native peaks lost mass corresponding to the mass of an iron atom as the pH was lowered from 6.0 to 4.0 (Figure 4B). These observations indicated that 12-lipoxygenase experienced a loss of iron as well as acid induced unfolding, but not necessarily in a coordinated fashion. This was quite different from similar experiments conducted on soybean lipoxygenases [20], and has important ramifications for the production of a soluble apoenzyme, and potential metal extraction/reconstitution experiments.

EPR measurements were carried out at 25 K on 12-lipoxygenase samples treated with solutions of 13-HPOD containing one equivalent or an excess of the peroxide (Figure 5). A solution of soybean lipoxygenase-1 at the same concentration was treated with the same solutions of 13-HPOD at the same concentrations for comparison purposes. The characteristic changes in the EPR spectrum of lipoxygenase-1 upon treatment with 13-HPOD were observed [18]. A single equivalent of 13-HPOD produced a heterogeneous signal at $g=6$, whereas an excess resulted in a large amplitude EPR signal at $g=4.3$ and a reduction in the amplitude and complexity of the $g=6$ signal. Neither of these features was manifest in the spectra of leukocyte 12-lipoxygenase treated in the same way. Instead, one equivalent of 13-HPOD caused a small increase in the amplitude of a $g=4.3$ signal that was evident in the untreated sample, and excess 13-HPOD produced a further increase in the signal at $g=4.3$, and a broad, poorly resolved signal

from $g=4.3$ to $>g=7$. Similar EPR experiments were conducted with the two lipoxygenases treated with 12- and 15-hydroperoxyeicosatetraenoic acids (HPETE). Interestingly, soybean lipoxygenase-1 consistently produced predominately the EPR signal at $g=6$ with either a stoichiometric or an excess quantity of either peroxide. There was some variation in the heterogeneity of the $g=6$ signals, but the large amplitude signal at $g=4.3$, which was the major feature of the spectrum, when lipoxygenase-1 was treated with 13-HPOD in excess was not observed. Treatment of 12-lipoxygenase with either 12- or 15-HPETE led to increases in the amplitude of the $g=4.3$ EPR signal present in an untreated sample, but little change in the $g=6$ region of the spectrum. Interestingly, solutions of 12-lipoxygenase treated with excess peroxide were consistently colorless.

DISCUSSION

The modest changes to the published procedure for the expression and purification of leukocyte 12-lipoxygenase made it possible to contemplate experiments that required large amounts of protein, e.g. EPR measurements, typically 3–5 mg of protein per sample. The final enzyme preparation was characterized by its specific activity toward the oxygenation of arachidonic acid, and by electrospray ionization mass spectrometry on the intact protein and trypsin-digested samples. The average specific activity for the expressed protein was $8.0 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ for determinations carried out at 25°C compared with the literature value of $8\text{--}12 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ when the assay was conducted at 30°C [8]. The intact protein under non-denaturing conditions had a mass of 75,007 amu on the basis of electrospray ionization mass spectrometry measurements. The calculated mass of the protein based on the database sequence (Swissprot accession **P16469**) was 75,099 amu. However, cDNA sequencing revealed a single amino acid difference at position-218 between the expressed protein (R) and the database (Q) sequences, resulting in a mass difference of +28 amu. If the N-terminal methionine of the expressed protein was removed, as is frequently the case during heterologous expression in *E. coli* [25], there would be an additional correction of -131 amu, for a final calculated mass of 74,996 amu for the intact protein. The experimental and calculated masses differ by 11 amu or 147 ppm. This value is very close to the experimental error for these measurements [20]. In addition, a nearly stoichiometric amount of iron, 0.94 atoms per molecule, was found associated with the protein by atomic absorption spectroscopy. The previously determined values for this quantity were “about 0.45” [22] and 0.70 ± 0.09 [26], or the value was not reported [8,9].

Dynamic light scattering measurements indicated that 12-lipoxygenase formed monomolecular solutions at 25°C with a hydrodynamic radius consistent with a 75 kDa globular protein. The circular dichroism spectra revealed an α/β protein, consistent with expectations based on the known structures of the soybean isoenzymes and rabbit reticulocyte 15-lipoxygenase [15–17]. Variable temperature circular dichroism at 222 nm, however, showed that the unfolding transition for the α -helix secondary structure in the leukocyte enzyme was almost 20°C lower than for soybean lipoxygenase-3 (53.6°C vs. 72.2°C) despite being roughly 20 kDa smaller [27]. The C-terminal domain of the lipoxygenase fold was composed of numerous long α -helices in all of the reported crystallographically determined three-dimensional structures. The significant difference in mass between the plant and mammalian lipoxygenases was attributed to the presence of additional loops in the former located at the periphery of the molecules, while the α -helical core of the C-terminal domain and the N-terminal β -barrel domain were largely conserved. The basis for the significant difference in thermal stability of lipoxygenases from different sources was not immediately apparent, but it could well correlate with some of the other differences in the properties between the soybean and leukocyte lipoxygenases that were discovered (*vide infra*).

The iron sites in lipoxygenases have been extensively characterized using low temperature EPR spectroscopy, as the high-spin ferric species produced signals that depended on the source of the enzyme and just how it was oxidized. The chemistry has great relevance because catalytic activity has been attributed to the iron(III) form of the enzyme, whereas the isolated enzyme usually contains iron(II). The lipoxygenases were oxidized, iron(II)→ iron(III), only by the hydroperoxide products of the catalyzed reactions. Observations of EPR signals at both g6 and g4.3 were frequently observed. The proportion of the signals obtained depended on the source of the sample, the stoichiometry of the redox reaction, and the pH of the solution [5,28–34]. For example, soybean lipoxygenase-1 treated with a single equivalent of 13-HPOD produced predominately g6 EPR signals, but a large amplitude g4.3 signal when treated with a molar excess of the peroxide [18]. This behavior was illustrated in Figure 5. Soybean lipoxygenase-3 treated with one equivalent of 13-HPOD at pH 6.5 produced a heterogeneous g6 EPR signal, but only a g4.3 signal when the same reaction was carried out at pH 9.5 [28]. The heterogeneity of the g6 signal of soybean lipoxygenase-1 was sensitive to the composition of the sample. For example, distinctively more homogeneous g6 signals were obtained when low molecular weight alcohols were present [35]. Human and potato 5-lipoxygenase both produced heterogeneous g6 EPR signals as well as signals at g4.3 upon oxidation [33,34]. In one instance the human enzyme oxidized with 5-HPETE gave EPR signals at g4.3, g5.2, g6.2, and g9.0, leading to the idea that numerous geometrical configurations were possible, and that the iron site in 5-lipoxygenase was inherently flexible [33]. This would be consistent with the three-dimensional structural information that is available for the iron(III) form of the enzyme. The ligand geometries for the iron in the 13-HPOD complex and in the cumene hydroperoxide oxidized form of soybean lipoxygenase-3 were significantly different [36,37].

The EPR spectrum for one sample of porcine leukocyte 12-lipoxygenase was reported previously [26]. The enzyme was isolated directly from leukocytes by immunoaffinity chromatography and gave a broad signal at g5.2 in the low temperature EPR spectrum. The signal was obtained from a sample that was not treated with a fatty acid hydroperoxide, and it was reported that the signal was not significantly changed by doing so. The recombinant 12-lipoxygenase reported on here showed only a small g4.3 signal in the EPR spectrum before being treated with 13-HPOD, which is a common observation. When first combined with a stoichiometric amount of 13-HPOD, the amplitude of the g4.3 signal increased (Figure 5A). The behavior most closely resembled what was observed for rabbit 15-lipoxygenase, which was a lot different finding than for lipoxygenase-1 or 5-lipoxygenase, but in studies of that enzyme there were also changes in the g6 region of the spectrum. The similarity with rabbit 15-lipoxygenase would be consistent with fact that on the basis of sequence alignment, 12-lipoxygenase like 15-lipoxygenase has histidine in a position to provide the sixth ligand in the iron coordination sphere. Upon treatment of leukocyte 12-lipoxygenase with an excess amount of 13-HPOD, there was an additional increase in the amplitude of the g4.3 signal and the appearance of a broad, unresolved EPR signal from g4.3 to >g7. While this was a new spectral signature for a high-spin ferric lipoxygenase, it also added to the notion that lipoxygenase iron had access to more than the two distinct coordination geometries characterized by the g4.3 and g6 signals, and quite likely an array of coordination configurations related to one another by the conformational flexibility of the protein. Interestingly, the new EPR feature was only obtained by oxidation with 13-HPOD. Treatment with neither 12-HPETE nor 15-HPETE resulted in a comparable EPR signal (Figure 5B). A further characteristic of 12-lipoxygenase was evident in the EPR samples prepared with excess hydroperoxides, which were all colorless. The soybean isoenzymes treated in this fashion produced a colored complex that was characterized as a metastable iron-peroxide complex [36].

Another interesting observation imbedded in the EPR data reported here was the difference in the result of the oxidation of soybean lipoxygenase-1 by an excess amount of 13-HPOD compared to either 12- or 15-HPETE. Neither of the 20-carbon fatty acid hydroperoxides

caused the EPR spectrum to shift in favor of the g4.3 signal from the g6 signal in the dramatic fashion caused by the 18-carbon hydroperoxide. The effect did not depend on the position of oxygenation as much as the overall chain length and degree of unsaturation, because both the ω -6 and the ω -9 hydroperoxide promoted the formation of the g6 EPR signal in samples of soybean lipoxygenase-1. These observations further illustrate just how sensitive the EPR spectra of high-spin ferric lipoxygenases can be to the conditions of their preparation.

The distinctive nature of the iron site in leukocyte 12-lipoxygenase was also evident in electrospray ionization mass spectrometry measurements. In a study of the pH dependence of the mass profile, there was evidence for both protein unfolding and iron loss, but not necessarily simultaneously. For example, ions characteristic of the folded protein but with the mass of the apoprotein were observed at pH 4.0. This was quite a different result from what was obtained earlier for the soybean lipoxygenases. The generation of a soluble apoprotein for the soybean lipoxygenases has been a considerable research challenge. We found a very narrow set of conditions for the extraction of iron from one of the soybean isoenzymes, lipoxygenase-3. Specifically, in sodium carbonate buffer at pH 8.0 in the presence of solid Chelex-100 the iron atom was removed from lipoxygenase-3 and a soluble apoprotein solution was obtained [19]. The apoprotein solution was not particularly stable, and the procedure was not successful with soybean lipoxygenase-1. When other conditions were evaluated, like treatment with soluble chelating agents or partial unfolding with chaotropic agents, a soluble apoprotein was not obtained for any of the soybean lipoxygenase isoenzymes. For example, when the pH dependence of the solution characteristics of the soybean lipoxygenases were evaluated by electrospray ionization mass spectrometry, we observed ions from natively folded protein containing iron or, at sufficiently low pH, unfolded protein devoid of iron, with no evidence for any intermediates in the process [20]. The reluctance of soybean lipoxygenases to give up the iron atom without unfolding was attributed to the unique nature of the iron-binding site. The C-terminal carboxylate serves as an iron ligand and the metal is situated quite centrally in the protein. The electrospray ionization mass spectrometry experiments conducted on porcine leukocyte 12-lipoxygenase indicate that this enzyme may be more favorable for iron extraction experiments than the ones from soybeans. Extraction/reconstitution experiments have been a mainstay in the investigation of the structure and mechanism of action of metalloenzymes [38]. Metal atom substitution, for example, was used to probe both redox and coordination chemistry in a variety of metalloproteins [39–43].

Acknowledgments

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Figure 1.

Final stage of the isolation of leukocyte 12-lipoxygenase. A, anion exchange FPLC chromatogram, Source 15Q, NaCl gradient, 4 °C. B, SDS PAGE lane 1, 0–40% ammonium sulfate fraction; lane 2, active fractions, anion exchange FPLC Q Sepharose FF; lane 3: active fractions, anion exchange FPLC Source 15Q. C, Isoelectric focusing, active fractions, anion exchange FPLC Source 15Q.

Figure 2. Electrospray ionization mass spectrometry of leukocyte 12-lipoxygenase. A, 0.010 M ammonium acetate buffer, pH 7.0, 3 μ M 12-lipoxygenase. B, Deconvolution of electrospray ionization mass spectrometry data in Panel A.

Figure 3.

Circular dichroism spectrometry of leukocyte 12-lipoxygenase. A, wavelength dependence of circular dichroism for 12-lipoxygenase, 8 μ M, 0.010 M Bis Tris propane buffer pH 7.0, 25 °C. B, temperature dependence for circular dichroism at 222 nm for 12-lipoxygenase, 8 μ M, 0.010 M Bis Tris propane buffer pH 7.0.

Figure 4. Electrospray ionization mass spectrometry of leukocyte 12-lipoxygenase at various pH values. 12-Lipoxygenase, 3 μ M, 0.010 M ammonium acetate buffer, room temperature. A, range 1000–6000 m/z units, pH 3–10. B, scale expansion around mass at m/z 4168, pH 4–6.

Figure 5.

EPR spectrometry at 25 K of leukocyte 12-lipoxygenase (PL 12-LOX) with soybean lipoxygenase-1 (SLOX-1) as a standard. A, 200 μ M lipoxygenase treated with 1X or 10X 13-HPOD, 0.10 M Bis Tris propane buffer, pH 7.0. B, 200 μ M lipoxygenase treated with 1X or 10X 12- or 15-HPETE, 0.10 M Bis Tris p ropene buffer, pH 7.0.