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X-ray absorption spectroscopy of soybean lipoxygenase-1

Influence of lipid hydroperoxide activation and lyophilization on the structure of the non-heme iron active site

Lambertus M. Van der HEIJDT [1], Martinus C. FEITERS [2], Suppiah NAVARATNAM [3], Hans-F. NOLTING [4], Christoph HERMES [4], Gerrit A. VELDINK [1] and Johannes F. G. VLIEGENTHART [1]

[1] Department of Bio-Organic Chemistry, Bijvoet Center for Biomolecular Research, Utrecht University, The Netherlands
[2] Department of Organic Chemistry, Nijmegen SON Research Center for Molecular Structure, Design and Synthesis, Catholic University of Nijmegen, The Netherlands
[3] North-East Wales Institute of Higher Education, Connah's Quay, Wales
[4] European Molecular Biology Laboratory, Outstation at Deutsches Elektronen Synchrotron, Hamburg, Federal Republic of Germany

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X-ray absorption spectra at the Fe K-edge of the non-heme iron site in Fe(II) as well as Fe(III) soybean lipoxygenase-1, in frozen solution or lyophilized, are presented; the latter spectra were obtained by incubation of the Fe(II) enzyme with its product hydroperoxide. An edge shift of about 2 – 3 eV to higher energy occurs upon oxidation of the Fe(II) enzyme to the Fe(III) species, corresponding to the valence change. The extended X-ray absorption fine structure shows clear differences in active-site structure as a result of this conversion. Curve-fitting on the new data of the Fe(II) enzyme, using the EXCURV88 program, leads to a coordination sphere that is in agreement with the active-site structure proposed earlier (6 ± 1 N/O ligands at 0.205 – 0.209 nm with a maximum variance of 0.009 nm, including 4 ± 1 imidazole ligands) [Navaratnam, S., Feiters, M. C., Al-Hakim, M., Allen, J. C., Veldink, G. A. and Vliegenthart, J. F. G. (1988) *Biochim. Biophys. Acta 956*, 70 – 76], while for the Fe(III) enzyme a shortening in ligand distances occurs (6 ± 1 N/O ligands at 0.200 – 0.203 nm with maximum variance of 0.008 nm) and one imidazole is replaced by an oxygen ligand of unknown origin. Lyophilization does not lead to any apparent differences in the iron coordination of either species and gives a much better signal/noise ratio, allowing analysis of a larger range of data.

Lipoxygenases (linoleate: oxygen oxidoreductases) are dioxygenases containing non-heme iron. They catalyze the regio- and stereo-selective dioxygenation of polyunsaturated fatty acids containing one or more (1*Z*,4*Z*)-pentadiene systems. The products are chiral *Z,E*-conjugated fatty acid hydroperoxides. In mammals, lipoxygenases are involved in the biosynthesis of physiologically active compounds, like leukotrienes and lipoxins from arachidonic acid [1]. In plants it is suggested that these enzymes play a role in germination and as possible anti-pathogens [2, 3]. Furthermore, they have been shown to be involved in the biosynthesis of 12-oxo-phytodienoic acid [4], a precursor of jasmonic acid [5]. Considerable attention has also been paid to these plant lipoxygenases because they are considered to be interesting models for mammalian lipoxygenases [6]. The soybean type-1 enzyme has been especially thoroughly investigated.

Correspondence to G. A. Veldink, Bijvoet Center for Biomolecular Research, Department of Bio-organic Chemistry, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands
Fax: + 31 30 540980.

Abbreviations. EXAFS, extended X-ray absorption fine structure; SERC, Science and Engineering Research Council; XAES, X-ray absorption edge structure; XAS, X-ray absorption spectroscopy.

Enzymes. Lipoxygenase, linoleate: oxygen oxidoreductase (EC 1.13.11.12); superoxide dismutase, superoxide: superoxide oxidoreductase (EC 1.15.1.1); protocatechuate 3,4-dioxygenase, protocatechuate: oxygen oxidoreductase (EC 1.13.11.3).

Soybean lipoxygenase-1 (94038 Da) [7] contains one mole iron/mole enzyme [8]. It catalyzes the production of (9*Z*,11*E*,13*S*)-13-hydroperoxy-9,11-octadecadienoic acid from linoleic acid. The native, colourless enzyme can be converted into a yellow enzyme form by addition of an equimolar amount of this hydroperoxide product. In the current mechanism of lipoxygenation this is a compulsory activation step [9]. The iron in the native enzyme is high-spin Fe(II) ($S = 2$) and EPR-silent, whereas in the yellow enzyme it is high-spin Fe(III) ($S = 5/2$) [10]. EPR and other spectroscopic studies have shown that the iron in Fe(III) lipoxygenase-1 is in an environment with a ligand field of nearly axial symmetry [9]. As lipoxygenase from soybeans consists of a single polypeptide chain and does not contain a porphyrin [9] nor a quinonoid cofactor [11], the iron is most probably ligated to amino acids of the polypeptide backbone [9]. On the basis of extended X-ray absorption fine structure (EXAFS) studies it has recently been proposed that the iron in the Fe(II) enzyme is coordinated by 6 ± 1 nitrogen and/or oxygen atoms at 0.205 – 0.209 nm, with a maximum variance of 0.009 nm [12]. This had led to a model for the active site of Fe(II) lipoxygenase consisting of four imidazole nitrogens at longer distances and two oxygens of unknown origin (possibly carboxylates) at a slightly shorter distance from the central iron atom [12]. Recent Mössbauer studies on both natural-abundance ^{57}Fe and ^{57}Fe-enriched Fe(II) lipoxygenase corroborate the coordination sphere of six nitrogen and/or oxygen atoms in a roughly

octahedral arrangement [13]. Since all lipoxygenases, of which the primary structure has been determined, were found to contain a conserved cluster of five histidines within a sequence of 38 highly conserved amino acids, these histidines have been proposed to include the ligands for the iron atoms [14]. Site-directed mutagenesis on human 5-lipoxygenase has shown at least two of these histidines (His368 and His373) to be essential for enzymic activity [15]. We report here the results of an EXAFS study on both Fe(II) and Fe(III) lipoxygenase-1 from soybeans [16].

EXPERIMENTAL PROCEDURES

Lipoxygenase-1 was isolated from soybeans (Williams, American Quality B) [17]. Before use, the enzyme preparation was dialyzed against 0.1 M sodium borate pH 9.0 and exhibited a specific activity exceeding 230 μmol O_2 min^{-1} mg^{-1}, as measured polarographically. Protein purity was checked by SDS/PAGE (BioRad mini-protean kit), which showed one major band with a molecular mass of about 100 kDa. The iron and manganese content, as determined by inductively coupled plasma-atomic emission spectrometry, were 0.97 and 0.04 mol/mol enzyme, respectively. Concentration to approximately 2.5 mM was performed by use of a collodion bag SM 13200 (Sartorius Membranfilter, Göttingen, FRG). (9*Z*,11*E*,13*S*)-13-Hydroperoxy-9,11-octadecadienoic acid was prepared by aerobic incubation of linoleic acid with lipoxygenase-1 at pH 9.0 and further purified by straight-phase HPLC as described earlier [18]. Fe(III) lipoxygenase-1 was prepared in 0.5 mM concentration through titration of the Fe(II) enzyme with the hydroperoxy-octadecadienoic acid [19]. Subsequently, the preparation was dialyzed to remove excess of the hydroperoxide and concentrated, as described for the Fe(II) enzyme. Lyophilized enzyme samples were prepared by freeze-drying their 0.1 M sodium borate pH 9.0 solutions on an Edwards EF05 freeze-dryer for approximately 20 h. Specific activities of all samples were determined before and soon after exposure to the high-intensity X-ray radiation. In addition to this, EPR spectra of exposed and unexposed Fe(III) enzyme samples at 9 GHz were recorded with a Varian E-9 spectrometer at temperatures between 8–20 K. Both methods showed the samples to be unaffected by the X-ray radiation.

X-ray absorption spectra at the Fe K-edge (approx. 7120 eV) of Fe(II) and Fe(III) lipoxygenase (both concentrated buffered solutions and lyophilized samples) were recorded in the fluorescence mode at the European Molecular Biology Laboratory EXAFS station at Hamburg Synchrotron Laboratory (Deutsches Elektronen-Synchrotron, Hamburg) [20]. The major components are an order-sorting Si(111) monochromator [21], a segmented, focussing Au-coated mirror and an energy calibration device [22]. For harmonic rejection the monochromator was detuned to about 50% of its peak intensity. The energy resolution of the spectrometer was 2.0 eV as established from the full width at half maximum of the calibration Bragg peaks. Sample cells of 1 mm thickness were filled and frozen only just before the X-ray absorption spectroscopy (XAS) measurements. During the experiments, the samples were located in the He exchange gas atmosphere (20 K) of a closed-cycle cryostat. Fluorescence radiation was detected with two plastic scintillators coupled to fast photomultipliers, mounted at 90° to the incoming beam. Each scan was individually checked and at least 15 good scans of each detector were averaged. All measurements were carried out during dedicated synchrotron radiation shifts with DORIS II working at an energy of 3.7 GeV and a current of 50–100 mA.

Additional X-ray absorption spectra of the Fe(III) lipoxygenase-1 [both concentrated and dilute (0.55 mM) buffered solutions] were recorded on the Anglo-Dutch EXAFS station 8.1 of the Science and Engineering Research Council (SERC) Synchrotron Radiation Source at the Daresbury Laboratory [23], in which case the configuration included a platinum-coated focussing mirror, a servo-controlled Si(111) monochromator detuned to approximately 80% of its peak intensity to suppress the harmonics, and a cryostat working at liquid nitrogen temperature. The storage ring was operated at 2.0 GeV at a ring current of 150–300 mA. For detection an energy-discriminating Canberra 13-elements solid-state fluorescence detector was used. For each scan all individual detector channels were checked and at least six scans of each sample were summed and averaged to obtain a satisfactory signal/noise ratio. Edge positions of samples measured on different instruments were related through comparison with data of iron polyimidazole model compounds collected on both instruments (Van der Heijdt, L. M. et al., unpublished results).

Data reduction was achieved by using computer programmes developed both at the EMBL Outstation (energy calibration, AUTCAL/AROT41/MOTCALF; averaging MEAN/MEANFINE; background subtraction, AREMPF1 and normalization, NLOOF) [24] and at SERC Daresbury Laboratory (Warrington, UK) (averaging, EXCALIB; background subtraction, EXBACK) while the final analysis of the processed data employed the SERC Daresbury Laboratory EXAFS analysis package, *viz.* the fast curved-wave EXAFS simulation and fitting programme EXCURV88 [25, 26], including MUFPOT for the *ab initio* calculation of phase shifts and backscattering factors. Fits were obtained by simulation of the EXAFS on the basis of models, varying occupancies and atom types, and iteratively refining of the shell radii and the Debye-Waller-type factors to a minimum in the fit index, as was done for the Fe(II) enzyme [12].

RESULTS

The EXAFS (k^3-weighted) and the X-ray absorption edge structure (XAES) of both Fe(II) and Fe(III) lipoxygenase-1 are shown in Fig. 1. Apparently, the edge spectra of the two enzyme species are different. There is a shift in edge energy of 2–3 eV to higher energy upon oxidation of the Fe(II) enzyme to the Fe(III) enzyme, corresponding to the valence change. The differences in the shape of the edges indicate a drastic change of the Fe-coordination geometry as is also reflected in the EXAFS spectra. The 'camel back' feature at 35–50 nm^{-1}, which is considered to be typical of imidazole backscattering [27], is different and much less pronounced in the fine structure of the Fe(III) enzyme. However, the overall beating patterns and amplitudes of the fine structure of the Fe(II) and Fe(III) enzyme species are alike. The Fourier transforms of the two species (Fig. 2), representing a radial distribution of backscattering atoms around the centralabsorber [28], show one major peak at about 0.20 nm and some minor peaks at 0.29–0.31 nm and 0.40–0.42 nm from the central iron. These features indicate that imidazoles are the main backscatterers also in the Fe(III) enzyme, despite the changes of the camel back as compared to the Fe(II) enzyme [12]. For transition metals with incompletely filled d shells, the intensities and shapes of

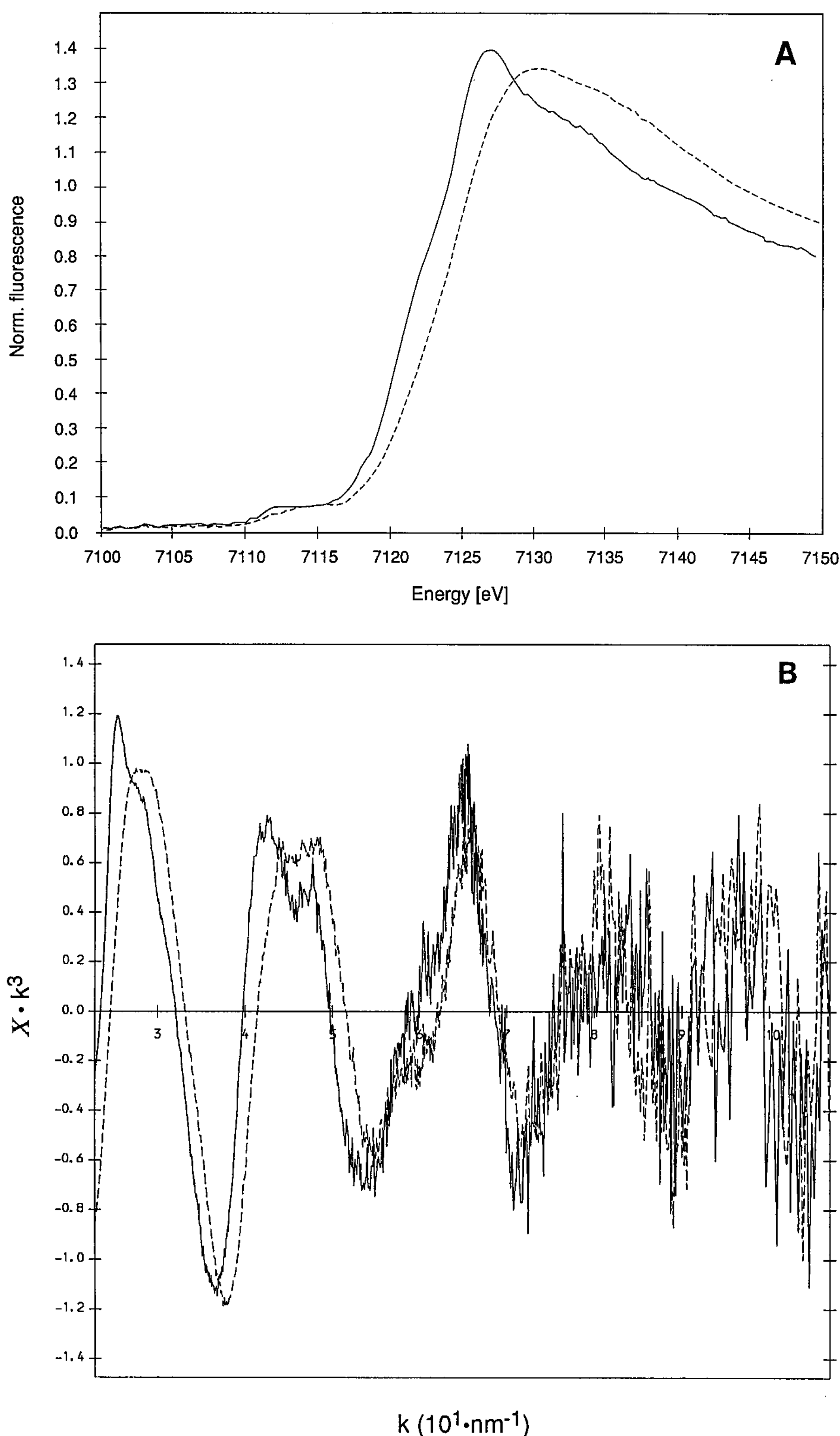


Fig. 1. XAS of concentrated buffered solutions of Fe(II) and Fe(III) lipoxygenase-1. (A) X-ray absorption near-edge structure of concentrated buffered solutions of Fe(II) (———) and Fe(III) lipoxygenase-1 (----). (B) Weighted fine structure (k^3) of concentrated buffered solutions of Fe(II) (———) and Fe(III) lipoxygenase-1 (----).

the pre-edge features are related to the 1s—3d transition [29] and to the symmetry of the coordination sphere of the central absorber [30]. As can be judged from Fig. 1 A, they are similar but not identical for the two enzyme species. This points to the same coordination number for both enzyme species [31], but to a different coordination geometry. On the basis of comparison with iron model compounds [30, 32] (Van der Heijdt, L. M. et al., unpublished results), hexa-coordination is most probable. This is in agreement with results from recent magnetic CD studies [33].

From previous EPR, light-absorption and magnetic-susceptibility studies [19], it has been concluded that the fraction

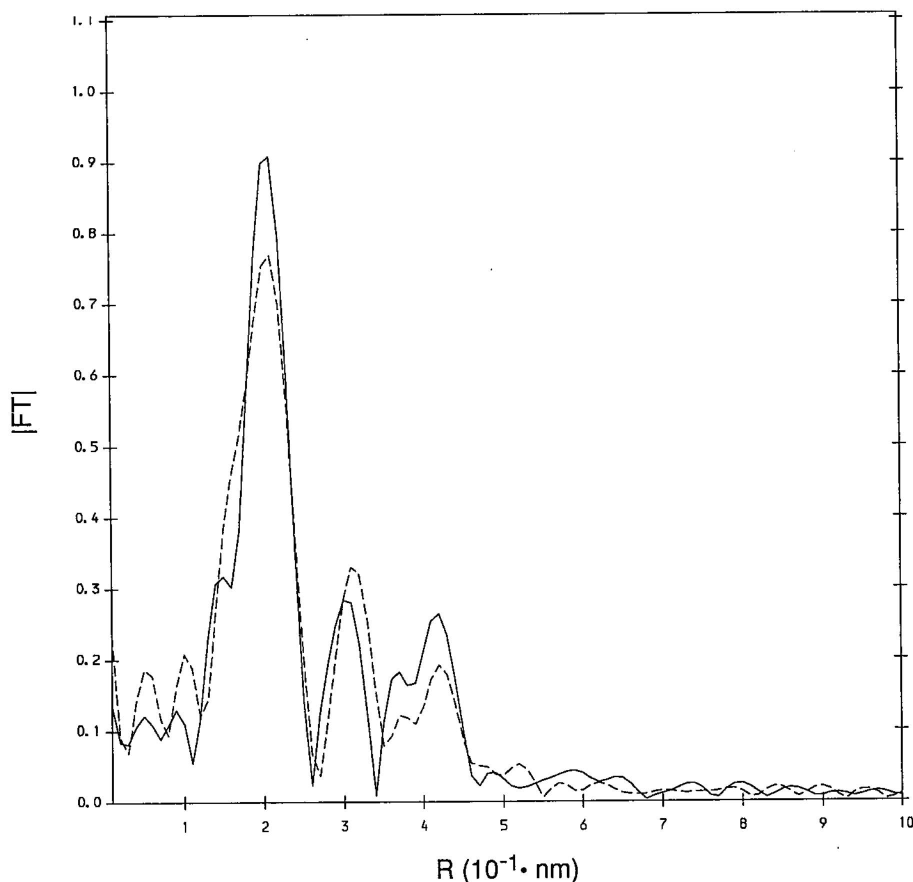


Fig. 2. Phase-corrected Fourier transforms of lyophilized samples of Fe(II) (——) and Fe(III) lipoxygenase-1 (----).

of high-spin, EPR-visible Fe(III) decreases with increasing enzyme concentration. Two possible explanations for the existence of an enzyme fraction with $S = 2$ have been suggested, i.e. the presence of Fe(III) antiferromagnetically coupled to an organic radical, or the presence of high-spin Fe(II) [19]. Because no deviation from Curie behaviour was observed at the high end (200 K) of the temperature range of the magnetic susceptibility measurements [19], the latter has been favoured. To verify whether XAS data of the Fe(III) enzyme, collected at 2.5 mM concentration, can be considered typical of data at lower concentrations, additional data were collected on samples of Fe(III) lipoxygenase at 0.55 mM concentration. Direct comparison of the edge position, EXAFS and XAES features of the dilute and concentrated buffered Fe(III) enzyme solutions shows that the collected data appear to be unaffected by the concentration procedure used and the amount of Fe(III) is the same at both concentrations (Fig. 3). This rules out the proposed reduction of Fe(III) lipoxygenase to Fe(II) lipoxygenase, at concentrations exceeding 0.5 mM [19]. The possibility of antiferromagnetic coupling of high-spin Fe(III) to a radical in Fe(III) enzyme preparations at such high concentrations should be considered seriously. Any conclusions on the active-site structure, drawn from the analysis of the data collected from concentrated Fe(III) enzyme samples, thus seem to apply to the structure of Fe(III) lipoxygenase at lower concentrations, which is closer to what can be considered physiologically relevant.

From the observation of line broadening in the low-field EPR signals of Fe(III) enzyme samples dissolved in $H_2{}^{17}O$, it was concluded that water or hydroxide is a direct ligand to the iron in this enzyme species [34]. As water is usually a readily displaceable ligand, lyophilization is expected to extract water from the metal coordination sphere or its environment, as has been demonstrated for the iron transport protein ovotransferrin [35] and for CuZn-superoxide dismutase [36]. Therefore, in addition to the experiments on concentrated solutions of Fe(II) and Fe(III) soybean lipoxygenase-1, XAS data for the corresponding lyophilized samples were collected as well. In comparison to the data taken on frozen and concentrated buffered solutions of lipoxygenase, removal of a water or a hydroxide ligand should lead to significant changes in the near-edge structure and possibly in the EXAFS as well. However, it can be seen from Fig. 4 that the principal features in the X-ray fluorescence spectra of both Fe(II) and Fe(III) lipoxygenase-1 are not influenced by lyophilization. The shape and intensities of the pre-edge features and the EXAFS (both shape and amplitude) are similar, indicating that the coordination numbers are unaltered. These results imply that the iron coordination sphere is not significantly affected by the lyophilization procedure and that certainly no ligand is lost. Whether this implies that H_2O or OH^- is not coordinated to the iron in lipoxygenase at all, remains to be established. As can be judged directly from the data presented in Fig. 4, the lyophilization procedure enhances significantly the signal/noise ratio of the fine structure data, permitting a more profound and detailed analysis, as compared to the data collected on frozen solutions.

In the initial step of the analysis, the k^3 weighted EXAFS of both the Fe(III) and the Fe(II) enzyme were Fourier-transformed in the range $k = 25-100\ nm^{-1}$, and the main shell

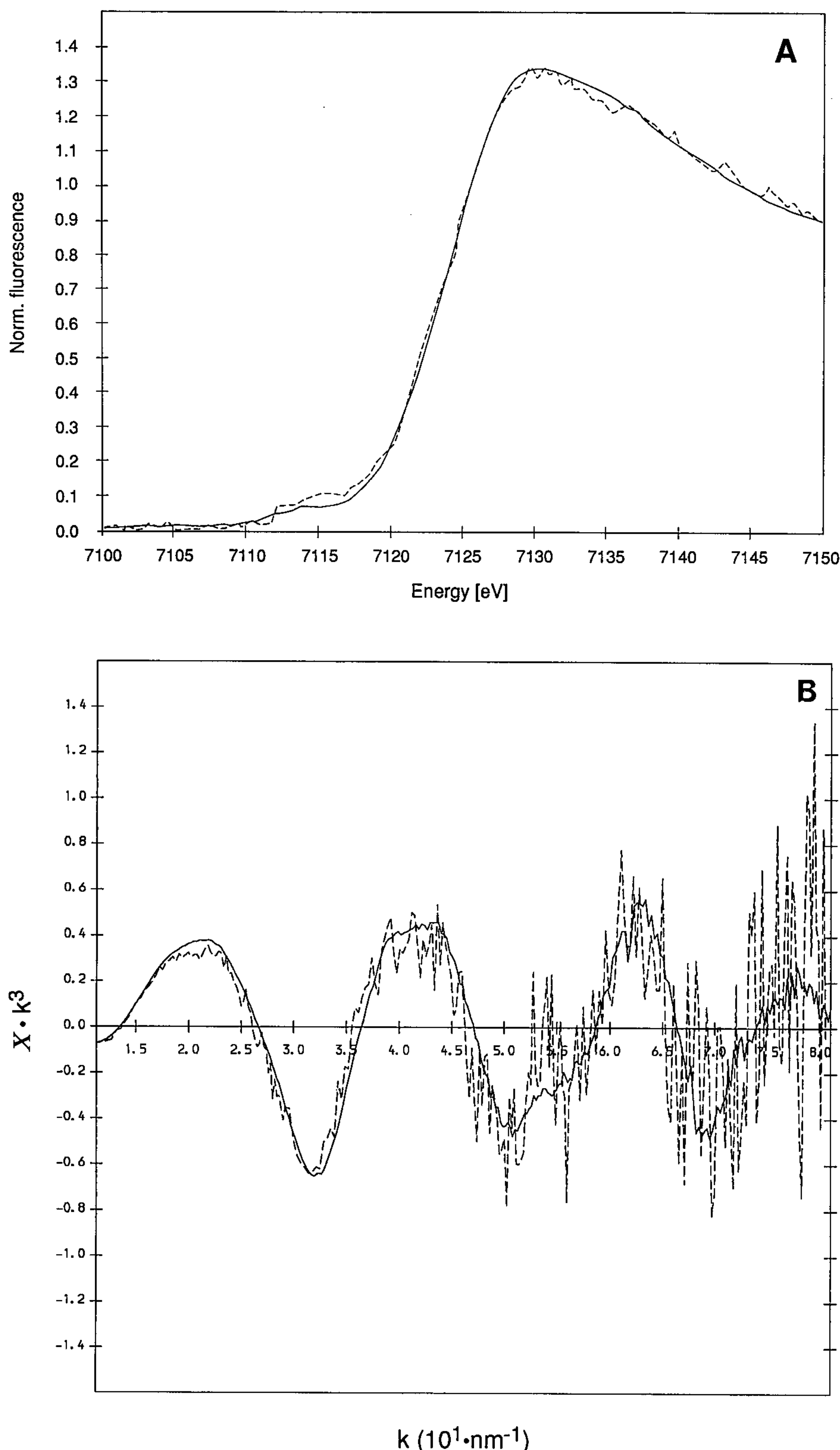
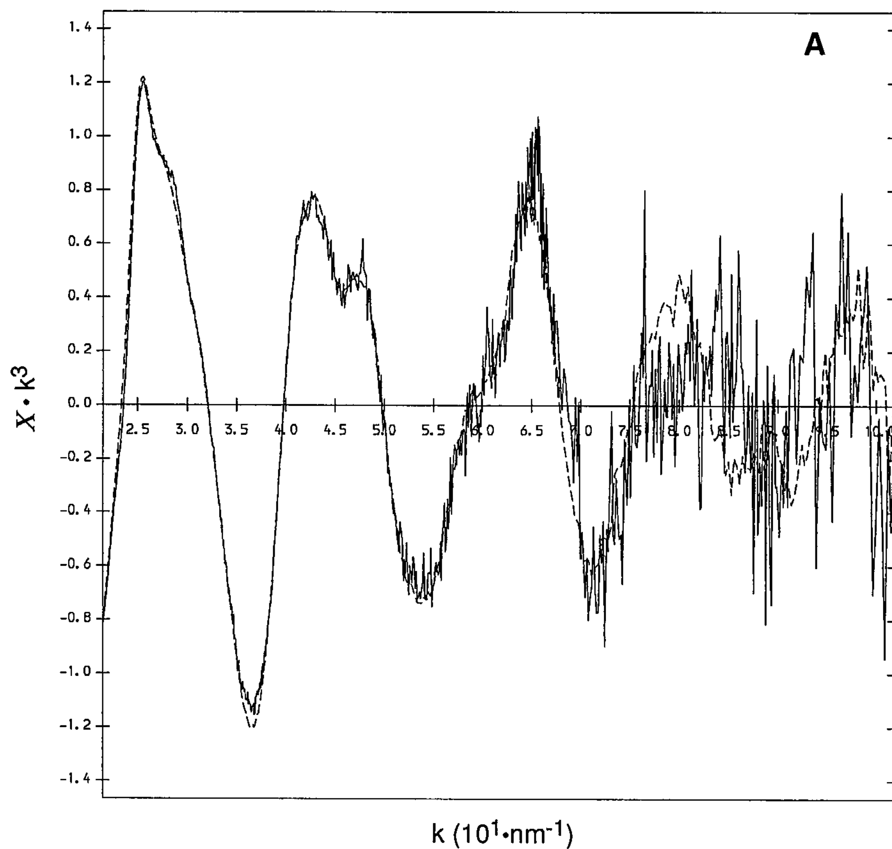


Fig. 3. XAS of dilute and concentrated buffered solutions of Fe(III) lipoxygenase-1. (A) X-ray absorption near-edge structure of dilute (0.55 mM) (----) and concentrated (2.5 mM (———) buffered solutions of Fe(III) lipoxygenase-1. (B) Weighted fine structure (k^3) of dilute (0.55 mM) (----) and concentrated (2.5 mM) (———) buffered solutions of Fe(III) lipoxygenase-1.

filtered and backtransformed. In the case of the Fe(III) enzyme, with the coordination numbers fixed at integer values, it could be fitted with either six nitrogens, at 0.203 nm (Debye-Waller-type factor, $2\sigma^2 = 0.00023$ nm^2) or six oxygens at 0.200 nm ($2\sigma^2 = 0.00029$ nm^2) or with combinations of nitrogens and oxygens. Best fits were obtained with a split first shell consisting of four nitrogens and two oxygens (4N at 0.208 nm/2O at 0.188 nm) or three nitrogens and three oxygens (3N at 0.209 nm/3O at 0.190 nm). Simulations of the EXAFS with oxygen rather than nitrogen ligands at the longer



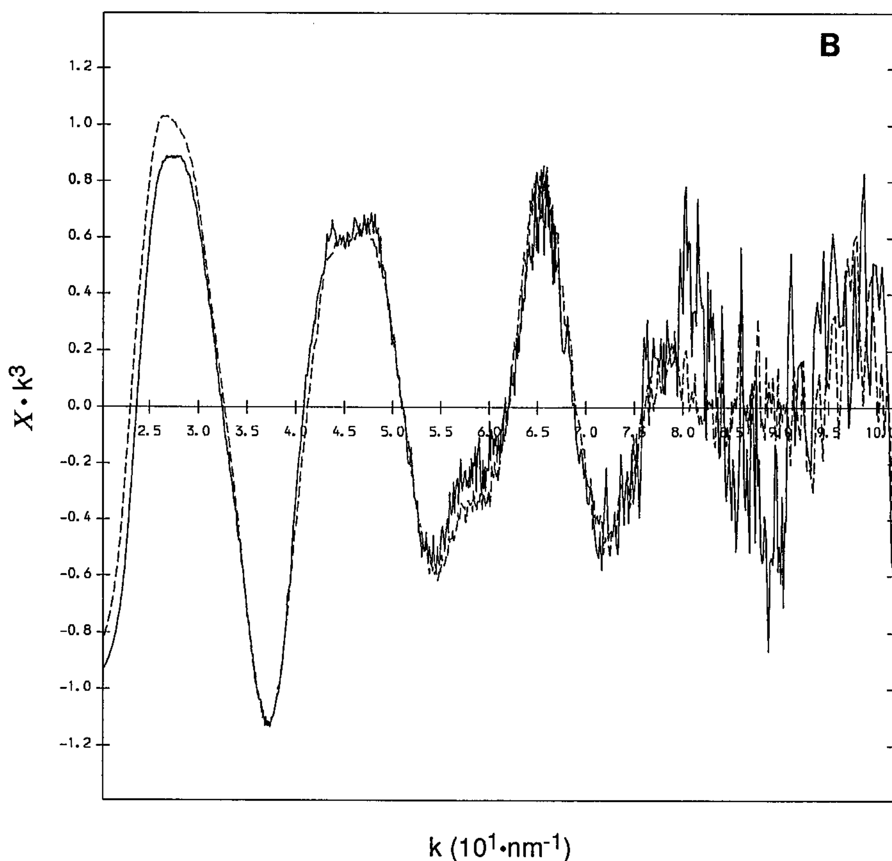


Fig. 4. Effect of lyophilization on the X-ray absorption spectra of Fe(II) and Fe(III) lipoxygenase-1. (A) Weighted fine structure (k^3) of Fe(II) enzyme; lyophilized (----) and frozen solution (———). (B) Weighted fine structure (k^3) of Fe(III) enzyme, lyophilized (----) and frozen solution (———). (C) Near-edge region of Fe(II) enzyme, lyophilized (----) and frozen solution (———). (D) Near-edge region of Fe(III) enzyme, lyophilized (----) and frozen solution (———).

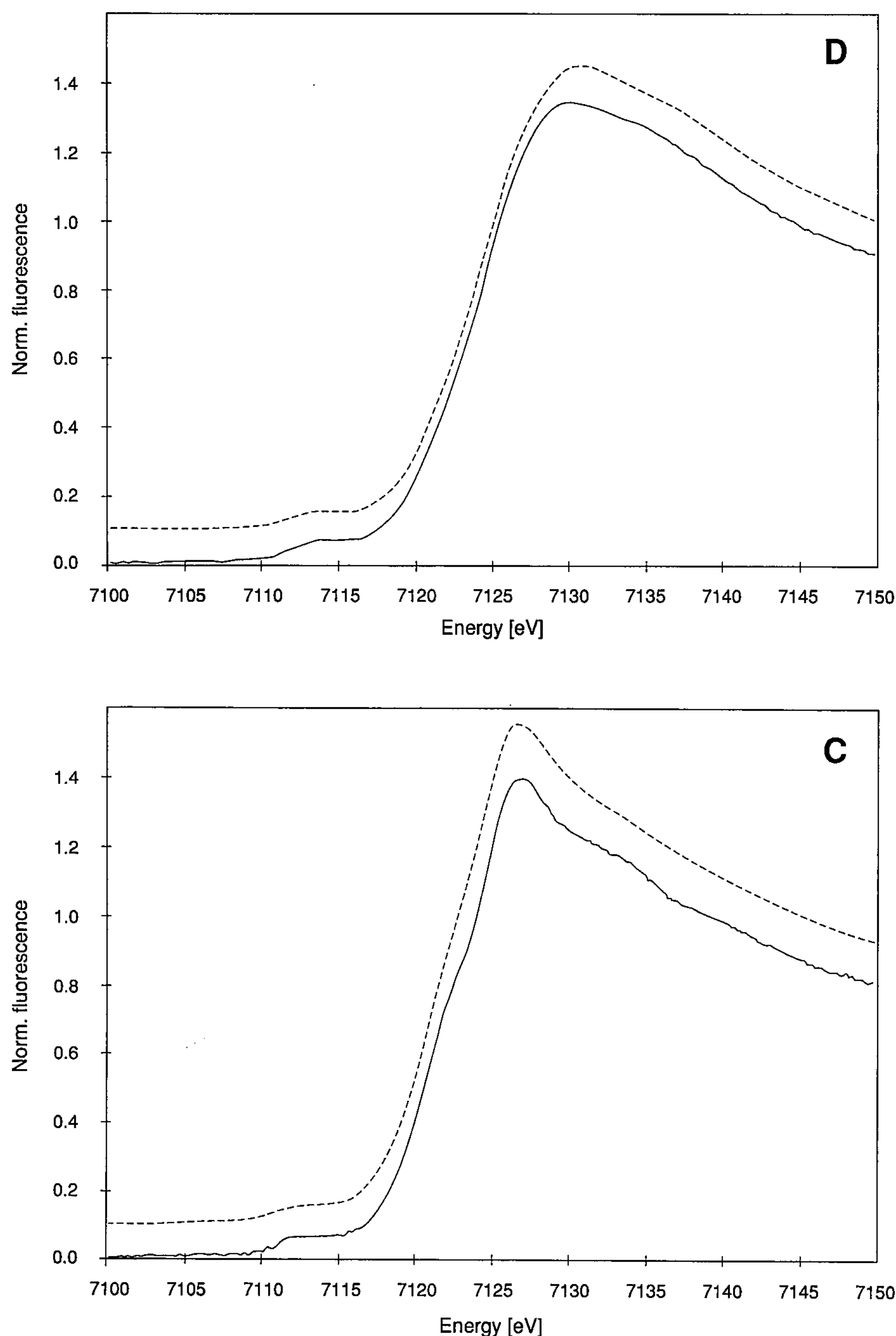


Fig. 4.

distance gave poorer fits to the experimental spectra. For the Fe(II) enzyme the first shell was best fitted with four nitrogens and two oxygens (4N at 0.212 nm/2O at 0.193 nm), while an attempt to fit the first shell with a combination of three nitrogens and three oxygens yielded a negative Debye-Waller-type factor for the oxygens. Thus, it seems that among other effects a shortening of the average ligand distance occurs as a result of the treatment of Fe(II) enzyme with product hydroperoxide. This is consistent with a decrease in ionic radius upon oxidation of Fe(II) to Fe(III). The change in average ligand distance by itself is expected to contribute to the observed edge shift in addition to the effect of the valence change [37].

In order to analyse the lipoxygenase EXAFS data with inclusion of the higher shells representing backscattering by the other atoms of imidazole rings, a multiple scattering approach was used, because for the higher shells large discrepancies in both phase and amplitude between theoretical and experimental EXAFS occur when only the single scattering theory is used [38, 39]. Since the data at low k ($k < 30-35\ \mathrm{nm}^{-1}$) are dominated by such multiple scattering events, the data range was extended to below $k = 25\ \mathrm{nm}^{-1}$ in this case. As the geometries of structures like histidines or pyrrole rings are known to vary only within a very limited range, in addition a method of constrained refinement [40] was used, correlating the internal bond angles and atomic distances for known rigid structures. This has the main advantage of reducing considerably the number of parameters being refined independently and, thereby, avoiding physically unrealistic structural interpretations. To estimate the number of imidaz-

Table 1. Parameters used for the simulation of the EXAFS. FI is the fit index, defined as the weighted sum of the squares of experimental minus theoretical data points: F.I. $= \Sigma_i[X(i)_{exptl} - X(i)_{theory}]^2 \cdot k^3/100 \cdot N$, where N is the number of data points in the experimental spectrum; ΔE_0 is the threshold energy, R is the distance of atom to Fe; the Debye-Waller-type factor is given as $2\sigma^2$; bond angle values refer to the angle Fe-N (at about 0.21 nm)-distant atom in the unit set up for multiple scattering calculations. The estimated accuracy is $\pm 10\%$ for coordination numbers and ± 0.003 nm for bond distances. Coordination numbers were fixed at integer values during all simulations.

Lipoxygenase	F. I.	ΔE_0	Atom type	No.	R	$100 \times 2\sigma^2$	Bond angle
		eV			nm	nm^{-2}	deg.
Fe(II) (concn solution)	1.96955	17.4	O	2	0.1938	0.011	
			N	4	0.2112	0.005	
			C	4	0.3100	0.010	−141
			C	4	0.2978	0.005	113
			C	4	0.4221	0.035	147
			N	4	0.4382	0.033	−174
Fe(III) (concn solution)	1.39941	13.43	O	3	0.1907	0.011	
			N	3	0.2110	0.001	
			C	3	0.3038	0.026	−120
			C	3	0.2991	0.017	121
			C	3	0.4248	0.030	161
			N	3	0.4412	0.030	−157
Fe(III) (lyophilized)	1.69743	14.65	O	3	0.1899	0.011	
			N	3	0.2097	0.001	
			C	3	0.3006	0.016	−119
			C	3	0.3025	0.016	121
			C	3	0.4230	0.030	160
			N	3	0.4361	0.030	−154

oles coordinating directly to the iron in both enzyme species, the numbers of imidazole and oxygen ligands were varied in the simulation allowing only integer values, while the overall coordination number was fixed at a value of six. The experimental EXAFS of lyophilized Fe(II) lipoxygenase was best fitted with a combination of four imidazoles/two oxygens, as was reported for this enzyme species in solution (2.5 mM) [12]. Small differences in ligand distances were observed, but they all fell within the variance calculated from the Debye-Waller-type factors for the different atoms. For Fe(III) lipoxygenase a simulation of the EXAFS with three imidazoles/three oxygens gave the best fit to the experimental data while fits involving four imidazoles/two oxygens or two imidazoles/four oxygens led to higher fit indices and/or to larger spreads in Debye-Waller-type factors for different atoms in the imidazole ring. As can be judged from Table 1, analysis of the EXAFS of the lyophilized Fe(III) enzyme yielded similar results.

DISCUSSION

Data analysis and curve fitting on the Fe(III) enzyme data using both single- and multiple-scattering approaches lead to a coordination sphere in which Fe(III) is ligated by three imidazole nitrogens and three oxygens, while for the Fe(II) enzyme the Fe(II) was proposed to be ligated by four imidazole nitrogens and two oxygens [12]. A recent spectroscopic study of the His−Fe(III) charge-transfer transition in Fe(III) lipoxygenase, combining magnetic CD and EPR, indicates the presence of two or three (but not necessarily four) histidine ligands coordinated in an equatorial plane [33]. Our data analysis indeed shows that one imidazole is replaced by an oxygen of unknown origin as an iron ligand, during activation of the enzyme by the product hydroperoxide. Furthermore, the ligand distances found for the Fe(III) enzyme are slightly shorter than those found for the Fe(II) enzyme. This is in agreement with the valence change of iron during activation. It is interesting to speculate that the gained oxygen stems from H_2O or OH^-. This would agree well with the observation of line broadening in the EPR spectrum of Fe(III) lipoxygenase samples prepared in $H_2{}^{17}O$ [34] and with the absence of line-broadening in the EPR spectrum of the nitrosyl complex of Fe(II) lipoxygenase [41]. However, the strong similarities which can be observed in both the EXAFS and XAES data of solutions and lyophilized samples of lipoxygenase in either of its oxidation states do not support such a possibility. Furthermore, no effects of ^{13}C substitution in cyanide on the EPR signal of the Fe(III) enzyme are observed [42], indicating that CN^- is not ligated to iron. As CN^- will readily exchange with coordinated H_2O, this makes it unlikely that H_2O or OH^- will be a ligand to iron. Another possibility could be that H_2O is not an easily replaceable ligand, implying that lyophilization of Fe(III) enzyme samples does not remove water or hydroxide as ligand, although it exchanges with $H_2{}^{17}O$ [34]. The significance of an exchangeable water molecule or hydroxide anion [34] in the catalytic mechanism of lipoxygenase is unclear. It has been argued that a water molecule coordinated to iron in lipoxygenase might be the acceptor of the proton formed upon reduction of iron in the free radical mechanism [6]. However, the present suggestion of dissociation of one histidine ligand prompts us to propose that this imidazole moiety serves as the proton acceptor, leading to the formation of the pentadienyl radical [43]. Because the pK_a of the imidazole ring is near physiological pH, histidine is an ideal residue as proton donor/acceptor in enzymic reactions [44]. An example of the involvement of histidines in enzymic catalysis is the dissociation from Cu of the imidazole bridging Cu and Zn in superoxide dismutase [37], and accommodation of a proton upon reduction [45, 46]. Likewise, EXAFS studies of both native protocatechuate 3,4-dioxygenase and its enzyme-substrate complex point to a mechanism in which one of the histidines, involved in the coordination of iron in the active site of the native enzyme,

dissociates upon interaction with its substrate, and acts as a base to deprotonate the catecholate complex [47]. The implication of dissociated His as the proton acceptor in lipoxygenase catalysis means that it would not be available for coordination to Fe(II) in the anaerobically substrate-reduced Fe(II) enzyme. This enzyme is indistinguishable from aerobic Fe(II) enzyme in light absorption, EPR spectroscopy and magnetic susceptibility studies [19]. Interestingly, recent Mössbauer studies [48] showed significant differences between the parameters for the aerobic Fe(II) enzyme and the anaerobically substrate-reduced Fe(II) enzyme, indicating that indeed alteration occurs in the coordination sphere of iron upon oxidation and subsequent reduction by substrate. Efforts are now under way to investigate whether the proposed difference in the number of coordinating imidazoles between Fe(II) and anaerobically substrate-reduced Fe(II) enzyme can be detected by EXAFS.

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