Characterization of the Non-Heme Iron Center of Human 5-Lipoxygenase by Electron Paramagnetic Resonance, Fluorescence, and Ultraviolet–Visible Spectroscopy: Redox Cycling between Ferrous and Ferric States†

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ABSTRACT: Purified human 5-lipoxygenase, a non-heme iron containing enzyme, has been characterized by electron paramagnetic resonance, (EPR), ultraviolet (UV)–visible and fluorescence spectroscopy. As isolated, the enzyme is largely in the ferrous state and shows a weak X-band EPR signal extending from 0 to 700 G at 15 K, tentatively ascribed to integer spin Fe(II). Titration of the protein with 13-HPOD (13-hydroperoxyoctadecadienoic acid) generates a strong multicomponent EPR signal in the $g' \approx 6$ region, a yellow color associated with an increased absorption between 310 and 450 nm ($\epsilon_{330nm} = 2400\ M^{-1}\ cm^{-1}$), and a 17% decrease in the intrinsic protein fluorescence. The multiple component nature of the $g' \approx 6$ signal indicates that the metal center in its oxidized state exists in more than one but related forms. The $g' \approx 6$ EPR signal and the yellow color reach a maximum when approximately 1 mol of 13-HPOD is added/mol of iron; the resultant EPR spectrum accounts quantitatively for all of the iron in the protein with a signal at $g' = 4.3$ representing less than 3% of the total iron in the majority of samples. Addition of a hydroxyurea reducing agent abolished the $g' \approx 6$ signal and yellow color of the protein and also reversed the decrease in fluorescence caused by the oxidant 13-HPOD. The results indicate that the $g' \approx 6$ EPR signal, the yellow color, and the decreased fluorescence are associated with the formation of the Fe(III) form of the enzyme. The spectroscopic data demonstrate that the Fe(III) center of 5-lipoxygenase can be redox-cycled, consistent with its postulated role in the mechanism of oxidation of unsaturated fatty acids to hydroperoxides. Studies using the irreversible inhibitor 4-nitrocatechol, which binds to the ferric form of the enzyme, are also consistent with the redox cycling of the iron and demonstrate that the oxidation state of the enzyme influences both its functional and physical properties.

Lipoxygenases are a class of non-heme iron dioxygenases which catalyze the oxidation of unsaturated fatty acids containing a 1,4-*cis*,*cis*-pentadiene unit to a hydroperoxy conjugated diene. Three major types of lipoxygenases have been identified, inserting dioxygen at the C-5, C-12, and C-15 positions of arachidonic acid. In human leukocytes, 5-lipoxygenase catalyzes the oxygenation of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HPETE)[1] and the dehydration of 5-HPETE to LTA_4 as the first committed steps in the synthesis of leukotrienes [see Yamamoto (1992) and DeWolf (1991) for reviews]. 5-Lipoxygenase is a soluble 78-kDa protein which becomes membrane-bound and activated following cell activation by various stimuli that raise intracellular calcium levels (Rouzer & Kargman, 1988; Wong *et al.*, 1991). The activity of the purified enzyme is strongly stimulated by physiological concentrations of Ca^{2+}, to a lower extent by ATP, and also by phospholipid vesicles or detergent micelles which presumably mimic the membrane environment in which cellular arachidonic acid oxidation occurs (Denis *et al.*, 1991; DeWolf, 1991; Percival *et al.*, 1992). The presence of 18-kDa membrane-bound protein (FLAP) is required for leukotriene biosynthesis to occur in intact cells (Dixon *et al.*, 1990). In light of their key role in leukotriene biosynthesis, both 5-lipoxygenase and FLAP have been the pharmacological targets for antiasthmatic and antiinflammatory agents (Batt, 1992).

All lipoxygenases characterized so far, including 5-lipoxygenase (Percival, 1991), have been shown to contain an essential iron atom which is believed to cycle between the ferric and ferrous forms during the catalytic cycle. In the case of soybean 15-lipoxygenase, X-ray absorption spectroscopy studies have led to the proposal that the non-heme iron is coordinated to the protein by 6 ± 1 nitrogen and/or oxygen ligands, four of them being imidazole ligands (Navaratnam *et al.*, 1988), one of which dissociates during the oxidation of the enzyme to the ferric state (van der Heijdt *et al.*, 1992).

Two crystal structures of the soybean 15-lipoxygenase have recently been solved and show that the iron atom in the ferrous state is coordinated by three histidines and the carboxylate of the C-terminal isoleucine (Boyington *et al.*, 1993; Minor *et al.*, 1993). A fifth ligand, an asparagine, is also present in one of the two structures (Minor *et al.*, 1993).

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CPHU, *N*-(chlorophenyl)-*N*-hydroxy-*N'*-(3-chlorophenyl)urea; DMSO, dimethyl sulfoxide; 13-HPOD, 13-(*S*)-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid; DTT, dithiothreitol; EPPS, *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid; EPR, electron paramagnetic resonance; LTA_4, (5*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); 5-HPETE, 5-(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; FerroZine, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p'*-disulfonic acid; FLAP, 5-lipoxygenase activating protein; NTA, nitrilotriacetate.

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The deduced primary amino acid sequences of plant and mammalian 15- and 12-lipoxygenases (Shibata *et al.*, 1988; Sigal *et al.*, 1988; Funk *et al.*, 1987) and of rat and human 5-lipoxygenases (Dixon *et al.*, 1988; Matsumoto *et al.*, 1988; Balcarek et al., 1988) have revealed the presence of a His motif of the form His-$(X)_4$-His-$(X)_4$-His-$(X)_{17}$-His-$(X)_8$-His (His-363 to His-400 for 5-lipoxygenase) and another His residue toward the C-terminal end (His-550 for 5-lipoxygenase) which are strictly conserved among the various enzymes. Three of these His residues (His-368, -373, and -550) have been shown to be essential for the activity of 5-lipoxygenase (Nguyen *et al.*, 1991; Zhang *et al.*, 1992; Ishii *et al.*, 1992) and two (His-373 and -550) for iron binding to the protein (Percival & Ouellet, 1992; Zhang *et al.*, 1993). The mutation of the corresponding three His residues in the soybean enzyme (Steczko *et al.*, 1992), which are the iron ligands identified in the crystal structures (Boyington *et al.*, 1993; Minor *et al.*, 1993) also lead to loss of activity. These results indicate that there are a number of structural similarities between the plant 15-lipoxygenase and mammalian 5-lipoxygenase although the Ca^{2+} and ATP requirements of the leukocyte enzyme demonstrate major differences in the regulation of their activities.

The soybean 15-lipoxygenase has been studied in detail using a variety of biophysical techniques including EPR, UV–visible, Mössbauer, X-ray absorption, fluorescence, and circular dichroism spectroscopy (Slappendel *et al.*, 1980, 1981, 1982a,b, 1983; de Groot *et al.*, 1975; Draheim *et al.*, 1989; Egmond *et al.*, 1975; Dunham *et al.*, 1990; Van der Heijdt *et al.*, 1992; Fieters *et al.*, 1990; Zhang *et al.*, 1991; Pistorius *et al.*, 1976; Funk *et al.*, 1990; Nelson & Cowling, 1990; Nelson *et al.*, 1990, 1991; Nelson, 1987, 1988a,b; Gaffney *et al.*, 1993). Similar studies of human 5-lipoxygenase have not been possible due to the limited amount of material available and the extreme lability of the purified enzyme. However, with the recent overexpression of the cDNA coding for human 5-lipoxygenase (Denis *et al.*, 1991) and the improvements in the procedures for obtaining purified and stable enzyme (Percival *et al.*, 1992), it is now possible to prepare human 5-lipoxygenase in sufficient quantities for biophysical studies. Such studies are of interest not only to define the structural similarities within the different types of lipoxygenases but also for the understanding of the mechanism of action of eventual therapeutic agents that have been developed based on potential and selective ligands of the active-site iron of 5-lipoxygenase (Carter *et al.*, 1991; Batt, 1992).

We report here the characterization of human 5-lipoxygenase by EPR, UV–visible and fluorescence spectroscopy. Evidence has been obtained that the non-heme iron of the enzyme can be redox-cycled reversibly between 13-HPOD-activated Fe(III) and Fe(II) forms. The EPR spectral properties of the oxidized human enzyme are characteristic of a high-spin ($S = {}^5/_2$) Fe(III) center with near axial symmetry and a zero-field splitting comparable to that of soybean 15-lipoxygenase. The EPR-active Fe(III) in the oxidized form of the protein accounts for all of the iron present in the sample.

MATERIALS AND METHODS

Source of Materials. Human 5-lipoxygenase was obtained from recombinant baculovirus-infected *Spodoptera frugiperda* cells and was purified in a single step using an ATP–agarose affinity column (Denis *et al.*, 1991) as recently modified to increase enzyme stability (Percival *et al.*, 1992). The enzyme was eluted with Chelex- (Bio-Rad) treated buffer containing 0.1 M Na-HEPES and 20 mM ATP, pH 7.3. The purified protein (>95% pure as judged by SDS–polyacrylamide gel electrophoresis) was frozen in aliquots in liquid nitrogen and was used without further purification for UV–visible and EPR titrations. For fluorescence experiments, the enzyme was desalted to remove ATP on a Pharmacia fast desalting column using 50 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA as buffer. For some EPR experiments the enzyme was simultaneously concentrated and dialyzed (Chelex-treated 5 mM KP_i, pH 7.7) using a collodion membrane (Schleicher & Schuell). Dithiothreitol was purchased from Research Organics, Inc., reagent-grade DMSO from Alfa Chemical Co., 4-nitrocatechol from Aldrich, and copper and iron atomic absorption standards from Fisher Scientific and EM Sciences, respectively. 13-HPOD was from Biomol Research Labs and CPHU was synthesized as previously detailed (Falgueyret *et al.*, 1992). The purity of the 13-HPOD was checked by HPLC on a Waters Nova-Pak C18 column using an isocratic mixture of 60:40:0.1 acetonitrile–water–acetic acid and detection at 235 nm. The purity was greater than 98%.

Iron Analysis. The iron content of the 5-lipoxygenase was assayed by the chromogen FerroZine method as previously reported (Percival, 1991). These results were confirmed using a second procedure in which a 100-μL aliquot of 5-lipoxygenase (0.65 mg) was added to 1 mL of 6 N HCl. The sample was heated at 100 °C for 24 h to digest the protein, followed by lyophilization to remove the HCl. The residue was then resuspended in 100 μL of buffer (0.1 M Na-HEPES, pH 7.0) and added to 100 μL of 0.12 M ascorbic acid, 100 μL of 0.25 mM FerroZine, plus 900 μL of water to bring the total volume to 1.2 mL. The absorbance of the iron–FerroZine complex was compared to that of standards as for the method above. The iron content of the protein determined by the two methods agreed within 4.4%.

EPR Spectroscopy. EPR measurements were performed on a Varian E-9 spectrometer using a TE104 dual rectangular cavity fitted with an Air Products Helitran low-temperature flow cryostat using a Cryo Industries of America transfer line. The temperature was measured before and after each spectral run with a carbon glass resistor (TRI Research) mounted in a 4-mm o.d. EPR tube. The EPR spectrometer was interfaced to an ISA standard Intel-based 80486 computer using data acquisition hardware and EPRWare software purchased from Scientific Software Services. Data manipulations and EPR spectral simulations were performed on this computer using the programs EPRWare and EPRPOW (White & Belford, 1976), respectively.

The amount of EPR-active iron(III) in the protein samples was determined relative to three standards, two copper and one diferric human serum transferrin standard. All standards were 0.50 mM in the respective metal and were derived from atomic absorption standards of known concentration. The two copper standards consisted of either 0.50 mM copper nitrate in 25% glycerol, pH = 2, or 0.50 mM copper nitrate, 1.0 mM EDTA, 0.01 M ascorbate, and 3 M $NaClO_4$, pH = 2. The transferrin standard was 0.36 mM in transferrin, 0.1 M HEPES and 0.01 M sodium bicarbonate, pH 7.5, and 0.50 mM in iron (~75% saturated). The iron was added to the apotransferrin using Fe–NTA (5 mM/10 mM), pH = 4. All EPR measurements were done with calibrated 3-mm i.d.–4-mm o.d. quartz EPR tubes. The EPR spectra of the copper and transferrin standards and lipoxygenase were measured at 15 K at microwave powers of 0.04, 0.25, and 10 mW, respectively, values determined to be well below levels of power saturation. The linearity of the power dial was verified by measuring the signal amplitude of the nonsaturating signal of strong pitch from 0.1 to 50 mW at room temperature; the expected square root of power dependence was observed. The

linearity of the instrument receiver gain and modulation dial settings were also checked. Copper standards were double-integrated between 1600 and 3400 G and transferrin between 1000 and 2300 G. The intensity factor g_P^{AV} was determined from the field setting corresponding to the half-maximum of the value of the double integral; the measured g_P^{AV} was found to be in excellent agreement with the value calculated using eq 6 of Aasa and Vanngard (1975). To correct for the thermal population of the other two doublets of the $S = {}^5/_2$ spin manifold of states, the double integral of the $g' = 4.3$ signal was multiplied by a factor of 3. After correction for power, receiver gain, and intensity factor g_P^{AV}, the double integrals of the copper and transferrin EPR standards were found to agree within 6.6%. The average value was used for quantification of the EPR-observable Fe(III) in lipoxygenase.

Oxidation and Reduction of 5-Lipoxygenase. For the titration with 13-HPOD, aliquots of stock concentrations of 13-HPOD in either DMSO (2.21 or 5.15 mM) or in 50 mM EPPS buffer, pH 9 (1.0 mM), were added consecutively to the EPR tube containing 250 μL of native 5-lipoxygenase at room temperature. After each addition, the tube was inverted and shaken several times before freezing and measurement of the EPR spectrum. To reduce the ferric enzyme, CPHU (0.1 M) and DTT (0.1 M) were added to the 5-lipoxygenase previously oxidized by 13-HPOD, shaken, and frozen. The DTT was dissolved in water and kept anaerobic until its addition to the sample along with CPHU in DMSO. DTT alone only slowly ($t_{1/2} \approx 7$ min) reduces ferric 5-lipoxygenase (K. Neden, unpublished data). The DTT was included since it has been previously shown to recycle oxidized CPHU (Falgueyret *et al.*, 1992) and therefore reduce the concentration of reactive species that may in some manner damage the enzyme. Additionally, the omission of DTT resulted in the formation of a strong chromophore in the region 280–600 nm which interfered with the UV–visible titrations described below.

UV–Visible Spectrophotometry. UV–visible spectrophotometry was carried out at 5 °C using a Hewlett-Packard 8452A photodiode array spectrophometer. Titrations were performed by the addition of 13-HPOD (3.2 mM in ethanol) or CPHU (6.1 mM in ethanol) to 0.20 mL of 5-lipoxygenase (6.6 mg/mL). After each addition and mixing, the solution was centrifuged for 3 min at 12000*g* to remove small amounts of precipitated protein prior to measurement of the spectrum. In the experiments in which CPHU was added, DTT (2 mM) and catalase (10 μg/mL) were also included. The catalase was present to protect 5-lipoxygenase against irreversible inhibition by H_2O_2 produced by thiol autoxidation (Percival *et al.*, 1992). The activity of the enzyme before and after the additions of 13-HPOD and CPHU was determined using an HPLC assay as previously described (Percival *et al.*, 1992).

Fluorescence Spectrophotometry. Fluorescence experiments were conducted at 5 °C using a Perkin-Elmer LS-5 fluorometer equipped with a stirring accessory. Desalted 5-lipoxygenase was diluted to 0.02 mg/mL in 2.0 mL of 50 mM Tris-HCl and 0.1 mM EDTA, pH 7.5, and aliquots of 13-HPOD or CPHU (87 and 174 μM in ethanol, respectively) were added. The excitation wavelength used was 280 nm.

Titrations Using 4-Nitrocatechol. (i) Reduced 5-lipoxygenase: Aliquots of 5-lipoxygenase (final concentration 0.065 mg/mL) were incubated at room temperature with 1 μM 4-nitrocatechol and 0–1 μM 13-HPOD in a final volume of 100 μL of buffer (10 mM Na-HEPES, pH 7.5, 0.1 mM EDTA, 5 μg/mL catalase, and 5 mM CHAPS). After 30 min the remaining activity was determined by diluting an aliquot (10 μL) into 1 mL of an assay mix as previously described (Percival *et al.*, 1992). (ii) Oxidized 5-lipoxygenase: Aliquots of 5-lipoxygenase were oxidized with 1.5 equiv (based on iron content) of 13-HPOD just prior to use. Alternatively, the enzyme was treated with 13-HPOD and the excess was removed by passage through a Pharmacia fast desalting column. CPHU (0–2 μM final concentration) was then incubated at room temperature with the oxidized enzyme (0.032 mg/mL) in a final volume of 100 μL of buffer (10 mM Na-HEPES, pH 7.5, 0.1 mM EDTA, 5 μg/mL catalase, and 5 mM CHAPS). After 1 min, 4-nitrocatechol (1 μM final concentration) was added and after a further 10 min an aliquot (5 μL) was assayed for enzyme activity as above.

The stabilities of 4-nitrocatechol and 13-HPOD in the presence of each other were confirmed by controls performed under conditions identical to those of the titration experiments. The stability of 13-HPOD (32 μM) in the presence of 4-nitrocatechol (20 μM) was determined by reversed-phase HPLC as described above. No destruction was observed over the same period as that of the experiments. Under the same conditions no change was observed in the absorption spectrum of 4-nitrocatechol.

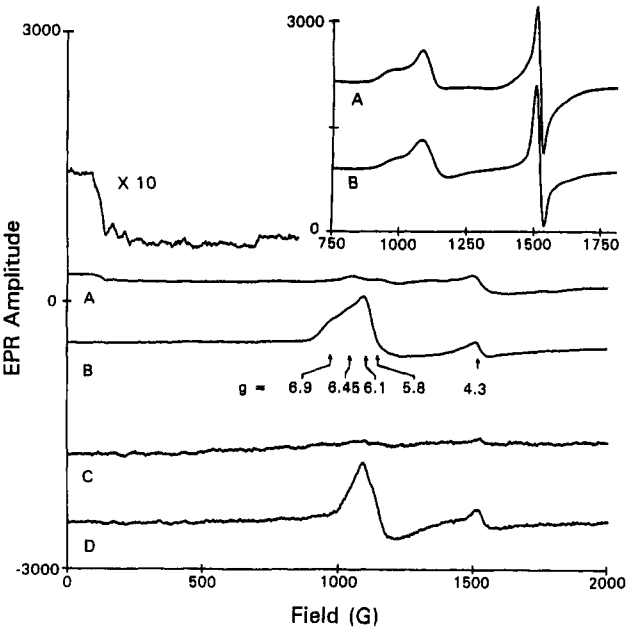

FIGURE 1: EPR spectra of human 5-lipoxygenase: (A) Native enzyme and (B) native enzyme with 1.0 equiv of 13-HPOD in DMSO added per iron; (C) native enzyme and (D) native enzyme with 1.0 equiv of 13-HPOD in 50 mM EPPS buffer, pH 9, added per iron. Inset: (A) 2.0 equiv of 13-HPOD per iron of native 5-lipoxygenase; (B) computer simulation of the spectrum. Spectrometer settings: field set = 1000 G, scan range = 2000 G, time constant = 3 s, modulation amplitude = 20 G, microwave power = 10 mW, frequency = 9.356 GHz, gain = 2500 (A, B), 10 000 (C, D) and 630 (inset A); temperature = 7.8 K for (A), 7.3 K for (B), 13.4 K for (C), 12.5 K for (D), and 15.3 K for inset (A). Enzyme concentrations: (A, B) 0.24 mM 5-lipoxygenase (0.2 Fe/enzyme); (C, D) 52 μM 5-lipoxygenase (0.38 Fe/enzyme); (inset A) 0.30 mM 5-lipoxygenase (0.49 Fe/enzyme); samples A, B, and inset A were in 5 mM KP_i, pH 7.7, and samples C and D were in 50 mM Na-HEPES, pH 7.3.

RESULTS

EPR Spectra of Native and Oxidized Human 5-Lipoxygenase. The EPR spectrum of the native enzyme (0.2 Fe/enzyme) in frozen solution at 7.8 K is shown in Figure 1 (trace A). The native form of the enzyme is mainly EPR-silent, indicating that the enzyme was isolated with the iron center in the reduced ferrous form. A weak EPR signal spanning the 0–710-G range is observed in the native sample and is tentatively ascribed to Fe(II). Low-field EPR signals from

integer spin Fe(II) in a number of enzymes and model compounds have been reported recently (Hendrich & Debrunner, 1989; Hendrich *et al.*, 1990, 1991). Low-intensity signals from high-spin Fe(III) are also observed in Figure 1 (trace A) at $g' \approx 4.3$, 5.80, and 6.35, but they represent only about 3.8% of the total iron in the sample.

The addition of hydroperoxides such as 13-HPOD has been shown to eliminate the kinetic lag phase of the 5-lipoxygenase reaction (Riendeau *et al.*, 1989), an effect which has been attributed to the oxidation of the enzyme to the ferric form by analogy with the results obtained for the soybean lipoxygenase (de Groot, 1975). To examine the effect of 13-HPOD on the EPR spectrum of the protein, a stoichiometric amount of 13-HPOD (1 equiv of 13-HPOD per Fe in 5-lipoxygenase) was added to the sample.[2] The putative Fe(II) signal in the 0–710-G range disappeared and a complex multicomponent signal developed in the $g' = 6$ region (Figure 1, *cf.* traces A and B). Addition of 13-HPOD in pH 9 EPPS buffer as opposed to DMSO results in a less complex $g' = 6$ signal (Figure 1, traces A and B *vs* traces C and D). The variation in the EPR signal of the human enzyme is similar to the natural variation in the EPR signal observed with different preparations of the soybean enzyme (Slappendel *et al.*, 1980). The effect of DMSO on the human protein is less pronounced than that seen with alcohols on the spectrum of the soybean enzyme (Slappendel *et al.*, 1982a). No influence of buffer on the EPR signal of the human protein was noted.

The observed Fe(III) spectra can be described by the following Fe(III) $S = {}^5/_2$ spin Hamiltonian:

$$\mathcal{H} = g\beta B\hat{S} + D[\hat{S}_z^2 - S(S+1)/3] + E[\hat{S}_x^2 - \hat{S}_y^2] \quad (1)$$

Here g is the electron g factor, β is the Bohr magneton, D and E are the axial and rhombic zero-field splitting constants, respectively, B is the applied magnetic field, and the $\hat{S}_i$ ($i = x$, y, and z) are the operators for electron spin angular momentum. The signal observed at the apparent isotropic $g' = 4.3$[3] (Figure 1B) arises from transitions within the $M_s = \pm{}^3/_2$ middle doublet of the $S = {}^5/_2$ manifold of states. Such a situation is obtained when the metal site has completely rhombic symmetry, *i.e.*, $E/D = 1/3$, and the zero-field splitting exceeds the microwave quantum of energy, namely, $D \geq h\nu$ (Yang & Gaffney, 1987; Scullane *et al.*, 1982; Aasa, 1970).

In contrast, signals with apparent anisotropic g'-factors of $g_\perp' = 6$ (Figure 1B) and $g_\parallel' = 2$ are expected from transitions within the lower $M_s = \pm{}^1/_2$ doublet when the metal site has axial symmetry, namely, $E/D = 0$ and $D \geq h\nu$ (Slappendel *et al.*, 1980). The signal at $g_\parallel' = 2$ is weak and is obscured in our samples by a sextet of lines from adventitious Mn^{2+} (spectrum not shown). Lines with g'-values near 6 are also observed when the metal site has near axial symmetry, *i.e.*, $E/D \leq 0.02$ (Slappendel *et al.*, 1981). Thus the lines at $g_\parallel'$ = 5.8, 6.1, 6.45, and 6.9 in Figure 1 (trace B) indicate that the EPR envelope at $g' \approx 6$ is composed of both "axial" and "near axial" components. Therefore, the EPR spectrum of 5-lipoxygenase has contributions from more than one form of the metal site, as observed also for the soybean enzyme (Slappendel *et al.*, 1981).

Quantification of the Iron Signals. Quantification of the amount of iron(III) contributing to the $g' \approx 6$ and $g' \approx 4.3$ signals is complicated by the fact that the $g' \approx 6$ signal is part of an EPR first-derivative envelope which extends from $g' \approx 6$ to $g' \approx 2$ and overlaps with the $g' \approx 4.3$ signal from the "rhombic Fe(III)" and with the $g' \approx 2$ signals from Mn^{2+}. Quantification of the EPR spectrum is difficult because of signal overlap and baseline error from double integration over such a large magnetic field range, 800–3400 G. Similar problems have been encountered with the soybean enzyme but have been overcome by the use of EPR spectral simulations (Slappendel *et al.*, 1981; Aasa & Vanngard, 1975). Here the spectrum of the axial and near axial Fe(III) species of 5-lipoxygenase was simulated over the field range from $g' \approx 6$ to $g' \approx 2$. Particular attention was paid to fitting the line shape of the $g' \approx 6$ multicomponent signal which accounts for most of the EPR intensity. The simulated spectrum was then integrated over the entire field range to obtain the total EPR intensity from transitions within the $M_s = \pm{}^1/_2$ Kramers doublet of the "axial" and "near axial" Fe(III). The simulated spectrum was then subtracted from the experimental spectrum to obtain the $g' = 4.3$ signal alone. This signal was then double-integrated to obtain the spin concentration of the "rhombic" Fe(III) species (see Materials and Methods).

Table I: EPR Simulation Parameters of 13-HPOD Oxidized Human 5-Lipoxygenase

species	g_x[a]	g_y[a]	g_z[a]	wt.
$g = 6$	6.04 (35)	5.90 (60)	2.00 (150)	0.24
$g = 6$	6.70 (50)	4.25 (85)	2.00 (150)	0.67
$g = 6$	6.17 (50)	5.24 (140)	2.00 (150)	0.09
$g = 4.3$	4.297 (60)	4.297 (60)	4.297 (60)	0.48
$g = 4.3$	4.347 (30)	4.347 (30)	4.347 (30)	0.26
$g = 4.3$	4.377 (16)	4.377 (16)	4.377 (16)	0.26

[a] Linewidths Γ in gauss at half-height of the absorption curve are given in parentheses.

To simulate the spectrum, a fictitious spin $S' = {}^1/_2$ spin Hamiltonian was assumed, *viz.*

$$\mathcal{H} = g_x\beta B_x\hat{S}_x + g_y\beta B_y\hat{S}_y + g_z\beta B_z\hat{S}_z \quad (2)$$

The results are shown in Figure 1 (inset) and the spin Hamiltonian parameters are given in Table I. Figure 1A (inset) is the experimental spectrum of a lipoxygenase sample (0.49 Fe/enzyme) which has been completely oxidized by the addition of an excess of 13-HPOD in DMSO (≈2 equiv of 13-HPOD/Fe) and is similar to that reported for some sample preparations of the soybean enzyme (de Groot *et al.*, 1975; Zhang et al., 1991). Figure 1B (inset) shows the simulated spectrum. In order to reproduce the $g' \approx 6$ line shape observed experimentally, it was necessary to include spectral contributions from three species in the simulation (Table I). Simulation of the $g' \approx 4.3$ signal also required three species, although the line shape was not as well reproduced as that of the $g' \approx 6$ signal (Figure 1B, inset), and consequently the $g' = 4.3$ spectrum obtained by subtracting the $g' = 2$–6 simulated EPR envelope from the experimental spectrum was used in the spin quantification as noted above. No claim is made to the uniqueness of the fit. Our principal objective is to use the double integration of the simulated $g' \approx 6$ spectrum to determine the spin population $N_{\pm 1/2}$ of the $M_s = \pm{}^1/_2$ doublet. The total spin population N_{total} from all three doublets is the quantity related to the concentration of EPR-active Fe(III) and is given by the following Boltzmann expression (Slappendel *et al.*, 1981):

$$N_{total} = N_{\pm 1/2}[1 + \exp(-2D/kT) + \exp(-6D/kT)] \quad (3)$$

A value of $D = 1.7$ cm^{-1} for the zero-field splitting (*vide infra*) was used in eq 3 to calculate N_{total} ($=2.12N_{\pm 1/2}$), from which the concentration of Fe(III) was determined by comparison with EPR intensity standards. A 50% error in the choice of

[2] 13-HPOD was used as the enzyme oxidant rather than 5-HPETE since 5-HPETE can be further converted by 5-lipoxygenase to LTA_4. This potential complication is avoided in the case of 13-HPOD since it does not contain the 8,11-*cis*,*cis*-pentadiene unit necessary for this reaction.

[3] For an S-state ion such as Fe(III), the true g-factor is taken as $g = 2.0$.

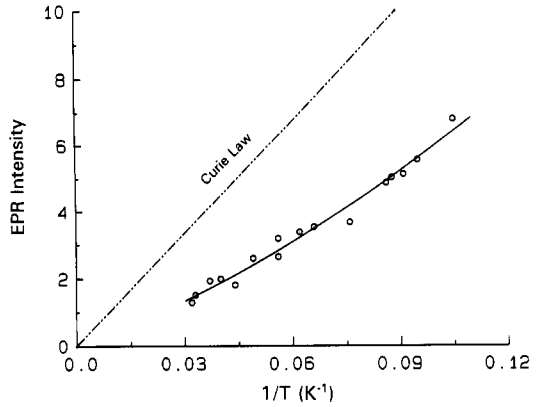

FIGURE 2: Temperature dependence of EPR signal intensity. Plot of EPR intensity vs $1/T$ at various temperatures (11.4–30.8 K). The solid line is the nonlinear least-squares fit to the data according to eq 4. The dashed line is Curie law behavior ($I = K/T$). Spectrometer settings: field set = 2000 G, scan range = 4000 G, modulation amplitude = 20 G, time constant = 1 s, scan time = 8 min, microwave power = 2 mW, gain = 4000, frequency = 9.356 GHz. Solution conditions: 84 μM 5-lipoxygenase (0.54 Fe/enzyme), 0.1 M Na-HEPES, and 2.1 mM ATP, pH 7.3.

D changes the Fe(III) spin concentration by only ≈15%. For the $g' = 4.3$ signal, a Boltzmann factor of 3 ($N_{total} = 3N_{\pm 3/2}$) was employed in computing the spin concentration, since at 15 K the zero-field splitting is much smaller than kT and all three doublets are equally populated (Slappendel *et al.*, 1980).

The results of the quantification showed that 89% of the EPR-visible iron was responsible for the signal near $g' \approx 6$, with the remaining 11% giving rise to the $g' = 4.3$ signal. In the majority of samples the $g' = 4.3$ signal constituted less than 3% of the iron. The large amplitude of the $g' = 4.3$ signal (Figure 1A, inset) is deceptive since its isotropic nature makes even a low concentration of the "rhombic" Fe(III) species highly visible in the spectrum. The concentrations of EPR-observable $g' \approx 6$ and $g' \approx 4.3$ species were found to be 128 ± 19 and 16 ± 2 μM, respectively, compared with the total iron concentration in the sample of 127 ± 6 μM. Similar quantitative results were obtained for samples oxidized by the addition of 13-HPOD in EPPS buffer, pH 9. Thus the EPR spectrum accounts for all of the iron in the protein.

Measurement of the Zero-Field Splitting. The zero-field splitting for human 5-lipoxygenase was measured from the temperature dependence of the EPR spectral intensity over the range 11.4–30.8 K for a sample in which the $g' \approx 6$ signal accounted for 99% of the iron. The double integral (I) was evaluated between 800 and 2600 G, just short of the interfering signals from adventitious Mn(II); the value obtained agreed with that from EPR simulation within 9%. The double integral versus $1/T$ (Figure 2) was fit to

$$I = \frac{K}{T[1 + \exp(-2D/kT) + \exp(-6D/kT)]} \quad (4)$$

by nonlinear regression with the proportionality constant K and zero-field splitting constant D as adjustable parameters. The deviation of the data from Curie law behavior given by $I = K/T$ is shown in Figure 2. A value of $D = 1.7 \pm 0.6$ cm^{-1} was obtained.

Titration of Spectral Changes with 13-HPOD. The results of an EPR spectrometric titration of the iron center of 5-lipoxygenase (0.54 Fe/enzyme) with 13-HPOD are shown in Figure 3. The addition of approximately 1 equiv of 13-HPOD results in maximal $g' = 6$ EPR signal intensity.[4,5] A UV–visible spectrophotometric titration with 13-HPOD on the same protein preparation results in the appearance of a broad absorption in the region 310–450 nm associated with the development of a yellow color in the protein solution (Figure 4). A plot of the absorbance at 330 nm as a function of the equivalents of 13-HPOD per Fe of lipoxygenase is shown in the inset of Figure 4. The maximal increase in absorbance was seen at about 1 equiv of 13-HPOD. The absorption coefficient at 330 nm, calculated in terms of iron-containing enzyme, for the difference between the yellow and native forms is 2400 M^{-1} cm^{-1}. From these data it is evident that 1 mol of iron is oxidized/mol of 13-HPOD and that the appearance of both the $g' \approx 6$ EPR signal and the 330-nm absorbance is correlated and arises from the oxidized state of the protein as found for the soybean enzyme (Slappendel *et al.*, 1983). Absorption in this spectral region has been ascribed to an

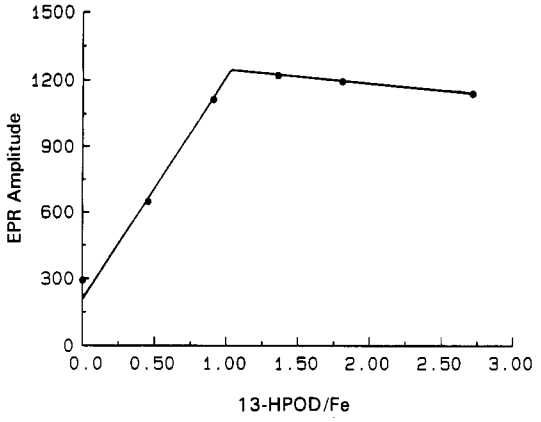

FIGURE 3: EPR spectrometric titration of 5-lipoxygenase with 13-HPOD. Spectrometer settings: Same as in Figure 1A,B except the measurements were made at 15.0 K and the gain = 4000. Solution conditions: Same as in Figure 2.

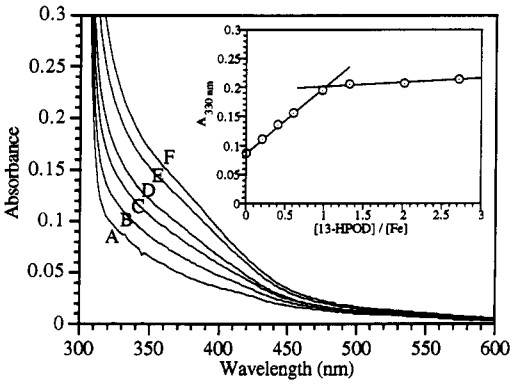

FIGURE 4: Effect of 13-HPOD on the UV–visible spectrum of 5-lipoxygenase. Spectra were measured after addition of aliquots of 13-HPOD (3.2 mM) to give final concentrations of (A) 0, (B) 9.3, (C) 18.6, (D) 27.9, (E) 44.8, and (F) 156.8 μM. (Inset) Plot of absorbance at 330 nm versus the ratio of 13-HPOD to iron concentration. Solution conditions: 6.6 mg/mL (84 μM) 5-lipoxygenase (0.54 Fe/enzyme), 0.1 M Na-HEPES, pH 7.3, and 2.1 mM ATP.

[4] Comparable results were obtained when the protein was titrated with 13-HPOD in 50 mM EPPS buffer, pH 9. Some turbidity was observed in the protein solution at the latter stages of the titration, suggesting some denaturation had occurred during the repetitive freeze–thaw cycling of protein with each addition of 13-HPOD.

[5] The dependence of the $g' = 4.3$ signal amplitude on the amount of added 13-HPOD was also examined. For seven different sample preparations, the signal amplitude was seen to increase 80% ± 64% and 23% ± 11% for the first and second equivalents of 13-HPOD added, respectively. The final $g' = 4.3$ signal amplitude represented 3% or less of the total iron in all instances.

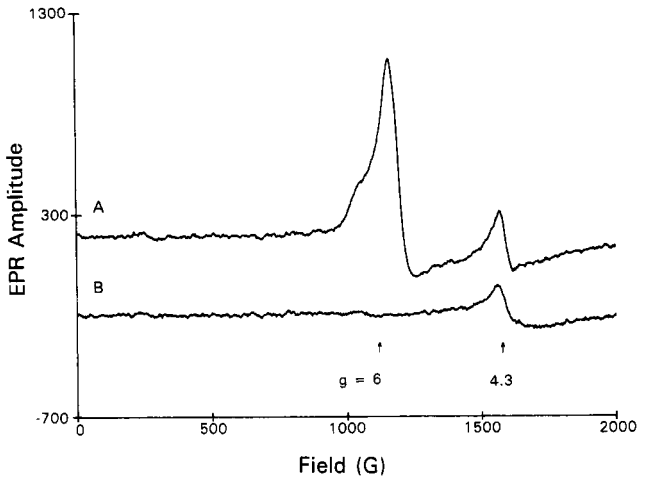

FIGURE 5: Effect of the reducing agent CPHU on the EPR spectrum of 13-HPOD-oxidized 5-lipoxygenase. (A) Addition of 2.0 equiv of 13-HPOD per Fe of native 5-lipoxygenase; (B) addition of 340 μM each CPHU and DTT. Spectrometer settings: field set = 1000 G, scan range = 2000 G, time constant = 1 s, modulation amplitude = 20 G, power = 10 mW, scan time = 8 min, frequency = 9.356 GHz, temperature = 12.2 K. Spectra are plotted scaled to the same gain. A cavity background signal was subtracted from both spectra, accounting for the reduced signal/noise ratio. Solution conditions: 84 μM 5-lipoxygenase (0.54 Fe/enzyme), 0.1 M Na-HEPES, and 2.1 mM ATP, pH 7.3.

endogenous ligand to iron(III) charge transfer transition, presumably involving a histidine ligand (or ligands) (Zhang *et al.*, 1991). The addition of an excess of 13-HPOD over iron-containing enzyme did not result in the formation of a purple color as it does in the case of the soybean lipoxygenase (Slappendel et al., 1983; Nelson et al., 1990). This result is consistent with the hypothesis that the purple enzyme consists of a mixture of enzyme-bound intermediates formed during the oxygenation of linoleic acid (Nelson et al., 1990). These reaction intermediates, and hence the purple color, cannot be formed in the case of 5-lipoxygenase since linoleic acid is not a substrate of this enzyme.

Effect of the Reducing Agent CPHU on the EPR and Absorption Spectra. The addition of a number of reducing agents has been shown to inhibit the activity of soybean lipoxygenase and of 5-lipoxygenase (Riendeau *et al.*, 1991b; Clapp *et al.*, 1985; Kemal *et al.*, 1987; Falgueyret *et al.*, 1992). Addition of CPHU (Falgueyret *et al.*, 1992) to the 13-HPOD-oxidized enzyme results in elimination of the $g' \approx 6$ signal (Figure 5, *cf.* A and B) and of the yellow color of the enzyme (Figure 6), indicating that the Fe(III) center has been reduced.

Fluorescence Spectroscopy of Reduced and Oxidized 5-Lipoxygenase. The fluorescence spectrum of native 5-lipoxygenase displays excitation and emission maxima at 280 and 338 nm, respectively (Figure 7, trace A). The addition of 13-HPOD to the sample reduces the relative fluorescence at 338 nm by 17% (Figure 7, trace B). Addition of a slight excess of the reducing agent CPHU results in an immediate restoration of native fluorescence spectrum (Figure 7, trace C). Again, these results support the findings that the iron in the active site of the enzyme can be cycled between ferrous and ferric states as further demonstrated below. Activity measurements at the completion of the UV–visible and fluorescence titrations demonstrated that the enzyme retained greater than 80% of its original activity.

Inactivation of the Ferric Form of 5-Lipoxygenase by 4-Nitrocatechol. Previous studies have shown that 4-nitrocatechol can inhibit the oxidized ferric form of the soybean lipoxygenase but not the reduced form (Galpin *et al.*, 1976). At pH 7, 4-nitrocatechol inhibits 5-lipoxygenase only when

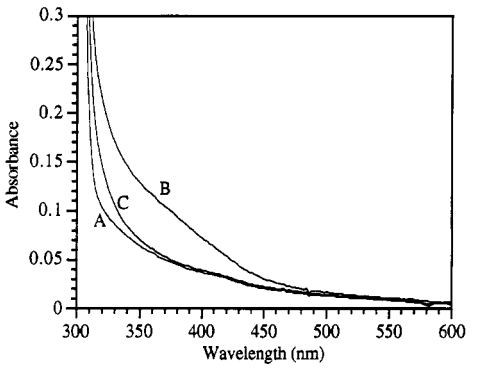

FIGURE 6: Effect of the reducing agent CPHU on the UV–visible spectrum of 13-HPOD-oxidized 5-lipoxygenase. (A) Native 5-lipoxygenase, (B) after addition of 43 μM 13-HPOD, (C) after further addition of 49 μM CPHU. Solution conditions: 6.6 mg/mL (84 μM) 5-lipoxygenase (0.54 Fe/enzyme), 0.1 M Na-HEPES, pH 7.3, 2.1 mM ATP, 2 mM DTT, and 10 μg/mL catalase.

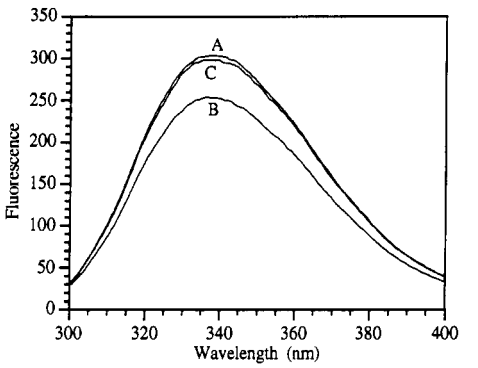

FIGURE 7: Effect of 13-HPOD and CPHU on the fluorescence spectrum of 5-lipoxygenase. (A) Native 5-lipoxygenase, (B) after addition of 0.4 μM 13-HPOD, (C) after further addition of 0.5 μM CPHU. Solution conditions: 0.02 mg/mL (0.3 μM) 5-lipoxygenase, 50 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA.

the enzyme has been pretreated with 13-HPOD or is in the presence of 13-HPOD, implying that the catechol interacts specifically with the oxidized form of the enzyme. The inactivation is slow and cannot be reversed (K. I. Skorey and M. J. Gresser, unpublished observations).

A titration of the reduced enzyme was performed by varying the concentration of 13-HPOD until complete inactivation by an excess of 4-nitrocatechol was observed. This titration reflects the amount of reduced enzyme capable of being oxidized by 13-HPOD and subsequently inactivated by 4-nitrocatechol. Similarly, a titration of the oxidized enzyme was obtained using CPHU to reduce the enzyme and prevent inactivation of the oxidized enzyme by 4-nitrocatechol. Shown are the plots of the recovery of 5-lipoxygenase activity versus [13-HPOD] and [CPHU] in Figures 8 and 9 for three different batches of enzyme of varying specific activities. For the 13-HPOD titration (Figure 8), the enzyme was incubated with 13-HPOD and 4-nitrocatechol for 30 min and the amount of active enzyme remaining was determined by measuring the total product formed after 100-fold dilution into the assay mixture containing the arachidonate substrate. For the CPHU titration (Figure 9), the oxidized enzyme was incubated with the reducing agent for 1 min, and then 4-nitrocatechol was added and the amount of active enzyme remaining after 10 min was determined as above. There was no inhibition of the enzyme in the assay by the 4-nitrocatechol at this diluted concentration (0.01 μM) since the 5-lipoxygenase reaction ceases due to the inactivation of the enzyme during the reaction before the slow development of the inhibition occurs.

A good correlation was found betwen the specific activities of the three enzyme preparations, their iron contents, and the

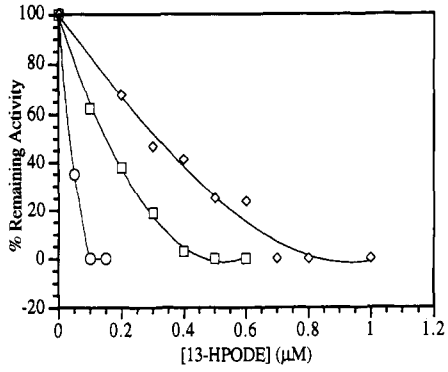

FIGURE 8: Titration of reduced 5-lipoxygenase with 13-HPOD. Three preparations of 5-lipoxygenase (0.065 mg/mL, 0.8 μM) with specific activities of 8 μmol of 5-HPETE/mg (0.11 Fe/enzyme) (○), 34 μmol of 5-HPETE/mg (0.36 Fe/enzyme) (□), and 70 μmol of 5-HPETE/mg (0.54 Fe/enzyme) (◇) were incubated with varying concentrations of 13-HPOD in buffer containing 1.0 μM 4-nitrocatechol. After 30 min an aliquot was assayed for the measurement of the remaining activity. Solution conditions: 10 mM Na-HEPES, pH 7.5, 0.1 mM EDTA, 5 μg/mL catalase, and 5 mM CHAPS.

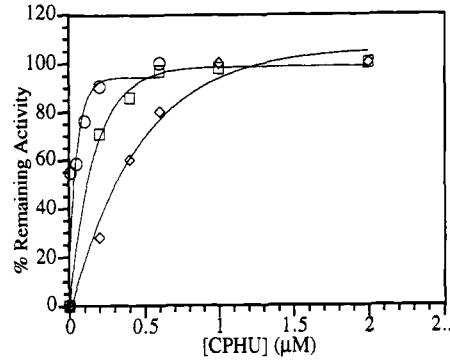

FIGURE 9: Titration of oxidized 5-lipoxygenase with CPHU. Three preparations of 5-lipoxygenase (0.032 mg/mL, 0.4 μM) with specific activities of 8 μmol of 5-HPETE/mg (○), 34 μmol of 5-HPETE/mg (□), and 70 μmol of 5-HPETE/mg (◇) were oxidized by addition of 1.5 equiv of 13-HPOD (based on iron content as given in the legend to Figure 8) and then incubated for 1 min with varying concentrations of CPHU. The enzymes was then incubated for 10 min with 1.0 μM 4-nitrocatechol prior to dilution for the measurement of activity. Solution conditions: 10 mM Na-HEPES, pH 7.5, 0.1 mM EDTA, 5 μg/mL catalase, and 5 mM CHAPS.

amounts of 13-HPOD and CPHU required to fully oxidize and reduce the enzyme, respectively (Figures 8 and 9). The results predict a maximal specific activity around 120 μmol of 5-HPETE/mg of protein for an enzyme containing 1 mol of iron/mol of protein under the present assay conditions.

These experiments demonstrate that the oxidation and reduction of 5-lipoxygenase by 13-HPOD and CPHU are reversible and also result in changes in the sensitivity of the enzyme to the inactivation by 4-nitrocatechol. This effect is in addition to the elimination of the kinetic lag phase of the 5-lipoxygenase reaction previously described (Riendeau *et al.*, 1989). Thus, the oxidation state of the iron determines the functional as well as physical characteristics of this enzyme.

DISCUSSION

The present study demonstrates that the non-heme iron center of 5-lipoxygenase can undergo reversible cycling between the Fe(II) state and an activated Fe(III) state. The titrations of 5-lipoxygenase with 13-HPOD indicate a stoichiometry of 1 mol of Fe(II) oxidized/mol of 13-HPOD added (Figures 3 and 4). The addition of the reducing agent CPHU reduces the Fe(III) of the 13-HPOD-activated enzyme to the Fe(II) state, virtually abolishing the $g' \approx 6$ signals from the high-spin Fe(III) (Figure 5). These results are consistent with previous EPR studies on the reduction of the soybean enzyme by alkylhydroxylamines and catechols (Clapp *et al.*, 1985; Kemal *et al.*, 1987). Indirect evidence has also been obtained for the reduction of 5-lipoxygenase by CPHU and other reducing agents since these compounds can stimulate the destruction of 13-HPOD catalyzed by the enzyme (Riendeau *et al.*, 1991a,b; Falgueyret *et al.*, 1992). The reduction of the active-site iron also returns the enzyme to its colorless form (Figure 6), further confirming that the iron in 5-lipoxygenase can be cycled between its Fe(II) and Fe(III) states.

The conclusion that the non-heme iron of 5-lipoxygenase can be reversibly oxidized is further supported by the experiments with 4-nitrocatechol, which demonstrate that the ability of the 4-nitrocatechol to irreversibly inhibit 5-lipoxygenase activity is clearly dependent on the oxidation state of the iron (Figures 8 and 9). The addition of 4-nitrocatechol to the native ferrous form of the enzyme results in irreversible inactivation of the enzyme only in the presence of 13-HPOD (Figure 8). In addition, the ferric form of 5-lipoxygenase can be effectively protected from 4-nitrocatechol inactivation by the presence of an excess of a reducing agent, in this case CPHU, which regenerates the enzyme to its ferrous state (Figure 9). In both cases, the concentrations of reductant (CPHU) or oxidant (13-HPOD) needed to protect or abolish activity was nearly equivalent to the concentration of iron of the enzyme preparation. It should be noted that high-expression systems for recombinant 5-lipoxygenase have been reported to yield proteins of variable iron contents (0.2–1.1 mol of Fe/mol of protein) and that the iron can dissociate from the protein during enzyme inactivation (Percival, 1991; Zhang *et al.*, 1992). Assuming that 5-lipoxygenase contains 1 atom of iron/mol of protein, it can be estimated that the maximal specific activity of the human enzyme (plateau assay) would be about 120 μmol of 5-HPETE/mg of protein.

The EPR spectrum of the ferric form of human 5-lipoxygenase indicates that the iron environment is very similar to that of the soybean enzyme (Pistorius *et al.*, 1976; de Groot *et al.*, 1975; Slappendel *et al.*, 1980, 1981). However, the spectrum is different from the broad signals reported for rabbit reticulocyte and porcine leukocyte lipoxygenases (Carroll *et al.*, 1993; Kroneck *et al.*, 1991). The multicomponent structure in the $g' \approx 6$ signal observed here reveals heterogeneity in the metal site, presumably from the presence of more than one ligand conformation in frozen solution. The detailed $g' \approx 6$ line shape depends on the sample preparation, as has been found for the soybean enzyme (Slappendel *et al.*, 1982a,b). Because of this variability between samples, a range of zero-field splittings, $D = 0.6$–3.1 cm^{-1}, has been measured for soybean lipoxygenase (Slappendel *et al.*, 1980; Zhang *et al.*, 1991). The zero-field splitting, $D = 1.7$ cm^{-1}, observed here for the human protein falls within this range.

The axial and near axial nature of the $g' \approx 6$ EPR signal is consistent with a metal-site geometry of the Fe(III) enzyme composed of three identical ligands in the equatorial plane, probably from histidine, with either nitrogen and/or oxygen donor ligands occupying the apical positions. Such a structure is in accord with recent EXAFS data (Van der Heijdt *et al.*, 1992; Fieters *et al.*, 1990) and spectroscopic data (Zhang *et al.*, 1991) on the soybean enzyme.

In the soybean enzyme, the $g' = 4.3$ signal has been variously suggested to arise from adventitious Fe(III) or perhaps active-site Fe(III) (Draheim *et al.*, 1989; Zhang *et al.*, 1991). Given the low integrated intensity of the $g' = 4.3$ signal,[5] it seems most likely that this signal is due to nonspecifically bound

Fe(III) or to Fe(III) in the specific iron binding site of denatured protein.

The deduced amino acid sequences of the soybean 15-lipoxygenase (Shibata *et al.*, 1988) and of mammalian 5-lipoxygenases (Balcarek *et al.*, 1988; Dixon *et al.*, 1988; Matsumoto *et al.*, 1988) demonstrate that the two enzymes share 42% amino acid identity and also a conserved His motif which has been discussed as a putative iron-binding site. The amino acid identity between the three classes of mammalian enzymes ranges from 40% to 85%. The differences between these classes of enzymes exist in the site of oxygen insertion into arachidonic acid, pH optima, their ability to use esterified fatty acids, and, in the case of 5-lipoxygenase, a specific requirement for ATP and Ca^{2+} (Yamamoto, 1992). The soybean 15-lipoxygenase and the leukocyte 5-lipoxygenase also differ in their sensitivity toward phenol and *N*-hydroxy inhibitors (Bélanger *et al.*, 1987; Falgueyret *et al.*, 1992). The similarities in the primary sequences of lipoxygenases and the observation that the soybean 15-lipoxygenase and the leukocyte 5-lipoxygenase both catalyze the reducing agent-dependent (Riendeau *et al.*, 1991b) and the anaerobic arachidonate-dependent pseudoperoxidase reactions (Percival & Ouellet, 1992) in addition to the oxygenation of arachidonic acid have led to the assumption that the different lipoxygenases follow similar basic mechanisms for their reactions, all involving the participation of the active-site non-heme iron. The present observations on the spectral properties of the human 5-lipoxygenase provide direct evidence of the similarity of the environment of the non-heme iron for the soybean and leukocyte enzymes. They also provide a basis for the comparison of the results of mutagenesis studies on the putative iron-coordinating histidines of 5-lipoxygenase (Nguyen *et al.*, 1991; Ishii *et al.*, 1992; Percival & Ouellet, 1992; Zhang *et al.*, 1992, 1993) with those of the soybean 15-lipoxygenase (Steczko *et al.*, 1992). These data established that the same three His residues in both enzymes are important for the maintenance of activity. Data from the two crystal structures of the ferrous form of soybean lipoxygenase which were recently published show that these three His residues are in fact ligands to the iron (Boyington *et al.*, 1993; Minor *et al.*, 1993).

The present results demonstrating the similarities between the environment of the active-site iron of the soybean and leukocyte enzymes suggest that the crystal structure data may allow a new, more rational approach to be taken in the design of therapeutic agents based on iron-coordinating moieties.

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