Eur. J. Biochem. 231, 186–191 (1995)
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Changes in the iron coordination sphere of Fe(II) lipoxygenase-1 from soybeans upon binding of linoleate or oleate

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(Received 6 February/26 April 1995) – EJB 95 0182/4

Fe K-edge X-ray absorption spectra of the non-heme iron constituent of lipoxygenase-1 from soybeans were obtained. The spectrum of 2.5 mM Fe(II) lipoxygenase, mixed with 1.2 M linoleate in the absence of O_2, was compared to the spectrum of the native (i.e. untreated) enzyme. In the lipoxygenase-linoleate complex, an edge shift to lower energy was observed. This indicated that the iron-ligand distances in this complex are slightly longer than those in the untreated enzyme species. The extended X-ray absorption fine strucutre spectrum of Fe(II) lipoxygenase, prepared by anaerobic reduction of 2.5 mM Fe(III) lipoxygenase with 1.2 M linoleate, was very similar to the spectrum of the anaerobic lipoxygenase-linoleate complex. We conclude that the conformational differences between the iron coordination spheres of native and cycled Fe(II) lipoxygenase must be ascribed to the presence of linoleate, and not to changes in the enzyme that occur only after one cycle of oxidation and reduction. Furthermore, spectra of 2.5 mM Fe(II) lipoxygenase mixed with 1.2 M oleate, either in the absence or in the presence of O_2, were also identical to the spectrum of the Fe(II) lipoxygenase-linoleate complex. This finding is in agreement with our observation that oleate is a competitive inhibitor of the lipoxygenase reaction. Moreover, the similarity of the lipoxygenase-oleate complexes in the presence and absence of O_2 excludes the possibility that O_2 binding to the iron cofactor is induced upon binding of a fatty acid to lipoxygenase.

Keywords. Extended X-ray absorption fine structure (EXAFS); unsaturated fatty acid; non-heme iron; X-ray absorption spectroscopy.

Lipoxygenases (linoleate:oxygen oxidoreductases) are non-heme-iron-containing enzymes that catalyze the dioxygenation of polyunsaturated fatty acids containing at least one 1,4-pentadiene system. The reaction products are chiral *E,Z*-conjugated hydroperoxy fatty acids. In mammalian tissues, lipoxygenases are involved in the initial steps of the biosynthesis of physiologically active compounds, such as leukotrienes and lipoxins (Samuelsson et al., 1987). The physiological function of lipoxygenases in plants is less clear. It has been suggested that they may play a role in germination, formation of anti-pathogens, and plant hormone biosynthesis (Ohta et al., 1986; Yamamoto et al., 1990; Zimmerman and Feng, 1978; Vick and Zimmerman, 1983). Lipoxygenases from plant and animal origin show considerable sequence similarity (Feiters et al., 1990). Soybean lipoxygenase-1 catalyzes the formation of 13*S*-hydroperoxy-9Z, 11*E*-octadecadienoic acid from linoleate at pH 9.0. This enzyme is readily purified in large quantities from resting soybean seeds, and has been studied extensively with several spectroscopic and kinetic techniques. It contains 1 mol non-heme iron/mol enzyme (Chan, 1973; Roza and Francke, 1973), and is isolated as a colorless, EPR-silent, high-spin Fe(II) species ($S = 2$). This native enzyme form is converted into a yellow, high-spin Fe(III) species ($S = 5/2$) by addition of an equimolar amount of the product hydroperoxide (Slappendel et al., 1980, 1982). Recently, the three-dimensional structure of native soybean lipoxygenase-1 was solved to 0.26-nm resolution with X-ray diffraction techniques (Boyington et al., 1993). The central Fe(II) appears to be coordinated by three histidine residues and a single carboxyl group. The coordination geometry is apparently octahedral, but with two adjacent positions not occupied by ligands. From the outside, two hydrophobic tunnels lead to the iron center. Boyington et al. proposed that these tunnels are the substrate-binding sites, and that the empty Fe(II) coordination sites are occupied by O_2 and the fatty acid 1,4-pentadiene system in the enzyme-substrate complexes (Boyington et al., 1993).

Extended X-ray absorption fine structure (EXAFS) is a powerful technique for studying the iron coordination sphere of lipoxygenase in solution, and for comparing various lipoxygenase complexes. In previous studies, it was shown that the iron coordination in Fe(III) lipoxygenase is different from that in the native enzyme (Navaratnam et al., 1988; Van der Heijdt et al., 1992). Magnetic circular dichroic studies suggest that one of the ligands is replaced by an exogenous ligand during the catalytic cycle (Zhang et al., 1991). Preliminary EXAFS measurements indicate that the iron coordination sphere in cycled Fe(II) lipoxygenase, i.e. Fe(II) lipoxygenase taken through one cycle of oxi-

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Abbreviations. EXAFS, extended X-ray absorption fine structure; XAES, X-ray absortion edge structure; SERC, Science and Engineering Research Council; EMBL, European Molecular Biology Laboratory.

Enzyme. Lipoxygenase (EC. 1.13.11.12).

dation with 13(*S*)-hydroperoxyoctadecadienoic acid and subsequent anaerobic reduction with linoleate, is different from that in native lipoxygenase. Significant differences between native and cycled Fe(II) lipoxygenase are also observed in Mössbauer studies on ^{57}Fe-enriched lipoxygenase (Funk et al., 1990). In a previous study, we suggested that the observed changes in the coordination might reflect conformational differences between native and cycled enzyme (Van der Heijdt et al., 1992).

Dioxygenation of linoleate, catalyzed by lipoxygenase-1, is highly stereospecific and regiospecific (Hamberg and Samuelsson, 1967). Although no O_2 seems to be present in the iron coordination sphere in native lipoxygenase (Petersson et al., 1985; Feiters et al., 1985; Boyington et al., 1993), it is possible that significant O_2 coordination does occur when the fatty acid binding site is occupied. A direct demonstration of an Fe(II) lipoxygenase$-O_2-$fatty acid complex by EXAFS studies will, however, only be feasible when dioxygenation of the fatty acid is prevented. Oleate (9*Z*-octadecenoic acid) lacks the 1,4-pentadiene system of linoleate, and cannot, therefore, be dioxygenated by lipoxygenase. Since oleate is a competitive inhibitor of the dioxygenation reaction (Lomnitski et al., 1993; this study), and because its structure is similar to that of linoleate, it is possible that the two compounds bind to the same site on lipoxygenase. In this study, we compare the X-ray absorption spectra of native lipoxygenase with those of the Fe(II) lipoxygenase-linoleate complex and of cycled Fe(II) lipoxygenase. Furthermore, we address the question of O_2 binding to the lipoxygenase-fatty acid complex by comparing the X-ray absorption spectra of Fe(II) lipoxygenase-oleate complexes in the presence and absence of oxygen.

EXPERIMENTAL PROCEDURES

Materials. Fe(II) lipoxygenase-1 was isolated from soybeans (White Hilum) as described by Slappendel (Slappendel, 1982). SDS/PAGE with Coomassie brilliant blue staining showed one band with a molecular mass of approximately 100 kDa. The preparation exhibited a specific activity exceeding 180 µmol $O_2 \cdot min^{-1} \cdot mg^{-1}$. Before use, the enzyme preparation was dialyzed against 0.1 M sodium borate, pH 9.0. A collodion bag (Sartorius Membranfilter SM 13200) was used to concentrate the protein to approximately 2.5 mM (250 mg/ml). 13(*S*)-Hydroperoxyoctadecadienoic acid was prepared as described earlier (Van der Heijdt et al., 1992). Fe(III) lipoxygenase-1 (0.5 mM) was prepared by titration of the Fe(II) enzyme with 13(*S*)-hydroperoxyoctadecadienoic acid (Petersson et al., 1987). The preparation was dialyzed to remove excess 13(*S*)-hydroperoxyoctadecadienoic acid and concentrated in the way described above.

Preparation of samples for X-ray absorption measurements. Parts of the Fe(II) and Fe(III) lipoxygenase preparations were transferred to bottles with an airtight sealcap and thoroughly flushed with argon under gentle stirring. Solutions of linoleate or oleate (100 mM in 1 M NH_4OH) were also flushed with argon, and added to the deaerated lipoxygenase solutions in a glove box under a N_2 atmosphere. The following solutions were used: A, native Fe(II) lipoxygenase; B, Fe(II) lipoxygenase and linoleate, deaerated; C, Fe(III) lipoxygenase and linoleate, deaerated; D, Fe(II) lipoxygenase and oleate, deaerated. The molar ratio of fatty acid/enzyme in the final solutions was 1.2. It should be noted that addition of excess linoleate under anaerobic conditions leads to reduction of Fe(III) lipoxygenase, and therefore the enzyme in solution C contains Fe(II). The solutions were transferred to the observation chambers for the X-ray absorption structure measurements in the same glove-box. The cells were sealed and the contents were immediately frozen in liquid N_2. Another sample of Fe(II) lipoxygenase and oleate (1.2 molar ratio) was prepared under air ($[O_2]$ = 240 µM) (solution E).

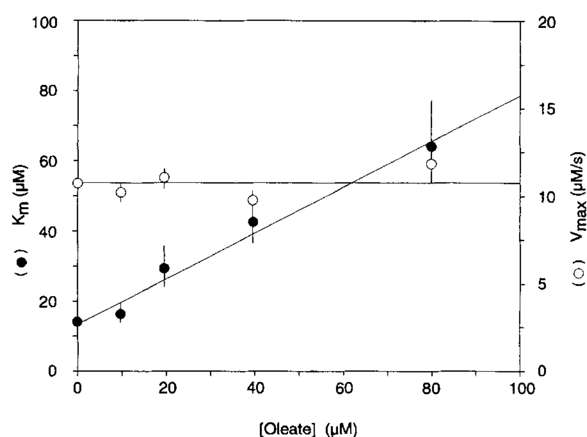

Fig. 1. Effect of oleate on the K_m and V_{max} values of the lipoxygenase-catalyzed dioxygenation of linoleic acid. [Lipoxygenase] = 35 nM; 2.5 < [linoleate] < 80 µM. K_m and V_{max} values were determined as described in the text.

X-ray absorption studies. The X-ray absorption spectra at the Fe K-edge (approximately 7120 eV) were recorded in the fluorescence mode, either at the European Molecular Biology Laboratory (EMBL) EXAFS station at Hamburg Synchrotron Laboratory (Deutsches Elektronen-Synchrotron, Hamburg) (Hermes et al., 1984; all solutions) or at the Anglo-Dutch EXAFS station 8.1 of the Science and Engineering Council (SERC) Synchrotron (Radiation Source at the Daresbury Laboratory (Van der Hoek et al., 1986; solution B). The conditions were as described elsewhere (Van der Heijdt et al., 1992) with the following modifications: (a) in both facilities, an energy-discriminating Canberra 13-element solid-state fluorescence detector was used for detection; (b) at the Hamburg Synchrotron Laboratory, the monochromator was detuned to approximately 70% of its peak intensity to suppress the harmonics; (c) at the Daresbury Laboratory, a servo-controlled Si(220) monochromator was used, which was detuned to approximately 80% of its peak intensity. Data reduction was achieved using computer programs developed at the EMBL Outstation (energy calibration: LOCREF/ROTAX/OFFEN; averaging: MEAN/MEANFINE; background subtraction: AREMPF1; normalization: NLOOF) (Nolting, 1988) or at the SERC Daresbury Laboratory (averaging: EXCALIB; background subtraction: EXBACK). Final analysis of the processed data was carried out using the SERC Daresbury Laboratory EXAFS analysis package, which consists of the fast curved-wave EXAFS simulation and fitting program EXCURV90 (Gurman et al., 1984, 1986), including MUFPOT for the *ab initio* calculation of phase shifts and backscattering factors.

Enzyme kinetics. The effect of oleic acid on lipoxygenase catalysis was studied using a Hi-Tech SF-51 stopped-flow spectrophotometer. The measurements were performed at 25°C in 0.1 M sodium borate, pH 10. The formation of 13(*S*)-hydroperoxyoctadecadienoic acid was followed by recording the absorbance at 243 nm in a 10-mm light path observation chamber. The reactions were initiated by mixing lipoxygenase with the appropriate amounts of linoleate and oleate. The final concentration of lipoxygenase was 35 nM, the final concentrations of linoleate were 2.5, 5, 10, 20, 40 or 80 µM. For each of these linoleate concentrations, curves were recorded with 0, 10, 20, 40 or

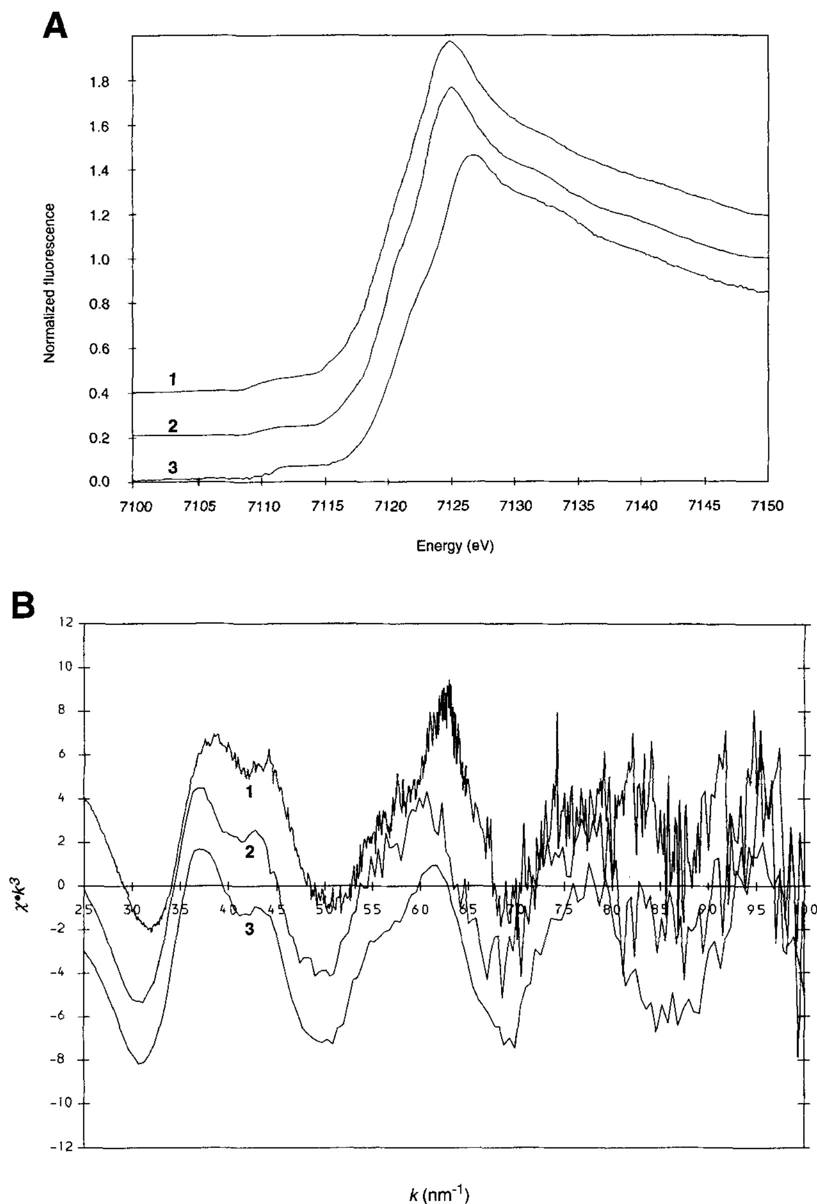

Fig. 2. XAES of concentrated buffered solutions of native Fe(II) lipoxygenase-1 (solution A), Fe(II) lipoxygenase-1 mixed with linoleate in the absence of O_2 (solution B), and Fe(III) lipoxygenase-1 mixed with linoleate in the absence of O_2 (solution C). (A) X-ray absorption near-edge structure of concentrated buffered solutions of native Fe(II) lipoxygenase-1 (trace 3), Fe(II) lipoxygenase-1 mixed with linoleate in the absence of O_2 (trace 2), and Fe(III) lipoxygenase-1 mixed with linoleate in the absence of O_2 (trace 1). The ordinate is displaced vertically by 0.2 units between successive traces. (B) Weighted fine structure (k^3) of concentrated buffered solutions of native Fe(II) lipoxygenase-1 (trace 1), Fe(II) lipoxygenase-1 mixed with linoleate in the absence of O_2 (trace 2) and Fe(III) lipoxygenase-1 mixed with linoleate in the absence of O_2 (trace 3). The ordinates of traces 1 and 3 are displaced vertically by 3.0 units and −3.0 units, respectively.

80 μM oleate. All observed curves showed an initial increase in rate (the induction period), followed by a brief period in which the rate is steady and maximal. The maximum rate, reached after the induction period, is referred to as *r*. Under the conditions at which *r* is measured, the effects of substrate and product inhibition are small, and the steady-state rate equation for lipoxygenase catalysis is well approximated by the Michaelis-Menten equation (Schilstra et al., 1992).

RESULTS

Effects of oleic acid on lipoxygenase catalysis. The effects of oleic acid on the K_m and V_{max} values for the lipoxygenase reaction (see Experimental Procedures section) are shown in Fig. 1. The values of V_{max} were constant throughout the range of oleate concentrations (10.8±0.8 μM/s), but the value of K_m increased linearly. The data are consistent with competitive inhibition of the linoleate dioxygenation reaction by oleate. In the equation for competitive inhibition, the expression for the apparent Michaelis constant is as follows: $K_m = K_{m,0} \cdot (1 + [I]/K_i)$. The slope of a straight line through the values of K_m is equal to the ratio of $K_{m,0}$, the Michaelis constant in the absence of the inhibitor I (oleate), and K_i, the dissociation constant for the lipoxygenase-oleate complex. The intercept is equal to $K_{m,0}$. The values of $K_{m,0}$ and K_i calculated from these data are 14±1 μM and 22±2 μM, respectively. Assuming that Fe(II) lipoxygenase and Fe(III) lipoxygenase have equal affinities for oleate, it was calculated that in the solutions used for the X-ray absorption measurements

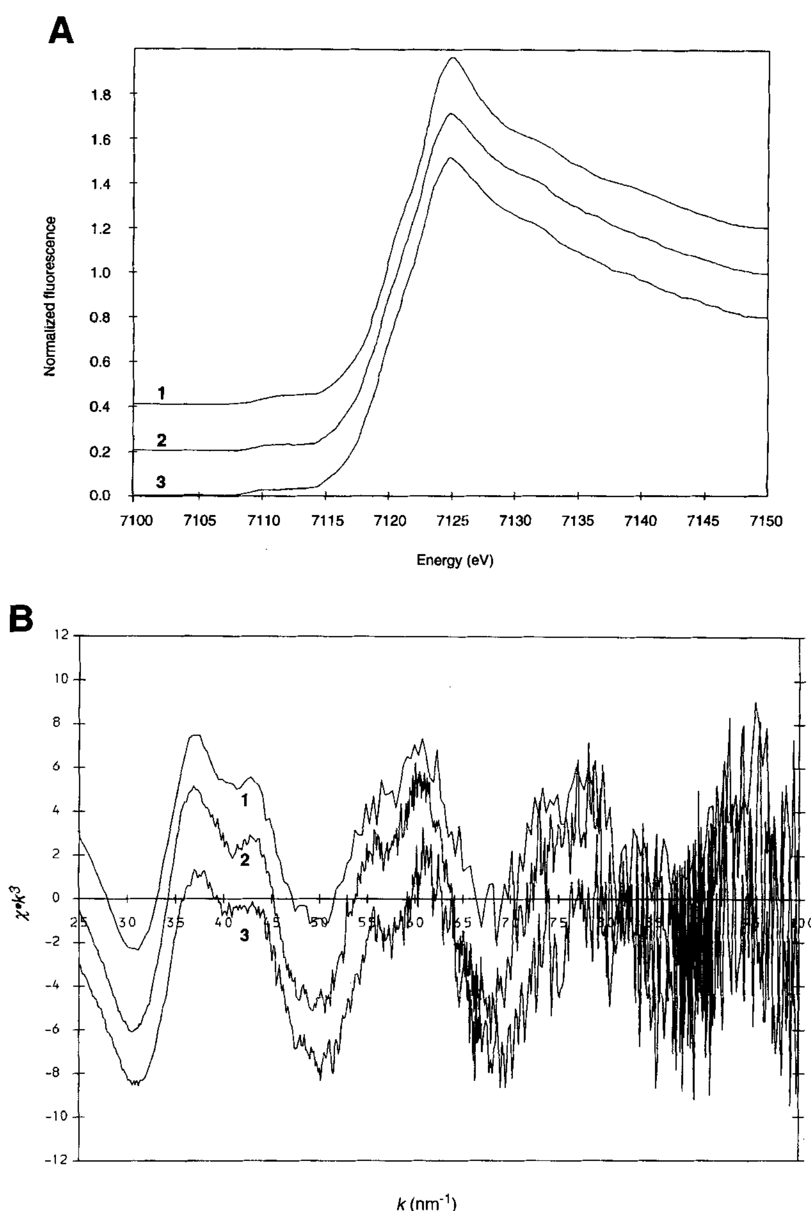

Fig. 3. XAES of concentrated buffered solutions of Fe(II) lipoxygenase-1 mixed with linoleate in the absence of O_2 (solution B), Fe(II) lipoxygenase-1 mixed with oleate in the absence of O_2 (solution D), and Fe(II) lipoxygenase-1 mixed with oleate in the presence of O_2 (solution E). (A) X-ray absorption near-edge structure of concentrated buffered solutions of Fe(II) lipoxygenase-1 mixed with linoleate in the absence of O_2 (trace 1), Fe(II) lipoxygenase-1 mixed with oleate in the absence of O_2 (trace 2), and Fe(II) lipoxygenase-1 mixed with oleate in the presence of O_2 (trace 3). The ordinate is displaced vertically by 0.2 units between successive traces. (B) Weighted fine structure (k^3) of concentrated buffered solutions of Fe(II) lipoxygenase-1 mixed with linoleate in the absence of O_2 (trace 1), Fe(II) lipoxygenase-1 mixed with oleate in the absence of O_2 (trace 2), and Fe(II) lipoxygenase-1 mixed with oleate in the presence of O_2 (trace 3). The ordinates of traces 1 and 3 are displaced vertically by 3.0 units and −3.0 units, respectively.

[2.5 mM Fe(II) lipoxygenase, 3 mM oleate], 99% of the lipoxygenase was present as a complex with oleate.

X-ray absorption spectroscopy. The X-ray absorption edge structures (XAES) of native lipoxygenase (solution A, see Experimental Procedures section) and of Fe(II) lipoxygenase mixed with linoleate in the absence of O_2 (solution B) are shown in Fig. 2A. The pre-edge feature of solution B was slightly less intense than that of solution A. This may suggest that in the lipoxygenase-linoleate complex, the Fe(II) coordination geometry is more symmetric than in the native enzyme. Furthermore, in solution B the absorption edge white line was shifted to a lower energy by approximately 1.5−2.0 eV, which is indicative of an increase of the average ligand distances. The corresponding EXAFS spectra (Fig. 2B) both exhibited a pronounced 'camel-back' feature at 35−45 nm^{-1}, which is typical of imidazole backscattering (Bordas et al., 1983), and an extra beat at 55−60 nm^{-1}. However, small but significant changes in the EXAFS were observed. Like the edge-shift in the XAES spectrum (Bianconi et al., 1983), the small reduction in the period of the main oscillation in the EXAFS spectrum of solution B suggested that the average ligand distances in the lipoxygenase-linoleate complex have increased with respect to those in the native enzyme. Previously, we suggested that the iron coordination in native lipoxygenase may be different from that in lipoxygenase that has undergone one or more cycles of oxidation and reduction (Van der Heijdt et al., 1992). The X-ray absorption spectra of native lipoxygenase (solution A, Fig. 2B, trace 1),

and lipoxygenase that was oxidized with 13(*S*)-hydroperoxyoctadecadienoic acid and subsequently anaerobically reduced with linoleate (solution C, Fig. 2B, trace 3), were indeed significantly different. However, the spectrum of solution C appeared to be very similar to that of the Fe(II) lipoxygenase-linoleate complex (solution B, Fig. 2B, trace 2). This means that the conformational changes in the iron coordination that are observed after one cycle of oxidation and reduction must be ascribed solely to the formation of a complex between the cycled Fe(II) lipoxygenase and linoleate, and not to changes of the enzyme that would occur exclusively after one cycle of oxidation and reduction.

The differences in the X-ray absorption spectra may be related to binding of fatty acid in the vicinity of Fe(II) only, but they may also be caused by a subsequent reaction step, for which the 1,4-pentadiene system is required. In order to investigate this, we recorded XAS spectra of Fe(II) lipoxygenase mixed with oleate in the absence of O_2 (solution D). Since oleate is a competitive inhibitor of the dioxygenation reaction (see above), it is likely that it binds to the same binding site on lipoxygenase as linoleate. A comparison of the XAES and EXAFS spectra of solutions B and D (Fig. 3, traces 1 and 2) showed that addition of oleate induced changes in the iron coordination sphere that are very similar to those induced by linoleate. This finding not only corroborates the hypothesis that linoleate and oleate bind to the same site on lipoxygenase, it also strongly suggests that the changes in the coordination sphere and the concomitant alteration in enzyme conformation are solely due to the presence of a fatty acid at the substrate-binding site.

In order to address the question of O_2 binding to the Fe(II) lipoxygenase-fatty acid complex, we also measured X-ray absorption spectra of Fe(II) lipoxygenase mixed with oleate in the presence of 240 μM O_2 (solution E, Fig. 3, trace 3). The XAES and EXAFS spectra of the complexes in the presence and absence of O_2 (traces 2 and 3) did not show any significant differences. Therefore, it is concluded that the iron coordination in Fe(II) lipoxygenase-oleate complexes is the same in the absence and in the presence of 240 μM O_2. This finding indicates that no O_2 binding in the vicinity of the iron center is induced upon binding of a fatty acid to lipoxygenase.

DISCUSSION

We have compared the X-ray absorption spectra of the following Fe(II) lipoxygenase species: (a) untreated native Fe(II) lipoxygenase; (b) native Fe(II) lipoxygenase to which, in the absence of O_2, an excess of linoleate was added (the lipoxygenase-linoleate complex); (c) Fe(II) lipoxygenase that was prepared by anaerobic reduction of Fe(III) lipoxygenase with an excess of linoleate [cycled Fe(II) lipoxygenase]; (d) native lipoxygenase to which an excess of oleate was added, both under aerobic and anaerobic conditions. The observations led to the following qualitative conclusions. (a) The iron coordination in native Fe(II) lipoxygenase is different from that in the Fe(II) lipoxygenase-linoleate complex. This observation is consistent with the results from Mössbauer studies on ^{57}Fe-enriched lipoxygenase (Funk et al., 1990). Boyington et al. have proposed that the fatty acid substrate may coordinate to one of the iron coordination sites that are empty in the structure of native lipoxygenase (Boyington et al., 1993). The results presented in this study support this view. We further found that treatment of the Fe(II) enzyme with oleate, a competitive inhibitor of linoleate dioxygenation, causes the same changes in the iron coordination sphere as treatment with linoleate. Apparently, the complete 1,4-pentadiene system is not required for the coordination of a fatty acid to the iron. (b) The EXAFS spectra show no differences between the iron coordination in the Fe(II) lipoxygenase-linoleate complex and in cycled lipoxygenase. Any conformational differences between the iron coordination spheres in native and cycled lipoxygenase must, therefore, be ascribed to the presence of linoleate in the fatty-acid-binding site of the cycled enzyme, rather than to conformational changes that may occur exclusively after one cycle of oxidation and reduction (compare Van der Heijdt et al., 1992). (c) Occupation of the fatty-acid-binding site with the non-reactive substrate analog oleate does not induce O_2 coordination.

The results presented in this study are in good agreement with the simple two-step model for lipoxygenase catalysis (De Groot et al., 1975a; Ludwig et al., 1987; Schilstra et al., 1992). A recent kinetic analysis of dioxygenation data on the basis of this model showed that, at $[O_2]$ = 240 μM, O_2 insertion must be two orders of magnitude faster than dissociation of the fatty acid radical from the enzyme. If dissociation of the lipoxygenase-radical complex occurred at the same rate as the estimated dissociation of the enzyme-fatty acid complex (at least 10^4 s^{-1}, estimated on the basis of data in Aoshima et al., 1978), then O_2 would be inserted at 10^6 s^{-1} (at $[O_2]$ = 240 μM). The association rate constant for O_2 binding is estimated to be in the 10^9 $s^{-1} \cdot M^{-1}$ range, which is close to the diffusion-controlled limit. In the three-dimensional structure of lipoxygenase, O_2 can diffuse in and out of a funnel-like channel that leads to one of the empty iron coordination sites (Boyington et al., 1993). O_2 insertion and iron oxidation may therefore occur almost immediately after O_2 has diffused into a catalytic site in which the substrate radical is present. In such a model, O_2 does not bind for any significant length of time to a specific site on the lipoxygenase molecule. Coordination of O_2 to the central iron will presumably elude detection. The absence of O_2 in the iron coordination sphere is, therefore, consistent with this model.

The authors wish to thank Mr M. Oversluizen (Science and Engineering Research Council/Netherlands Organisation for Scientific Research Daresbury Laboratory) for assistance with the collection of X-ray absorption data. The European Molecular Biology Organisation, the Netherlands Organisation for the Advancement of Pure Research, and SERC are acknowledged for the provision of facilities either at the EMBL Outstation at Hamburg, Germany, or at the Daresbury Laboratory, Warrington, England.

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