Crystallographic Determination of the Active Site Iron and Its Ligands in Soybean Lipoxygenase L-1†

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ABSTRACT: Five ligands of the active site iron atom in soybean lipoxygenase L-1 have been identified from the electron density map of the crystallized enzyme. The position of the iron atom can be readily and independently located from an anomalous difference electron density map. The ligands identified are His-499, His-504, His-690, Asn-694, and Ile-839, the carboxy-terminal residue. Our previous view that these three histidines are essential for activity and binding of iron, based on site-specific mutation studies, is confirmed. A sixth protein ligand is not present, and the sixth coordination site opens into a wide cleft. The structure of the soybean lipoxygenase was solved by multiple anomalous isomorphous replacements.

Lipoxygenases are non-heme, non-sulfur iron dioxygenases which act on fatty acids containing a *cis,cis*-1,4-pentadiene moiety to form hydroperoxides. The enzymes are widely distributed in the plant and animal kingdoms. [For reviews, see Vliegenthart and Veldink (1982), Gardner (1991), and Siedow (1991).] Lipoxygenase substrates comprise most of the common polyunsaturated fatty acids and include arachidonic acid, the precursor of a number of physiologically potent effectors critical in human physiology.

Soybean seed lipoxygenase L-1 (L-1),[1] the best characterized of all lipoxygenases, was found about 20 years ago to contain one atom of iron per molecule of protein (Chan, 1973; Pistorius & Axelrod, 1974). Subsequently, all lipoxygenases of both plant and animal origins that have been examined have been found to contain iron. Some years earlier it had been noted that L-1 is activated by its product, *e.g.*, 13-hydroperoxy-9-*cis*,11-*trans*-octadienoic acid, when its substrate is linoleic acid (Haining & Axelrod, 1958). It was later shown that the activated enzyme exhibits a strong EPR signal at $g \cong 6$ at liquid helium temperatures, in contrast to the resting enzyme which is EPR-silent under the same conditions (Pistorius & Axelrod, 1974; de Groot *et al.*, 1975; Pistorius *et al.*, 1976). This change is accompanied by a conversion of high-spin Fe(II) to high-spin Fe(III) in the activated enzyme, suggesting that the metal is an octahedral complex and participates in the reaction (Slappendel *et al.*, 1982; Cheesbrough & Axelrod, 1983).

The chemical and physical properties of the active site Fe atom, as well as the identity of its protein ligands, have been a continuing focus of research on lipoxygenases in recent years. A variety of studies support the view that spherically coordinate iron is present in L-1. Van der Heijdt *et al.* (1992), using X-ray absorption spectra from the Fe K-edge of the iron site, have verified previous EXAFS results (Feiters *et al.*, 1990; Navaratnam *et al.*, 1988) which indicated that Fe(II) in native L-1 is held in a coordinate sphere containing 4 ± 1 N (imidazole) atoms and 2 ± 1 O atoms. They found, however, that when the enzyme is "activated" with hydroperoxide product, one of the imidazole bonds is replaced with an O bond. Mössbauer spectroscopy indicated spherically coordinate iron (Funk *et al.*, 1990; Dunham *et al.*, 1990). Magnetic circular dichroism studies (MCD) indicate an octahedral 6-coordinate system with a small rhombic distortion and at least two histidine residues as ligands (Whittaker & Solomon, 1988; Zhang *et al.*, 1991). MCD studies ruled out the possibility that O_2 is a ligand. Data from magnetic susceptibility measurements are compatible with an iron environment of largely axial symmetry and provide no evidence for the possibility of O_2 coordination (Petersson *et al.*, 1987). The possibility that carboxylate ions or H_2O might serve as ligands has been suggested (Nelson, 1988; Navaratnam *et al.*, 1988). It has been proposed on the basis of resonance Raman spectroscopy of the catechol–L-1 complex that iron coordination involves a carboxylate ligand and three neutral ligands, "probably histidine" (Cox *et al.*, 1988). It has been suggested that in the activated, *i.e.*, the Fe(III), form of the enzyme one of the N (imidazole) ligands may be replaced by an exogenous ligand, *e.g.*, product hydroperoxide (Van der Heijdt *et al.*, 1992; Zhang *et al.*, 1991). The native, *i.e.*, Fe(II), form of L-1 was used in the present studies.

A reasonable mechanism, compatible with the spectral and kinetic evidence, has been proposed for the role of iron (Petersson *et al.*, 1987). Critical discussions of the mechanism appear in recent reviews (Gardner, 1991; Siedow, 1991). The resting enzyme is activated when a trace of lipid hydroperoxide oxidizes the Fe(II) to the Fe(III). The Fe(III), in turn, oxidizes the central methylene group in the pentadiene region of the fatty acid, causing the release of a proton and leaving an intermediate with an unpaired electron. The resulting free radical undergoes rearrangement, perhaps in a concerted

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[1] Abbreviations: L-1, soybean seed lipoxygenase L-1; EXAFS, extended X-ray absorption fine structure.

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fashion, with the addition of the dioxygen to form a hydroperoxyl radical. Electron pairing is then satisfied by reduction of the radical intermediate to the hydroperoxide anion by the Fe(II) which is thus restored to the Fe(III) form, completing the iron cycle. The substrate and its intermediates must be under a steric restraint in the enzyme complex since both the release of the proton and the addition of the dioxygen occur in a stereospecific manner.

The three major isozymes of soybean seed, lipoxygenase L-1 (Shibata *et al.*, 1987), lipoxygenase L-2 (Shibata *et al.*, 1988), and lipoxygenase L-3 (Yenofsky *et al.*, 1988), were the first lipoxygenases to be sequenced. We noted a strongly conserved motif of 38 amino acid residues which included 5 conserved histidines and suggested this region as a putative iron binding site (Shibata *et al.*, 1988). A sixth conserved histidine occurs about 190 residues closer to the carboxyl terminus. Sequences of 13 lipoxygenases from both higher plants and mammalian species are now available. They all contain a well-conserved version of the 38-residue region and have retained all of the conserved histidines. The conservation of these features must be regarded as highly significant since the plant enzymes are about 25–30% larger than the animal enzymes and show homology with them in limited regions. Site-specific mutants of L-1 in which the conserved histidine residues, His-499, His-504, and His-690, were replaced one at a time with glutamine, or in some cases with serine, did not display detectable enzyme activity (Steczko *et al.*, 1992) nor did they contain iron (Steczko & Axelrod, 1992). Replacements of the other three conserved histidines yielded products which were active albeit at reduced levels. Similar indications concerning the importance of the corresponding histidines in the human 5-lipoxygenase have been reported (Nguyen *et al.*, 1991; Zhang *et al.*, 1992, 1993; Percival & Ouellet, 1992; Ishii *et al.*, 1992).

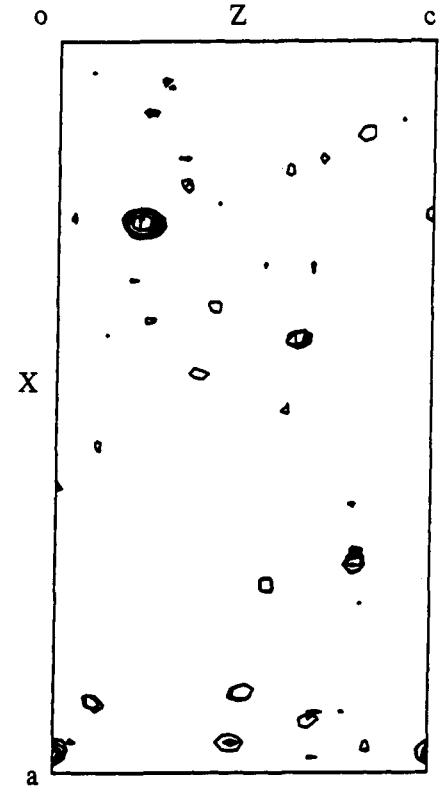

FIGURE 1: Asymmetric unit of an anomalous difference electron density map. The plot is a superposition of the contours from all unique sections of the map, which was calculated on a 1-Å grid using data between 30- and 60-Å resolution as described in the text. Contours were drawn at intervals of the root mean square (rms) density of the map beginning at 3 rms. The largest and highest density feature, at fractional coordinates (x = 0.24, z = 0.21), corresponds to the active Fe atom.

MATERIALS AND METHODS

L-1 Crystals. Crystals used were monoclinic, belonging to space group $P2_1$ and having the cell constants a = 95.6 Å, b = 94.2 Å, and c = 50.3 Å and β = 91.3°. The calculated V_M, 2.41 Å^3/Da is consistent with the presence of one molecule of enzyme in the crystallographic asymmetric unit (Z = 2) (Matthews, 1968). The crystallization procedure, using sitting drops, was described earlier (Steczko *et al.*, 1990). Two derivatives suitable for isomorphous replacement studies were prepared, one by cocrystallization with 2.5 mM methylmercury hydroxide, as previously described, and the other by soaking in K_2OsCl_6. To prepare the latter, a 10 mM stock solution of the osmium salt was made in the same starting buffer as used in growing the crystals (0.2 M sodium acetate buffer, pH 5.6, containing 8% PEG 3400). Sufficient stock solution of the osmium salt was added to the crystallization droplet to give a final concentration of 2 mM. The treated droplet was stored at 22 °C for 48 h prior to X-ray diffraction experiments.

X-ray Diffraction. The native and mercury derivative diffraction data were collected on an RAXIS-II imaging plate system (Molecular Structures Corp.) using the rotation method. Crystal rotations were 2° and the crystal–detector distance was 100 mm. The osmium derivative data were collected on a Xuong–Hamlin area detector (San Diego Multiwire Systems) with an oscillation angle of 0.1° per frame, using swing angles of 15.5° and 34.0° and crystal–detector distances of 460 and 547 mm, respectively. In both experiments the X-ray source was monochromatized Cu Kα radiation from a Rigaku RU200 X-ray generator operated at 50 kV and 50 mA. The native and derivative data were processed to 2.6- and 3.0-Å resolution, respectively. RAXIS data were initially integrated with the package supplied by the manufacturer. Fully recorded reflections were merged and scaled using the ROTAVATA/AGROVATA programs (CCP4, 1979). Later, data were reprocessed with the DENZO program (Otwinowski, 1988), and partially recorded reflections were utilized and used for all subsequent operations. Data containing all full and partial reflections were scaled with the SCALEPACK program (Otwinowski, 1988). The Xuong–Hamlin area detector data were processed with the suite of programs supplied by the manufacturer and scaled again with SCALEPACK. The overall R_{sym} factors for native, mercury, and osmium derivatives were 6.6%, 4.9%, and 3.9%, respectively. The R factor between the mercury derivative data and the native data set was 20.0%.

The positions of mercury-binding sites were determined by inspection of difference and anomalous Patterson maps (Rossmann, 1961) calculated with data from 15.0- to 3.0-Å resolution. The positions of osmium-binding sites were calculated from difference Fourier maps. Positions, occupancies, and thermal parameters for the heavy atoms were refined with the MLPHARE program (Otwinowski, 1991). The phasing included anomalous scattering information from both the mercury and osmium data. Multiple isomorphous replacement phases were calculated to 3.0-Å resolution. Phase improvement and extension to 2.6 Å were performed by a solvent flattening program that incorporates a maximum entropy procedure (Otwinowski, to be published). The resulting electron density map confirmed the choice of enantiomorph for the heavy atom positions by inspection of

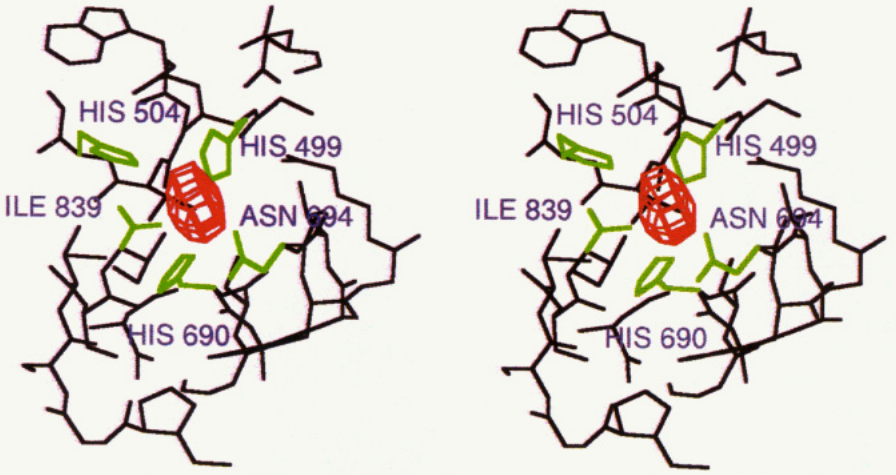

FIGURE 2: 2. Stereoscopic view of the active Fe center of lipoxygenase-1. The Fe atom is represented in red by a single three-dimensional contour (at five times the rms density) from the anomalous difference map shown in Figure 1. The five protein groups that act as ligands are represented by green bonds, whereas other protein groups are shown in black. Note that one of the five ligands is the C-terminal carboxylate of Ile-839 whereas the other ligands are the side chains of the indicated residues.

the stereochemistry of the α-helices, β-sheets, and amino acid residues.

The position of the active site Fe atom was located in an anomalous difference electron density map based on the improved multiple isomorphous replacement phases (Figure 1). Reflections in the range of 30.0–6.0 Å were used to calculate the map. Roughly 60% (1370 of 2276) of the possible reflections were used; the largest 10% of the differences as well as all differences derived from reflections with $|F|\sigma < 1.0$ were omitted from the calculation. The Fe atom is represented by a large, nearly spherical feature with a maximum value 5.8 times the root mean square (rms) density of the map; this peak is the highest density in the map. The asymmetric unit (Figure 1) contains only one additional feature of comparable maximum value (4.7 rms).

RESULTS AND DISCUSSION

Interpretation of electron density maps derived from the above experiments allowed us to determine the location of the active site Fe atom and to identify five protein groups which act as its ligands: the C-terminal carboxylate group of Ile-839, the side chains of His-499, His-504, and His-690, and the side chain of Asn-694.

Identification of the active site Fe atom and its ligands was achieved as follows. Examination of the binding sites found for the methylmercury hydroxide derivative as possible markers for cysteine residues led to the location of Cys-492 on the basis of local agreement between the amino acid sequence and the electron density map. In fact, Cys-492 is near the middle of a long helix (helix I) spanning roughly 30 residues. Extension of the interpretation from Cys-492 toward the C-terminal end of helix I was straightforward and led to the recognition of a feature wherein density assigned to the side chains of His-499 and His-504 was continuous with density for two side chains from an adjacent helix (helix II). This feature was interpreted as the likely Fe binding site. Examination of an anomalous difference electron density map, which had its strongest peak at the putative Fe position, supported this assignment, as did consideration of comparative sequence information (see below) and the results of mutagenesis experiments (see above).

The assignment of the amino acid sequence for helix II was based on the identification of density corresponding to the hexapeptide Phe-Gly-Gln-Tyr-Pro-Tyr, residues 695–700, just to the C-terminal side of the two residues that interact with the Fe atom. Extension of the interpretation in the N-terminal direction identified these residues as His-690 and Asn-694. The fifth protein ligand was identified as the C-terminal carboxylate group of Ile-839 by recognition of the fact that its density was consistent with a chain terminus as well as agreement of the density and amino acid sequence through a segment of more than 40 residues. A definitive heptapeptide included residues 816–822 of sequence Pro-Tyr-Thr-Leu-Leu-Tyr-Pro.

The density suggests that each histidine binds the Fe through its Nϵ2 atom, whereas Asn-694 binds through Oδ1. Coordination of the terminal carboxylate appears to be unidentate; however, the two oxygen atoms are not resolved, and this interpretation is subject to revision. The arrangement of the five ligands is consistent with approximate octahedral coordination. The sixth octahedral site is vacant in the sense that it is not occupied by a protein group. However, we cannot exclude or confirm the binding of a solvent molecule to this site, which is at the border of a deep crevice leading to the surface of the molecule. It seems likely that this crevice is the substrate binding site. In Figure 2 we show an portion of an unrefined model which includes the immediate environment of the active site Fe atom and its ligands.

As noted above, the three histidine residues implicated in the coordination sphere of the iron are conserved in all of the 13 sequences examined. Asn-694, in L-1, is conserved in homologous positions with two exceptions. In these instances, 15-lipoxygenases from human and rabbit erythrocytes, the corresponding position is occupied by histidine, which, it may be assumed, can serve as a functional substitute. We plan to examine the effect of substituting His for Asn-694 on the activity of L-1.

Concerning Ile-839, the C-terminal residue of L-1 is also a ligand. It has been suggested (Navaratnam *et al.*, 1988) that one or two of the ligands may be a carboxylate ion. It is noteworthy that L-1 terminates with the five-residue sequence Asn-Ser-Ile-Ser-Ile. This pattern is conserved with either total identity or strong homology, in 12 of 13 lipoxygenases (Figure 3). The exception is 5-lipoxygenase from rat leukemic cells. Here the corresponding sequence has been reported to terminate with Arg-Phe-Glu-Thr-Val, as deduced from its DNA sequence (Balcarek *et al.*, 1988).

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2190    AGA TCC CAA ACA GTG TAG CCA TCT AAG

666     Arg Phe Gln Thr Val *** Ser Ile ***
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A		Asn-Ser-Ile-Ser-Ile
B		Asn-Ser-Val-Ala-Ile
C		Asn-Ser-Val-Thr-Ile
D	(a)	Arg-Phe-Gln-Thr-Val
	(b)	Gln-Thr-Val-Ser-Ile

FIGURE 3: 3. Comparison of sequences of the five terminal amino acids in 13 lipoxygenases: (A) soybean lipoxygenase-1 (Shibata *et al.*, 1987), soybean lipoxygenase-2 (Shibata *et al.*, 1988), soybean lipoxygenase-3 (Yenofsky *et al.*, 1988), pea seed lipoxygenase "L-2-like" (Ealing & Casey, 1988), pea seed lipoxygenase "L-3-like" (Ealing & Casey, 1989), rice seed lipoxygenase (Shibata, personal communication), soy cotyledon lipoxygenase (Shibata *et al.*, 1991); (B) human reticulocyte 15-lipoxygenase (Sigal *et al.*, 1988), rabbit 15-lipoxygenase (Fleming *et al.*, 1989), human 5-lipoxygenase (Dixon *et al.*, 1988; Matsumoto *et al.*, 1988), porcine leukocyte 12-lipoxygenase (Yoshimoto *et al.*, 1990); (C) human platelet 12-lipoxygenase (Funk *et al.*, 1990); (D) (a) rat leukocyte 5-lipoxygenase (Balcarek *et al.*, 1988); (b) adjusted by deletion of the first stop codon.

Note that the first stop codon is followed by codons for Ser-Ile-stop. Were the first stop codon an inadvertent insertion, the terminal five residues would become Gln-Thr-Val-Ser-Ile, which bears reasonably good homology with the conserved pattern (Figure 3).

It has been reported that human 5-lipoxygenase deprived of its terminal six amino acid residues is inactive (Zhang *et al.*, 1992). It will be interesting to see if the terminal isoleucine is required for activity of L-1 by replacing it with other monoamino monocarboxylic acids or by deleting it.

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